Univerzita Karlova v Praze

1. lékařská fakulta

Studijní program: biomedicína

Studijní obor: Fyziologie a patofyziologie člověka

MUDr. MARTINA NOVÁKOVÁ

VLASTNOSTI A REGULACE MUSKARINOVÝCH A ADRENERGNÍCH RECEPTORŮ

Podtitul: Působení stresu na vlastnosti muskarinových a adrenergních receptorů v plicích a srdci

Characterisation and regulation of muscarinic and adrenergic receptors

Subtitle: The effect of stress on muscarinic and adrenergic receptors in the lung and in the heart

Disertační práce

Vedoucí závěrečné práce: doc. MUDr. Jaromír Mysliveček, Ph.D

Fyziologický ústav 1. LF UK v Praze

Praha, 2011

Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem řádně uvedla a citovala všechny použité prameny a literaturu. Současně prohlašuji, že práce nebyla využita k získání jiného nebo stejného titulu

Souhlasím s trvalým uložením elektronické verze mé práce v databázi systému meziuniverzitního projektu Theses.cz za účelem soustavné kontroly podobnosti kvalifikačních prací.

V Praze, 22.9.2011

MUDr. NOVÁKOVÁ MARTINA

Podpis

Poděkování

Ráda bych poděkovala především školiteli doc. MUDr. Jaromíru Myslivečkovi, Ph.D za pečlivé a svědomité odborné vedení, za veškerou podporu a nedocenitelnou pomoc, bez níž by tato práce nikdy nespatřila světlo světa.

Velký dík patří RNDr. Richardu Kvetňanskému DrSc. za poskytnutí tkání výzkumných zvířat a především za množství cenných rad a postřehů. Dík patří i všem členům výzkumného týmu Dr. Kvetňanského z Ústavu experimentálnej endokrinológie SAV v Bratislavě za pomoc při experimentální práci i za příjemné zázemí, které mi poskytli v době mé stáže na jejich pracovišti.

V neposlední řadě děkuji i všem kolegům z laboratoře docenta Myslivečka za pomoc a přátelskou podporu.

Za výbornou a precizní technickou pomoc děkuji laborantkám Bc. Evě Šimákové a Petře Svatošové.

Identifikační záznam

NOVÁKOVÁ, Martina. *Vlastnosti a regulace muskarinových a adrenergních receptorů: Působení stresu na vlastnosti muskarinových a adrenergních receptorů v plicích a srdci.[Characterisation and regulation of muscarinic and adrenergic receptors: The effect of stress on muscarinic and adrenergic receptors in the lung and in the heart]*. Praha, 2011. 113s. Disertační práce. Univerzita Karlova v Praze, 1. Lékařská fakulta, Fyziologický ústav. Vedoucí práce Mysliveček, Jaromír.

Použité zkratky

AAR	**α-adrenergní receptory**
ACTH	**adrenokortikotropní hormon**
AD	**adenylátcykláza**
ANS	**autonomní nervový systém**
AP 1	**activator protein 1**
BAR	**β-adrenergní receptory**
cAMP	**cyklický adenosinmonofosfát**
CRE	**glucocorticoid respond element**
CREB	**cAMP rerspond element binding protein**
CRH	**kortikotropin releasing hormon**
CRH KO	**vyřazení genu pro CRH, myši s vyřazeným genem pro CRH**
DAG	**diacylglycerol**
HPA	**hypotalamus – hypofýza-adrenokortikální systém**
IP_3	**inositoltrifosfát**
KO	**knock out: vyřazení specifického genu**
MR	**muskarinové receptory**
mRNA	**mesengerová ribonukleová kyselina**
PKA	**proteinkináza A**
PKC	**proteinkináza C**
PLC	**fosfolipáza C**
PLD	**fosfolipáza D**
RT-PCR	**real time polymerázová řetězová reakce**
PNMT	**fenyletanolamin-N-metyl transferáza**
WT	**wild type: kontroly**

Obsah

Souhrn v češtině

Cílem předkládané práce bylo objasnění, zda a jakým způsobem se mění množství adrenergních a muskarinových receptorů v srdci a plicích pod vlivem stresu. K výzkumné práci jsme měli k dispozici plíce a srdce laboratorních potkanů a plíce CRH KO myší. Nejprve jsme ověřili expresi mRNA pro jednotlivé podtypy α_1- a β-adrenergních receptorů a pro receptory muskarinové (zde i zastoupení jednotlivých podtypů) v srdci a plicích. Následně jsme stanovili množství vazebných míst odpovídajících příslušným receptorům pomocí specifických radioligandů. V plicích potkanů jsme prokázali přítomnost všech tří podtypů α_1-adrenergních receptorů. V plicích u WT myší se nám podařilo prokázat pohlavní rozdíly v množství α_1-adrenergních a muskarinových receptorů, u samic byla zvýšená denzita α_1-adrenergních receptorů oproti samcům, naopak množství vazebných míst pro muskarinové receptory bylo vyšší u samců. Pohlavní rozdíly v distribuci β-adrenergních receptorů jsme v plicích myší neprokázali. U CRH KO myší bylo množství vazebných míst pro sledované receptory sníženo oproti WT myším (vyjma β_1-adrenergních receptorů u samic).

Hlavním záměrem práce bylo zjistit imobilizací, tj. stresorem, navozené změny v množství výše uvedených receptorů v plicích WT myší a současně sledovat vliv vyřazení genu pro CRH (CRH KO myši). Krátkodobá i dlouhodobá imobilizace způsobila u WT myší výrazné snížení všech podtypů α_1-adrenergních receptorů u samic, zatímco u samců se snížilo pouze množství α_{1A}-adrenergních receptorů. Množství β_1-adrenergních receptorů se snížilo u samců, u samic zůstalo nezměněno. Množství β_2-adrenergních receptorů a muskarinových receptorů bylo sníženo u obou pohlaví proporčně srovnatelně. Pokles v množství příslušných receptorů u CRH KO myší pod vlivem imobilizace byl ve srovnání s WT zvířaty méně výrazný a to především u samců. Aktivita AC byla u WT vlivem imobilizace snížena, aktivita PLC zůstala beze změn. U CRH KO myší nedošlo k žádné změně v aktivitě AC.

Dále jsme detekovali změny exprese a množství receptorů navozené působením imobilizace v srdcích u myší a potkanů.

Souhrn v angličtině

The aim of this thesis was to clarify the influence of the stress on the adrenergic and muscarinic receptors in the heart and in the lungs. Research was performed on rat hearts and lungs and on the hearts and lungs of the CRH KO mice. First, we assessed mRNA levels of all α- and β-adrenergic receptor and muscarinic receptor subtypes. Subsequently, we performed the radioligand-binding studies to determine densities of these receptors.

We identified all three α_1-adrenergic receptor subtypes in the rat lungs. In the lungs of WT mice, we found that the amount of α_1-adrenergic and muscarinic receptors was sex-dependent. Densities of the former were higher in females and those of the latter were higher in males. There was no difference between males and females in β-adrenergic receptor density. As for CRH KO mice, the basal densities of studied receptors were lower than in WT mice (except β_1-adrenergic receptors in females).

The main purpose of the thesis was to detect immobilization-induced changes in the studied receptors in the control (WT) and CRH KO mice. Short-term and long-term immobilization caused decrease in all α_1-adrenergic receptor subtypes in females, whereas only α_{1A}-adrenergic receptors decreased in males. The amount of β_1-adrenergic receptors decreased in males and remained without change in females while β_2-adrenergic receptors decreased in both sexes equally. Decrease of the receptors after the stress exposure was more striking in WT mice than in those with disrupted CRH gene. Moreover, the decrease of the receptors in CRH KO males was less prominent than in CRH KO females. Adenylyl cyclase activity was decreased in WT mice after the immobilization, the PLC activity in WT mice did not change. In CRH KO mice there was no change in AC and PLC activity.

Futhermore, we detected immobilization-induced changes in expression and amount of the receptors in the rat and murine hearts.

1. ÚVOD

1.1 Zastoupení receptorů pro transmitery autonomního nervového systému v plicích

Správná funkce respiračního systému je pro zachování života zcela nezbytná. Ačkoliv existují vnitřní mechanismy, které determinují rytmické změny zodpovědné za dechovou automacii (jedná se o pacemakerovou aktivitu specializovaných buněk), akutní změny plicních funkcí jsou kontrolovány prostřednictvím autonomního nervového systému. Tato část nervového systému zprostředkovává své účinky cestou dvou téměř antagonisticky působících neurotransmiterových systémů: cestou sympatických a parasympatických nervů. Oba tyto systémy působí na úrovni orgánů prostřednictvím receptorů spřažených s G proteiny. Neurotransmitery sympatického oddílu aktivují adrenergní receptory, parasympatický oddíl účinkuje přes receptory muskarinové.

Adrenergní receptory mohou být rozděleny na α- a β-adrenergní (Ahlquist 1948), které se vyskytují v mnoha podtypech: α-adrenergní receptory se dělí na α_1- a α_2-adrenergní receptory, které se dále dělí na následující podtypy (Guimaraes and Moura 2001): α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B} a α_{2C}.

β- adrenergní receptory mohou být rozděleny na β_1-, β_2- a β_3-adrenergní receptory (Lohse, Engelhardt et al. 2003). Muskarinové receptory se vyskytují v následujících podtypech: M_1, M_2, M_3, M_4 a M_5 podtyp (Wess, Duttaroy et al. 2003). Strukturálně všechny tyto receptory patří do rozsáhlé rodiny receptorů spřažených s G proteiny. Tyto receptory jsou bílkovinné molekuly sedmkrát procházejících cytoplasmatickou membránou. Vazba příslušného specifického transmiteru vede ke konformační změně receptorové bílkoviny, následuje zvýšení afinity receptoru ke G proteinu, štěpení GTP a následná aktivace/inhibice dalších intracelulárních poslů jako jsou např. enzymy adenylátcykláza (AD), fosfolipáza C (PLC).

Všechny tyto změny vedou ke změně koncentrace nitrobuněčných druhých poslů jako např. cyklický adenosinmonofosfát (cAMP), diacylglycerol (DAG) a inositoltrifosfát (IP_3). Tyto molekuly poté ovlivňují řadu nitrobuněčných regulačních a funkčních kaskád od akutních změn v propustnosti cytoplasmatické membrány až po ovlivnění genové exprese: např. cestou AP-1 (activator protein 1), CREB (cAMP response element binding protein) či mnoha dalších molekul.

1.1.1 Adrenergní receptory v plicích

Na úrovni plic rozlišujeme tkáň dýchacích cest, jejichž vlastnosti, především průměr, mohou být ovlivněny prostřednictvím muskarinových i adrenergních receptorů a tkáň plicních alveolů, kde dochází k vlastní výměně plynů.

1.1.1.1 α-adrenergní receptory v plicích

α-adrenergní receptory se v plicích vyskytují v zastoupení podtypů α_{1A}, α_{1B}, α_{1D} (Zhong and Minneman 1999). Všechny tyto podtypy aktivují G protein z rodiny $G_{q/11}$. Následně dochází k aktivaci enzymu fosfolipáza C (PLC), který konvertuje fosfatidylinositol na inositoltrifosfát a diacylglycerol. Obě tyto molekuly působí jako druzí poslové v nitrobuněčné signalizaci.

Nicméně kolem existence všech tří podtypů α_1-adrenergních receptorů v plicích nadále panují jisté pochybnosti. Některým autorům (Yang, Reese et al. 1998) se podařilo v plicní tkáni myší prokázat pouze podtypy α_{1A} a α_{1B}, ovšem nikoliv podtyp α_{1D}. Hiramatsu a jeho tým (Hiramatsu, Muraoka et al. 1994) detekovali v roce 1994 v plicní tkáni potkana mRNA pro všechny 3 podtypy α_1-adrenergních receptorů. Obdobně i Faure et al.(Faure, Pimoule et al. 1994) a Alonso-Llamazares et al. (Alonso-Llamazares, Zamanillo et al. 1995) identifikovali mRNA pro všechny tři podtypy α_1-adrenergních receptorů včetně α_{1D} podtypu v plicích u potkana.

Obecně je přijímána role α_1-adrenergních receptorů v plicích a jejich podíl na řízení ventilačních funkcí jako minoritní až zanedbatelná. Některé studie však dokumentovaly roli nízkoafinních vazebných míst pro prazosin odpovídajících α_1-adrenergním receptorům (α_{1L}-adrenergní receptory) v procesu alergické bronchokonstrikce (Nobata, Fujimura et al. 2002). Zde je důležité, že ligand (JTH-601) použitý v této studii vykazuje vysokou afinitu k α_{1A} a α_{1B}-adrenergním receptorům a velice nízkou afinitu k α_{1D}-adrenergním receptorům (Takahashi, Taniguchi et al. 2000).

Některé studie naznačují, že tkáně plic obsahují i α_2-adrenergní receptory (Calzada and De Artinano 2001).

1.1.1.2 β-adrenergní receptory v plicích

Funkce a distribuce β-adrenergních receptorů v plicích byla intensivně studována Amrahamem a kol. (Abraham, Kottke et al. 2003). Hlavní závěry této studie byly následující: a) počet β-adrenergních vazebných míst je vyšší v plicích než v tkáni bronchů a trachey, b) ve všech oddílech výrazně převažují β_2-adrenergní receptory. β_2-adrenergní receptory jsou zastoupeny především v hladkých svalových buňkách, kde jsou zodpovědné za bronchodilataci, ale jsou současně exprimovány také v oblasti alveolů. V alveolech se vyskytuje i další podtyp adrenergních receptorů: β_1-adrenergní receptory (Mak, Nishikawa et al. 1995). Poměr β_1/β_2-adrenergních receptorů vykazuje mezidruhové rozdíly. Pro příklad, u prasat činí 58:42 (Liang and Mills 2001), u myší 14: 86 (Ota, Matsui et al. 1993), u koní 20-26/74-80 (Abraham, Kottke et al. 2003), což dále závisí také na typu tkáně (trachea, bronchiální tkáň, aleveoly). V dýchacím traktu je β_2-adrenergní receptory možno nalézt i na epiteliálních buňkách (Aksoy, Mardini et al. 2002). U prasat byly identifikovány mRNA pro $\beta_1/\beta_2/\beta_3$ v poměru 3.8:1.9:0.1 z celkové RNA, v plicní tkáni potkana to bylo 2.5:3.8, nicméně v dalších studiích β_3 mRNA nalezena nebyla (McNeel and Mersmann 1999). Příčinou

hyperreaktivity dýchacích cest může být i funkční disbalance způsobená nepoměrem jednotlivých podtypů β-adrenergních receptorů, podobně jako je tomu při nepoměru v zastoupení muskarinových receptorů. Celkové množství adrenergních receptorů se zvyšuje a procento β-adrenergních receptorů odpovídající vysokoafinitnímu vazebnému místu pro agonistu se naopak snižuje vlivem zrušení vazby receptoru s G_s (Emala, Clancy et al. 1997).

1.1.2 Muskarinové receptory v plicích

Muskarinové receptory jsou v dýchacím systému reprezentovány M_1, M_2 a M_3 podtypy. Aktivace všech těchto podtypů vede v konečném důsledku k bronchokonstrikci, nicméně poněkud odlišným mechanismem. Zatímco aktivace muskarinových receptorů patřících k podtypu M_3 vyvolává jejich kontrakci, inhibice M_2 podtypu je zodpovědná za uvolnění acetylcholinu a relaxaci hladkých svalových buněk dýchacích cest (Fryer and Jacoby 1998). Nesmírně důležitým faktem je, že změny v poměrném zastoupení v expresi M_1 a M_2 podtypu by mohly hrát klíčovou roli ve zvýšené reaktivitě dýchacích cest (Kadota, Kuwahara et al. 2001). V hladké svalovině trachey je poměrné zastoupení M_1 a M_2 podtypu muskarinových receptorů prakticky vyvážené, v oblasti alveolů však značně dominuje podtyp M_3 (Kadota, Kuwahara et al. 2001). Zatímco M_2 muskarinové receptory jsou zodpovědné za inhibici relaxace vyvolané aktivací β adrenergních receptorů, aktivace M_3 muskarinových receptorů vede přímo ke kontrakci hladkých svalových buněk bronchů (Fryer and Jacoby 1998). Obdobně jako β-adrenergní receptory mohou být tyto receptory nalezeny na epiteliálních buňkách (Fryer and Jacoby 1998), což by mohlo nasvědčovat, že i na úrovni těchto buněk by mohl existovat funkční antagonismus. V epiteliálních buňkách je zastoupen především M_1 podtyp muskarinových receptorů.

Exprese a popřípadě funkce dalších podtypů muskarinových receptorů (M_4) v tkáních plic je nejistá (Bymaster, McKinzie et al. 2003), (Stengel, Gomeza et al. 2000) a (Stengel and Cohen 2002).

1.1.3 Regulace plicních funkcí receptory ANS

Shrneme-li funkční aspekty, na procesu bronchodilatace se podílejí receptorové systémy aktivující Gs protein a dále adenylátcyklázu, především β_2-adrenergní receptory. Adenylátcykláza prostřednictvím zvýšení koncentrace cAMP aktivuje proteinkinázu A (PKA) a dochází k disociaci katalytické podjednotky této kinázy od podjednotky regulační. Katalytická podjednotka je schopna fosforylovat cílové struktury v hladké svalové buňce klíčové pro její relaxaci (Hall 2000) a dojde tedy k bronchodilataci. M_2 muskarinové receptory jsou zodpovědné za inhibici relaxace vyvolané právě aktivací β_2 adrenergních receptorů.

Aktivací receptorů signalizujících přes PLC, tj. M_1 a M_3 muskarinové receptory a α_1-adrenergní receptory, dochází ke zvýšení intracelulární koncentrace Ca^{2+} prostřednictvím IP_3 a tento děj v konečném důsledku vede ke kontrakci hladkých svalových buněk a bronchokonstrikci (Hall 2000).

Jak již bylo zmíněno, hlavní pozornost byla vždy věnována především vlastnostem a funkci β-adrenergních receptorů a podrobnosti funkce α_1-adrenergních receptorů v plicích jsou nadále nejasné. Výsledky zabývající se rolí α_1-adrenergních receptorů v patofyziologii astma bronchiale je v tuto chvíli nadále obtížné interpretovat (Goldie, Paterson et al. 1990). Aktivace α_1-adrenergních receptorů může vest ke kontrakci hladkých svalových buněk bronchiální stěny, ale pouze za specifických podmínek: bronchokonstrikce indukovaná fenylefrinem může být demonstrována pouze u astmatiků s předchozí blokádou β-adrenergních receptorů (Goldie, Paterson et al. 1990). Při absenci tohoto predisponujícího faktoru podání fenylefrinu vyvolá bronchodilataci. Je nutné zmínit, že u zdravých jedinců netrpících astmatem efekt podání fenylefrinu na hladké svalstvo dýchacích cest není přítomen. Preuss a kol. v roce 1998 pozoroval, že schopnost kontrakce hladkých svalových buněk ve stěně trechey jako odpověď na podání α_1-adrenergních agonistů je závislá na

anatomické lokalizaci: v horních oddílech trachey je kontrakce výraznější než v dolních oddílech (Preuss, Rigby et al. 1998). Goldie a kol. prokázal (Goldie, Paterson et al. 1990), že vzájemný poměr β-adrenergních receptorů a α_1-adrenergních receptorů se mění při astma bronchiale/alergiích. Za fyziologických okolností je poměr β-adrenergních receptorů a α_1-adrenergních receptorů u člověka 6:1, ale za patologických podmínek klesá na 1:1. Stejná situace nastává i v plicích morčat (Goldie, Paterson et al. 1990).

Z těchto skutečností vyplývá, že rozhodující je vzájemný poměr a funkční vyváženost α_1-adrenergních receptorů a β-adrenergních receptorů, což hraje roli především za patologických okolností jako např. při astma bronchiale. Možným mechanismem vzájemného ovlivnění je potenciace funkcí zprostředkovaných β-adrenergními receptory aktivací α_1-adrenergních receptorů, což se domnívají někteří autoři (Nousiainen, Arnala et al. 1977; Goldie, Paterson et al. 1990), dále (Thiele, Nemergut et al. 2011).

Antagonistické působení muskarinových a adrenergních receptorů na plicní funkce je tedy více než pravděpodobné (Johnson 1998).

1.2 Zastoupení receptorů pro transmitery autonomního nervového systému v srdci

Správná regulace srdečních funkcí je jednou ze základních podmínek pro přežití organismu. Adekvátně fungující regulační mechanismy jsou schopné zajistit za podmínek co nejnižší energetické náročnosti maximálně efektivní a včasný funkční výstup.

Hlavními regulátory srdečních funkcí jsou dva typy receptorů: β-adrenergní receptory (BAR) a muskarinové receptory (MR), které mají prakticky protichůdné účinky na srdeční funkce. V srdci jsou zastoupeny jak majoritní podtypy, tak v menší míře i podtypy minoritní, které nejspíše přispívají k jemnějšímu doladění regulačních mechanismů.

1.2.1 Adrenergní receptory v srdci

Schématické zobrazení funkcí adrenergních receptorů v srdeční fyziologii je na obrázku 1. Jsou na něm zobrazeny všechny adrenergní receptorové podtypy s nejdůležitějšími intracelulárními signalizačními cestami.

1.2.1.1 α-adrenergní receptory v srdci

V srdci se kromě majoritně zastoupených β-adrenergních receptorů vyskytují i α-adrenergní receptory. Jedná se především o α_1-adrenergní podtyp (Brodde O.E. and M.C. 1999), ale v malé míře také α_2-adrenergní podtyp.

Ačkoliv u lidí jsou v buňkách myokardu α_1-adrenergní exprimovány výrazně méně než β-adrenergní receptory, u hlodavců, především potkanů, je tomu naopak. Navzdory malému zastoupení α_1-adrenergních receptorů v kardiomyocytech, není jejich význam zanedbatelný. Z funkčního hlediska jsou α_1-adrenergní receptory schopny zvyšovat srdeční ionotropii a současně zvyšovat citlivost myokardiálních myofilament k Ca^{2+}. Vedle těchto krátkodobých efektů, může dlouhodobá stimulace zprostředkovaná aktivací α_1-adrenergních receptorů indukovat změny charakteru hypertrofie, jak bylo prokázáno na potkaních srdcích (Zimmer 1997), (Noguchi H 1993). Nutné je ovšem pamatovat, že tyto změny byly prokázány pouze u hlodavců a nelze je extrapolovat na lidskou fyziologii.

α_1-adrenergní receptory patří do rodiny receptorů spřažených s G proteiny, v tomto případě se jedná o Gq/11 (viz tabulka 1). Následné kroky intracelulární signalizační kaskády jsou aktivace fosfolipázy C (PLC) a zvýšení intracelulární koncentrace diacylglycerolu (DAG) a inozitoltrifosfátu (IP_3). Proteinkináza C (PKC) a IP_3 a přenášejí signál na další intracelulární struktury. Aktivace PLC představuje pouze jednu z cest přenosu signálu při aktivaci α_1-adrenergního receptoru v kardiomyocytech. Spuštěna může být i signalizační dráha zahrnující aktivaci fosfolipázy D (PLD), což vede z ovlivnění L-typu Ca^{2+} kanálů a

změně membránového potenciálu a další aktivaci napěťově řízených membránových receptorů. (Endoh, Hiramoto et al. 1991). Dále může být aktivována i Na^+/H^+ pumpa a Na^+/K^+- ATPáza (Endoh, Hiramoto et al. 1991).

Role α_2-adrenergních receptorů v srdci je především modulační, nepůsobí zde jako primární cílové struktury pro přímý účinek katecholaminů. Aktivace α_2-adrenergních receptorů vede k inhibici presynaptického uvolnění noradrenalinu na synapsích. Všechny podtypy α_2–adrenergních receptorů vykazují po vazbě specifického transmiteru děje vedoucí k inhibici adenylátcyklázy a tedy v konečném důsledku snížení intracelulárního cAMP.

1.2.1.1.1 Nízkoafinní stav α_1 – adrenergních receptorů v srdci

Nejprve byla vazebná místa, charakterizovaná jako α_{1L}-adrenergní receptory, detekována farmakologicky a předpokládalo se, že se jedná o nový podtyp α_1-adrenergních receptorů s nízkou afinitou k prazosinu (Muramatsu 2006). Na základě funkčních studií bylo prokázáno, že tento podtyp vyvolává kontrakci v oblasti dolních močových cest u člověka, ale podařilo se ho identifikovat i v srdci (Maruyama, Nakamura et al. 1998). Nakonec bylo rozhodnuto na základě experimentů s klonovanými α-adrenergními receptory, že se nejedná o nový podtyp α-adrenergních receptorů, ale pouze o nízkoafinní stav α_1-adrenergních receptorů (Ford, Daniels et al. 1997). Nadále ovšem o tomto závěru existují pochybnosti – farmakologický profil těchto receptorů se poněkud liší od profilu membránových α_1-adrenergních receptorů, ale spíše se zdá, že by se mohlo jednat o funkční izoformu α_1-adrenergních receptorů než o samostatný podtyp (Marti, Miquel et al. 2005) a v poslední době je ověřována hypotéza, zda by se mohlo jednat o výsledek dimerizace α_1-adrenergních receptorů. Důležitým zjištěním v této oblasti je, že α_1-adrenergní receptory mohou tvořit homo- i heterodimery, ovšem tyto komplexy nevykazují sníženou afinitu k prazosinu (Ramsay, Carr et al. 2004).

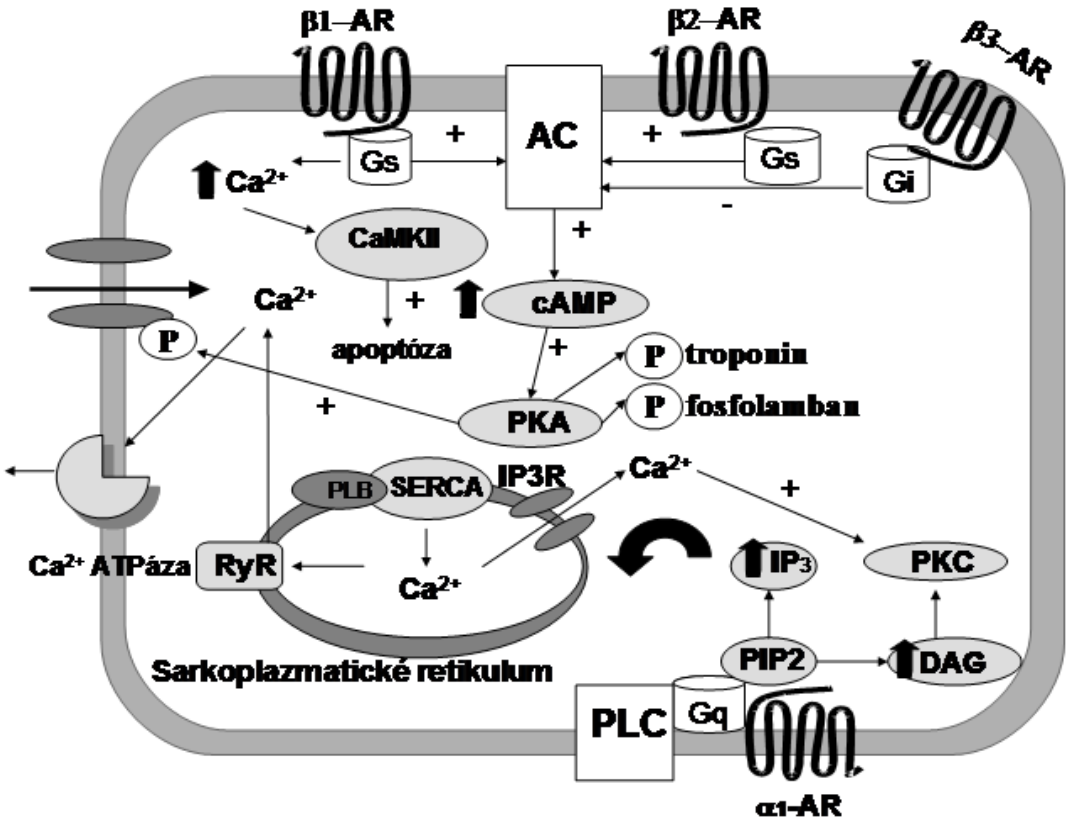
1.2.1.2 β – adrenergní receptory v srdci

Srdeční buňky exprimují β_1 a β_2 podtyp adrenergních receptorů, ovšem relativní množství β_1-adrenergních receptorů je mnohem vyšší než množství podtypu β_2-adrenergních receptorů. Oba podtypy stimulují adenylátcyklázu (AC). Hlavní funkcí β-adrenergních receptorů v srdci je působit pozitivně ionotropně a chronotropně (Brodde O.E. and M.C. 1999), (Kaumann and Molenaar 1997). Další důležitou funkci β-adrenergních receptorů (především receptorů β_1-adrenergních) představuje stimulace Ca^{2+} kanálů *via* proteinkináza A (PKA) nebo přímým navázáním G proteinu ($G_s\alpha$). Výskyt dalších podtypů β-adrenergních receptorů, β_3-adrenergních receptorů a jejich mRNA byl popsána již dříve v srdečních síních i komorách (Gauthier, Tavernier et al. 2000), kde zprostředkovávaly kardioinhibiční působení cestou G_i proteinu. Jejich existence však také byla řadou autorů zpochybněna (Kaumann and Molenaar 1997). Další významnou skutečností je, že exprese β_3-adrenergních receptorů vykazuje značnou mezidruhovou variabilitu. Také nám se podařilo identifikovat mRNA pro β_3-adrenergní receptor v jednotlivých srdečních oddílech u laboratorního potkana (včetně odlišení částí obsahujících ganglia převodního systému srdečního od částí negangliových; (Myslivecek, Novakova et al. 2006).

Současná představa regulace na úrovni srdečních receptorů předpokládá vzájemné regulační propojení buněčných funkcí prostřednictvím různých receptorových podtypů. Pro tuto teorii svědčí např. existence jednak adrenergních podtypů, které mohou působit kardiostimulačně (β_1 a β_2-adrenergní receptory) a současně podtypů, které mají kardioinhibiční účinky (β_3-adrenergní receptory). Tato problematika byla před nedávnem dopodrobna zpracována (Gauthier, Tavernier et al. 2000), (Brodde O.E. and M.C. 1999) a(Granneman 2001)).

Posledním podtypem β-adrenergních receptorů, který byl v srdci identifikován, je tzv. čtvrtý podtyp. Je označován také jako atypický kardiostimulační β-adrenergní receptor a dříve byl považován za samostatný podtyp, β_4-adrenergní receptor (Kaumann 1997). Toto vazebné

místo vykazuje zcela jedinečné vazebné charakteristiky a je nyní považováno za atypický vysokoafinitní stav β_1-adrenergního receptoru (Granneman 2001). Změny u "propranolol/bupranolol-insensitivní formy β_1-adrenergního receptoru" (Brodde O.E. and M.C. 1999) vykazují velmi podobné charakteristiky, jaké byly prokázány při sledování β_1-adrenergních receptorů. Nicméně za určitých podmínek je možné spekulovat, jak jsme prezentovali nedávno (Myslivecek, Ricny et al. 2003), že β_4-adrenergní receptory se při podávání hydrokortizonu mění vzhledem k β_1-adrenergním receptorům recipročně.



Obrázek 1: Schématické znázornění hlavních signalizačních kaskád zprostředkovaných aktivací adrenergních receptorů v srdci
AC – adenylátcykláza, DAG – diacylglycerol, Gs, Gi, Gq – G proteiny, IP3 – inozitol 1,4,5-trifosfát, IP3R – IP3 receptor, PKA - proteinkináza A, PKB - proteinkináza B, PKC – proteinkináza C, PLC – fosfolipáza C, PIP2 – fosfoinozitol 4,5-difosfát, RyR – ryanodinreceptor, PLB fosfolamban, SERCA – sarkoplazmatické retikulum kalcium ATPáza, CaMKII – calmodulin-dependentní kináza II, PI3K – fosfatidylinositol 3-kináza

1.2.1.3 Rozdílná afinita adrenergních receptorů ke katecholaminům

Funkční výstup vyvolaný aktivací receptoru závisí na více skutečnostech. Nejdůležitější je relativní množství daného typu receptoru na buňkách příslušné tkáně, dále afinita receptoru k příslušnému transmiteru a v neposlední řadě schopnost receptoru aktivovat intracelulární signalizační kaskádu. Chceme-li zhodnotit efekt aktivace receptoru v dané tkáni, musíme vzít v úvahu všechny tyto body, jinak by v důsledku zjednodušení mohlo dojít k významnému zkreslení výsledků (Kenakin 2004).

Hodnotíme-li tedy účinek katecholaminů zprostředkovaný srdečními nebo plicními adrenoceptory musíme vzít v potaz následující fakta:

1) Relativní množství daných receptorů v příslušné tkání včetně region-specifických rozdílů. Například: β_1-adrenergní receptory jsou v kardiomyocytech více zastoupeny než β_2-adrenergní a dále jsou výrazně početnější než α_1-adrenergní a β_3-adrenergní receptory (Brodde O.E. and M.C. 1999). Nicméně v některých srdečních oblastech je relativní množství β_2-adrenergních receptorů výrazně vyšší než β_1-adrenergních (Myslivecek, Novakova et al. 2006).
2) Specifická afinita receptoru k ligandu. Existují významné rozdíly v afinitě adrenergních receptorů k adrenalinu a noradrenalinu. Například: β_2-adrenergní receptory mají vyšší afinitu k adrenalinu než k noradrenalinu, viz tabulka 1.
3) Receptorová funkce. Adrenergní receptory mají rozdílný vliv na srdeční fyziologii, aktivace β_3-adrenergních receptorů působí kardioinhibičně, aktivace β_1-adrenergních receptorů kardiostimulačně. Některé podtypy mají komplexnější funkce, např. α_1-adrenergní receptory.

Receptor	adrenalin	noradrenalin	G protein	2. posel
α_{1A}	0.3	1.2-2.8	G_q/G_{11}	IP_3/Ca^{2+}; DAG
α_{1B}	6	10	G_q/G_{11}	IP_3/Ca^{2+}; DAG
α_{1D}	0.016	0.012	G_q/G_{11}	IP_3/Ca^{2+}; DAG
α_{2A}	8.3-5.6*	8.4-5.6*	G_i/G_o	Snížení cAMP
α_{2B}	6.2-5.2*	9.1-5.6*	G_i/G_o	Snížení cAMP
α_{2C}	6.2-5.8*	8.7-5.9*	G_i/G_o	Snížení cAMP
β_1	1.0 0.0027#	0.5 0.0008#	Gs	Zvýšení cAMP
β_2	0.9±0.7 0.0022#	10±6 (9.7) 0.036#	Gs	Zvýšení cAMP
β_3	4.7-3.9* 0.049#	3.8-5.3* 0.0063#	Gi Gs	Snížení cAMP Zvýšení cAMP

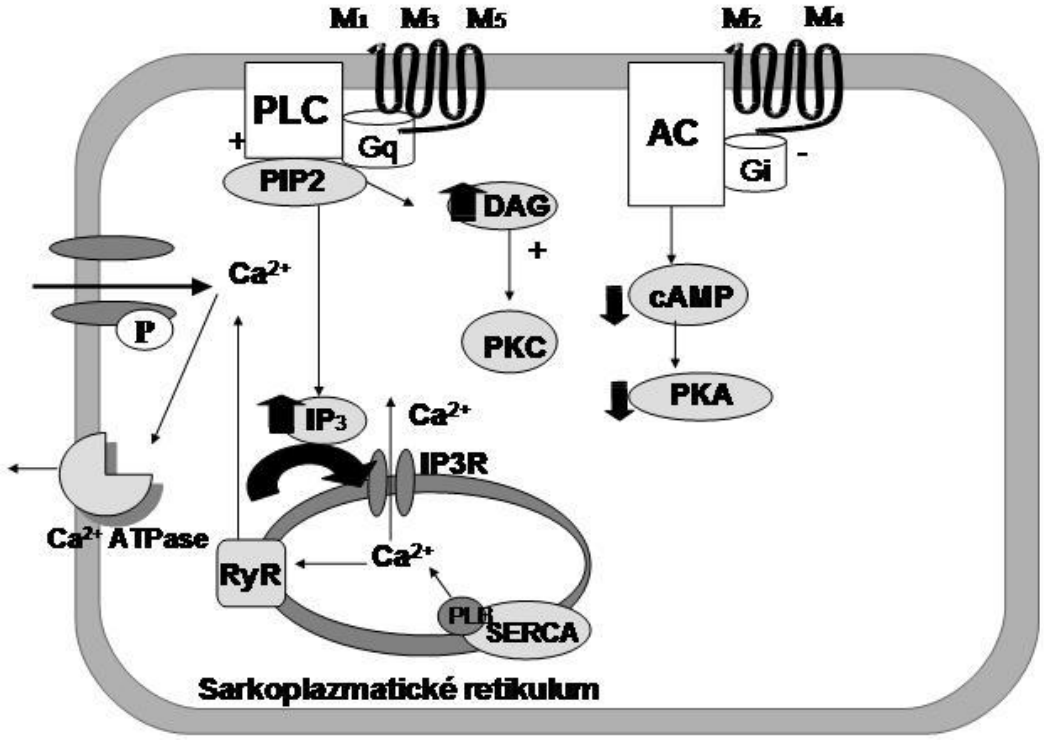
Tabulka 1: Vlastnosti adrenergních receptorů
Afinita adrenergních receptorů k adrenalinu a noradrenalinu vyjádřená hodnotou EC50 (□mol.l-1) nebo pKi. (Bylund, Eikenberg et al. 1994), (Skeberdis 2004) a IUPHAR receptor databasis. (Germack and Dickenson, 2006).

1.2.2 Muskarinové receptory v srdci

Muskarinové receptory jsou v myokardu savců reprezentovány především podtypem M_2 (Brodde O.E. and M.C. 1999). Funkce zprostředkované MR jsou antagonistické k funkcím vyvolaným aktivací β-adrenergních receptorů (tj. negativně ionotropní a chronotropní). M_2 receptory jsou spřaženy s G_i proteiny (inhibují AC), ale výrazná aktivace těchto receptorů vede k interakci s G_q proteinem a následné aktivaci fosfolipázy C (PLC): (Dhein, van Koppen et al. 2001). Dalším významným dopadem aktivace MR je ovlivnění prostupnosti membrány pro ionty a to cestou aktivace/inhibice příslušných iontových kanálů (viz Obrázek 2). Aktivace M_2 muskarinových receptorů inhibuje Ca^{2+} kanály nepřímo cestou snížení produkce cAMP a tudíž snížené aktivity PKA; hlavním výsledkem aktivace muskarinových receptorů na úrovni iontových kanálů je ale aktivace acetylcholin-senzitivních draslíkových kanálů (K_{ACh}). Aktivace tohoto kanálu vede k hyperpolarizaci buněk myokardu a působí na svalovinu síní negativně ionotropně. Dále se tyto kanály podílejí také na negativně chronotropním působení. Jiná situace je v oblasti pacemakeru: SA uzel (sinoatriální uzel), kde negativně

chronotropní efekt je vyvolán muskarinovými receptory zprostředkovanou inhibicí adenylátcyklázy.

Představa, že M_2 receptory nejsou jediným podtypem MR vyskytujícím se v srdci je široce podporován, ale potíže s identifikací a kvantifikací zatím nebyly uspokojivě vyřešeny. Mezi všechny non-M_2 receptory identifikované farmakologicky a/nebo elektrofyziologicky v srdci do současné doby (M_1, M_3, M_4, (Brodde O.E. and M.C. 1999), jsou M_1 a M_3 podtypy (kardiostimulační, působící přes Gq/PLC) zatím zdokumentovány nejlépe (detekce M_1 mRNA pomocí RT-PCR (real time polymerase chain reaction)), (Colecraft, Egamino et al. 1998)), ačkoliv nedávné poznatky tuto hypotézu zpochybňují (Hamilton, Hardouin et al. 2001). Pro podrobnosti týkající se výskytu M_3 podtypu muskarinových receptorů v srdci (Wang, Shi et al. 2004). Pokoušeli jsme se identifikovat minoritní podtypy v srdci u laboratorního potkana a podařilo se nám detekovat populaci pravděpodobně odpovídající M_1 muskarinovým receptorům (Myslivecek, Klein et al. 2008).



Obrázek 2: Schématické znázornění hlavních signalizačních kaskád zprostředkovaných aktivací muskarinových receptorů v srdci
AC – adenylátcycláza, DAG – diacylglycerol, Gs, Gi, Gq – G proteiny, IP3 – inozitol 1,4,5-trifosfát, IP3R – IP3 receptor, PKA - proteinkináza A, PKB - proteinkináza B, PKC – proteinkináza C, PLC – fosfolipáza C, PIP2 – fosfoinozitol 4,5-difosfát, RyR – ryanodinreceptor, PLB fosfolamban, SERCA – sarkoplasmatické retikulum kalcium ATPáza, CaMKII – calmodulin-dependentní kináza II, PI3K – fosfatidylinositol 3-kináza

1.3 Receptorové regulace

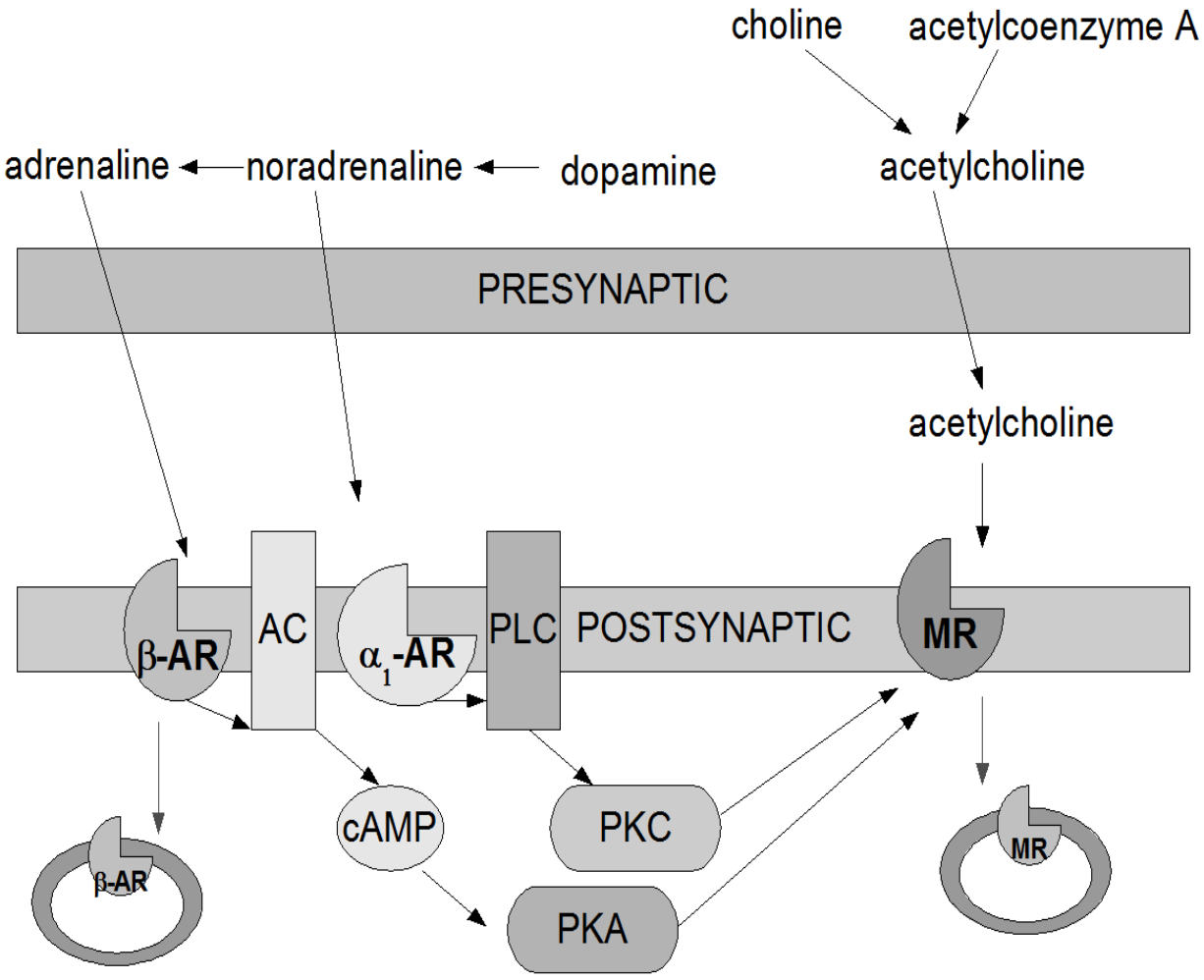
Receptory jsou vysoce dynamické proteinové struktury, které jsou schopny měnit úroveň intensity přenášeného signálu v různých oddílech v řádu sekund. Senzitivita k extracelulárním působkům může být modifikována nejen jednoduše jako důsledek masivní aktivace příslušného receptorového typu (homologní regulace, viz (Morris and Malbon 1999)), ale také aktivací nebo inhibicí dalších receptorů exprimovaných v buňce (heterologní regulace, viz (Selbie and Hill 1998); (Bunemann and Hosey 1999) a (Cordeaux and Hill 2002)). Nepřímé

důsledky vyvolané aktivací receptoru mohou být chápány jako "jemné dolaďování" receptorem zprostředkovaného signálu (Selbie and Hill 1998) a (Cordeaux and Hill 2002).

Heterologní regulace receptorů spřažených s G proteiny (GPCR) je proces, při němž proteinkinázy (PKA, PKC) „označí", nafosforylují, všechny receptory bez ohledu na to, jsou-li obsazeny agonistou anebo jsou-li volné. Tato skutečnost zásadně odlišuje heterologní a homologní regulaci (zprostředkovávána cestou GRKs, kinázy receptorů spřažených s G proteiny). Heterologní regulace může v konečném důsledku zesilovat účinek signálu přenášeného označeným receptorem či tento účinek naopak zeslabovat Zkřížená regulace je dobře dokumentována mezi receptory spřaženými s G_i- and G_q-proteiny a G_s- and G_q-proteiny viz (Cordeaux and Hill 2002, Hur and Kim 2002). Konečně je pravděpodobné, že nikoliv pouze fosforylace receptorů, ale také fosforylace $G\alpha_i$ proteinu by mohla hrát určitou roli v regulaci inhibičního působení G_i proteinu. Fosforylace GPCRs prostřednictvím receptorů s vnitřní tyrosinkinázovou aktivitou je další formou tzv "jemného dolaďování" signálu přenášeného GPCRs (Selbie and Hill 1998; Cordeaux and Hill 2002; Hur and Kim 2002). Analogicky (Morris and Malbon 1999) může být jako zkřížená regulace chápána jednak posttranslační regulace (fosforylace proteinů, tj.desensitizace), posttranskripční regulace (destabilizace mRNA, tj. down-regulace) a také transkripční regulace (kdy jde o složitou regulaci označených, fosforylovaných, genů).

Mechanismus heterologní regulace není v současné době zcela přesně znám. V mnoha případech je heterologní regulace mezi receptory exprimovanými v buňkách kardiovaskulárního systému studována za použití buněk se stálou expresí receptorů pro neurotransmitery autonomního nervového systému (Bunemann and Hosey 1999). Toto použité zjednodušení si však vynucuje otázku, do jaké míry výsledky získané na takto upravené tkáni odpovídají fyziologickým podmínkám.

Existence heterologní regulace mezi adrenergními a muskarinovými receptory již byla demonstrována mnohokrát (pro přehled (Cordeaux and Hill 2002; Hur and Kim 2002) a (Myslivecek and Trojan 2003). Několikrát bylo prokázáno, že v srdci jsou tyto receptory ve vzájemné funkční vazbě (J.Myslivecek 1996; Myslivecek 1996; Myslivecek 1998; Myslivecek and Trojan 2000; Myslivecek 2003). Nicméně mechanismům této vzájemné regulace rozhodně nebyla doposud věnována dostatečná pozornost



Obrázek 3: Schéma možných receptorových regulací včetně předpokládané aktivace receptorů vlivem zvýšených hladin cirkulujících katecholaminů. Podle (Myslivecek, Tillinger et al. 2008).

1.4 Vliv stresu v plicích a srdci, význam vyřazení CRH pro stresovou reakci

Pojem stres byl poprvé použit v třicátých letech dvacátého století Hansem Seleym (Seley 1936) a to, jak uvádí sám Seley spíše neznalostí přesné anglické terminologie (stress, tj. tlak, napětí vs. strain, tj. nápor, zátěž). Jde o slovo, které je v současnosti nadužíváno a velmi často řečník, který toto slovo užívá, jeho přesný význam nezná. Na druhou stranu přesná definice stresu neexistuje. V nejméně rigidní definici je stres vše, co vede ke vzniku jakékoliv nemoci u člověka. Obecně lze chápat stres dvěma způsoby: jednak (v rámci Seleyho konceptu) jako nespecifickou odpověď organismu, jednak jako specifickou odpověď, která je specifická v závislosti na typu stimulu (Pacak and Palkovits 2001). Koncept Pacáka a Palkovitse je dnes chápán jako odpovídající skutečnosti.

1.3.1 Stres, stresory

Zjednodušeně lze říci, že stres je situace, v níž je organismus ovlivňován stresorem (tedy stresovým podnětem). To vyvolává změny v organismu, které vedou ke změnám výstupních veličin organismu. V tomto kontextu podává zajímavý pohled na stresovou reakci Mravec (Mravec 2008), který popisuje stres jako specifický „reflexní oblouk“. Tak se lze na stresovou reakci dívat jako na reflexní reakci či chování, kde je *aferentace* reprezentována aktivací receptoru(-ů), *centrum* je komplikovaná neuronální síť mezi specifickými oblastmi CNS (které zahrnují emoční, neuroendokrinní, neuronální integraci) a *efektor* (eferentace) jsou systémy aktivované s cílem udržet homeostázu.

Stresory, dle své povahy, mohou být děleny do následujících skupin (Myslivecek and Kvetnansky 2006) (viz též Tabulka 2):

1) fyzikální,
2) chemické,
3) psychologické

4) sociální,

5) porušující kardiovaskulární/metabolickou rovnováhu,

6) ovlivňující více systémů.

Stresorová skupina	**Typ stresoru**	**Stresorová skupina**				
		fyzikální	chemické	psychologické	sociální	kardiovaskulární
fyzikální	**chlad**					✓
	teplo		✓			✓
	Intenzivní radiace					
	hluk			✓		
	vibrace					✓
chemické	**hypoglykémie**					✓
	Oxidativní stres	✓				
	otravy	✓				
	hypoxie					✓
psychologické	**Omezení příjmu potravy**		✓			
	Spánková deprivace					
	Emoční stres				✓	
	deprese				✓	
sociální	**tlačenice**			✓		
	izolace			✓		
	Umístění do teritoria dominantního jedince	✓		✓		
porušující kardiovaskulární/metabolickou rovnováhu	**krvácení**	✓	✓			
	cvičení			✓		
	orthostáze	✓				
	Svislý sklon	✓				
Ovlivňující více systémů	**Imobilizace**	✓		✓	✓	✓
	Omezení pohybu	✓		✓	✓	✓
	bolest	✓	✓	✓		
	Potravová a vodní deprivace	✓	✓	✓		✓
	Změny v režimu světlo/tma	✓		✓		
	Vynucené plavání	✓		✓		✓

Tabulka 2: Typy stresorů. Nejsou uvedeny všechny typy stresorů, v případě stresorů ovlivňujících více systémů jsou uvedeny příslušné hlavní účinky. Lze si všimnout, že žádný stresor neovlivňuje pouze jeden systém (podle Myslivečka a Kvetňanského, 2006).

Ve vztahu k trvání může stresor ovlivňovat organismus buď akutně (někdy se hovoří o akutním, jednorázovém, intermitentním stresu) či chronicky/opakovaně (pak hovoříme o chronickém, opakovaném či dlouhodobém stresu).

Jak je vidět z Tabulky 2, neexistuje stresor, který by ovlivňoval organismus pouze jedním způsobem. Proto mohou být v experimentálním stresu používány pouze stresory, které ovlivňují více systémů.

1.3.2 Vliv stresu v plicích

Opakovaně bylo prokázáno, že stres ovlivňuje imunitní funkce (viz předchozí kapitola) a nejinak je tomu i v plicích (Kanemi, Zhang et al. 2005). Nicméně změny podtypů adrenergních receptorů a muskarinových receptorů vlivem stresu nebyly dosud do hloubky studovány. Právě s ohledem na vlastnosti adrenergních receptorů představuje navození stresu významný výzkumný prostředek, jelikož se při stresu zvyšuje uvolňování noradrenalinu a adrenalinu (Pacak and Palkovits 2001). Hlavním zdrojem cirkulujícího adrenalinu během stresu je dřeň nadledvin stimulovaná působením sympatiku, ovšem přibližně 70% noradrenalinu v cirkulaci pochází přímo ze sympatoneuronálního systému (Kvetnansky, Weise et al. 1979). Stejně tak může být stresem ovlivněn i periferní cholinergní systém: právě cestou paralelní regulace muskarinových a adrenergních receptorů (Myslivecek and Kvetnansky 2006). Mimo přímých účinků, které jsou dány vyplavením katecholaminů, mohou být adrenergní receptory (ale též receptory muskarinové, pravděpodobně na základě heterologní regulace – viz diskuse k jednotlivým pracím) ovlivněny také množstvím působících glukokortikoidů. Sekrece glukokortikoidů vzrůstá s peakem za 30 minut (pro většinu stresorů) po začátku působení stresoru. Vysoké hladiny glukokortikoidů mohou ovlivnit expresi adrenergních i muskarinových receptorů cestou cytoplasmatických receptorů pro glukokortikoidy, které následně vstupují do buněčného jádra a ovlivňují expresi příslušných genů. Tyto účinky mohou být jednak přímé, cestou GRE (glucocorticoid response elements), které se vyskytují v promoterové oblasti genů β_2-adrenergních receptorů (Cornett, Hiller et al. 1998) a β_1-adrenergních receptorů (Tseng, Stabila et al. 2001). Jednak mohou být tyto účinky na genové sekvence receptorů nepřímé, jako je tomu u muskarinových M_2 receptorů (Zhou, Fryer et al. 2001). Sekvence GRE je také možné najít na v promoterové oblasti genu α_{1B}-adrenergních receptorů (Gao and Kunos 1993), ale vazba a funkční následky takové vazby nejsou doposud známy. Promoterová oblast α_{1A}-AR genu obsahuje šest GRE

sekvencí (Scanga and Schwinn 1998). Nicméně je třeba poznamenat, že výskyt GRE se může druhově značně odlišovat (Tseng, Stabila et al. 2001).

Glukokortikoidy jsou nezbytné pro aktivaci genové transkripce fenyletanolamin N-methyltransferázy (PNMT) a její aktivaci během působení stresoru (Kvetnansky, Kubovcakova et al. 2006). PNMT je klíčovým enzymem v přeměně noradrenalinu na adrenalin.

Zajímavou a jistě důležitou skutečností je, že schopnost odpovědi na působení stresu může být pohlavně závislá. Příkladem jsou například pohlavní rozdíly v prevalenci astmatu, dále pohlavní rozdíly v průměru dýchacích cest v souvislosti s aktivací adrenergních či muskarinových receptorů v respiračním systému (Almqvist, Worm et al. 2008). U člověka je vývoj a manifestace astmatu prokazatelně spojena s přítomností stresu (Vig, Forsythe et al. 2006), přičemž ale souvislosti se změnami muskarinových a adrenergních doposud důkladněji studovány nebyly.

1.3.3 Vliv stresu v srdci

1.3.3.1 Vliv stresu na periferní adrenergní receptory

Data o změnách adrenergních receptorů za stresových podmínek nejsou četná a rozhodně je nelze označit za kompletní a vyčerpávající. V prvních pracích (U'Prichard 1980; Torda, Yamaguchi et al. 1981; Yamaguchi, Torda et al. 1981), které se týkaly sledování vlivu stresu na periferní adrenergní receptory, popsali autoři změny těchto receptorů v srdci. Další práce pak ukázaly, že pokles adrenergních receptorů v srdci (Torda T. 1984; Torda, Kvetnansky et al. 1985) není inhibovatelný chemickou sympatektomií (Torda T. 1985). Bylo také ukázáno, že za stresových podmínek hrají významnou úlohu β_2-adrenergní receptory (Kirby and Johnson 1990). Podobně, krátkodobá imobilizace měnila selektivně množství β_1- a β_2-adrenergních vazebných míst v různých oblastech srdce (Myslivecek, Ricny et al. 2004). U ryb může být počet β-adrenergních receptorů snížen vlivem hypoxie (Gamperl, Vijayan et al.

1998). Na druhou stranu, oxidativní stres (konkrétně aplikace H_2O_2) také snižoval množství srdečních β-adrenergních vazebných míst (Persad, Panagia et al. 1998). Krátkodobá imobilizace vedla i k poklesu α_1-adrenergních receptorů (Meerson 1991). Další autoři pak sledovali změny v senzitivitě srdečních receptorů, které byly vyvolány stresem – viz přehledný článek Santos a Spadari-Bratfisch (Santos and Spadari-Bratfisch 2006).

1.3.3.2 Vliv stresu na periferní muskarinové receptory

Mezi řadou stresorů tak, jak byly zmíněny v předchozí kapitole, je nejvíce prozkoumán vliv oxidačního stresu na periferní muskarinové receptory. Stimulace srdečních muskarinových M_2 receptorů vedla ke zvýšení negativně inotropní odpovědi v isolovaných srdečních předsíních potkana po expozici chlornanem indukovaného oxidativního stresu (Peters, Sand et al. 2001). Tento jev nebyl pozorován po stimulaci adenosinových A_1 receptorů, které jsou stejně jako M_2 muskarinové receptory spřaženy s Gi-proteiny. V potkaní ocasní arterii nebyla kontraktilní odpověď (zprostředkovaná stimulací M_3 receptor muskarinových receptorů) zvýšena. Autoři proto předpokládají, že M_2 muskarinové receptory jsou specifická pro chlornanem indukované efekty (Sand, Peters et al. 2002). Stimulace elektrickým polem zvýšila vaskulární tonus v závislosti na frekvenci a byla inhibovatelná acetylcholinem. Může být tedy uzavřeno, že rozdílná citlivost neuronálních a srdečních M_2 muskarinových receptorů k chlornanu může být vysvětlena rozdílnostmi v podjednotkách G-proteinu, které se uplatňují v aktivaci následné signalizační kaskády. Na druhou stranu, eventualita, že M_2 muskarinové receptory v srdci nemusí být nutně lokalizovány pouze "pre-synapticky" (myšleno na varikozitách nervových cholinergních zakončení), ale i na kardiomyocytech (což by odpovídalo nervové lokalizaci post-synaptické), jak je tomu právě u srdeční tkáně, nebyla vůbec v této práci diskutována (Sand, Peters et al. 2002).

Další práce (Kubera, Skowron-Cendrzak et al. 1992) ukázala, že β-adrenergní receptory se mění v lymfocytech jako odpověď na akutní imobilizaci. Tyto změny ale nebyly sledovány u

muskarinových receptorů. Chronický imobilizační stres (5 dní, v trvání 2 h) snížil vazbu muskarinových receptorů na lymfocytech odebraných ze sleziny a krve, ale nikoliv na lymfocytech odebraných přímo z tymu, Ke změnám β-adrenergních receptorů v tymocytech ani lymfocytech získaných z krve a sleziny nedošlo.

Krátkodobá imobilizace vedla k poklesu muskarinových receptorů (Myslivecek, Ricny et al. 2004). Existují ale i dřívější práce, které naopak dokumentovaly zvýšení muskarinových receptorů po působení krátkodobé imobilizace (Meerson 1991).

1.3.4 Význam vyřazení CRH pro stresovou reakci

Vzhledem k významu osy hypotalamus-hypofýza-nadledviny se předpokládalo, že kortikotropin releasing hormon (CRH) bude mít pro stresovou reakci zásadní význam. Proto, když to ke konci minulého století pokrok v genetických manipulacích umožnil, byla zásadní otázka, jak bude vypadat stresová reakce u zvířat (myší), u nichž CRH chybí. Při chybění CRH (tedy u CRH KO myší) se množství cirkulujících glukokortikoidů v průběhu stresové reakce dramaticky snižuje (Jeong, Jacobson et al. 2000; Kvetnansky, Kubovcakova et al. 2006). Kromě toho u CRH KO myší nedochází k cirkadiánním změnám v hladinách glukokortikoidů (Muglia, Jacobson et al. 1997). Vzhledem k tomu, že glukokortikoidy, jak již bylo zmíněno dříve, jsou důležité pro expresi PNMT, není překvapivé, že exprese tohoto enzymu je u těchto myší snížena a noradrenalin není účinně methylován na adrenalin. To vede k tomu, že plasmatické hladiny adrenalinu jsou sníženy, naopak hladiny noradrenalinu se zvyšují (Jeong, Jacobson et al. 2000). Nicméně je třeba si i uvědomit, že působení dalších hypothalamických hormonů může částečně u CRH KO myší kompensovat vliv na sekreci ACTH.

Z hlediska plicních funkcí je potřeba ještě zmínit jeden důležitý, vývojový fakt. CRH KO (-/-) myši, které vznikly křížením mezi homozygotními CRH KO samci a samicemi mají zhoršené plicní funkce a umírají krátce po narození (první postnatální den) (Venihaki and Majzoub

1999). Toto fatální respirační selhání nastává proto, že CRH je nezbytný pro sekreci dostatečných koncentrací glukokortikoidů a tím pádem zprostředkovaně stimuluje produkci surfaktantu. Proto, pokud chceme získat jedince homozygotní na nepřítomnost genu pro CRH, je třeba křížit CRH+/- samce a CRH+/- samice. Díky tomu nedochází vývojově (prenatálně) ke snížení hladiny glukokortikoidů, snížení produkce surfaktantu a tak u takto narozených jedinců homozygotních CRH-/- není zvýšena postnatální mortalita. To navíc ukazuje, že heterozygotní matky produkují takové množství glukokortikoidů, které je dostačující k fyziologické syntéze surfaktantu u mláďat a umožní tak adekvátní vývoj jejich plic. Obdobně lze vývoj surfaktantu a tím i mortalitu ovlivnit i přidáním glukokortikoidů do pitné vody CRH-/- březím myším.

2. CÍLE PRÁCE A HYPOTÉZY

Hlavními cíly naší práce, jejichž naplnění je dokumentováno v zařazených publikovaných pracích, bylo přispět k detailnějšímu poznání mechanismů, regulujících množství muskarinových a adrenergních receptorů v buňkách myokardu a plicích. Tyto zákonitosti a změny ve zmíněných receptorových systémech nás zajímaly jak za fyziologických podmínek, tak především pod vlivem stresorů. Pochopení vzájemné regulace těchto dvou receptorových typů je zásadní vzhledem k jejich významu v modulaci srdeční činnosti a vlivu na plicní funkce a také proto, že přesné zastoupení jejich podtypů a především vliv stresu na tyto receptory nebyl dosud přesněji objasněn.

Položili jsme si tyto specifické otázky:

a) Jaké podtypy α_1-adrenergních receptorů se vyskytují v plicní tkáni?

b) Jaké podtypy muskarinových a adrenergních receptorů jsou exprimovány srdeční tkání a to jednak na úrovni genové exprese (mRNA), jednak na úrovni vazby?

c) Jak se mění množství muskarinových a adrenergních receptorů vlivem stresu v plicích a srdeční tkáni?

d) Jak tyto změny v plicní tkáni ovlivní nepřítomnost důležitého hormonu účastnícího se humorální regulace za stresových podmínek (CRH)? Jsou tyto změny závislé na pohlaví?

V souvislosti s těmito cíli jsme si vytýčili následující hypotézy:

a) V plicní tkáni budeme schopni na základě vazebných studií identifikovat všechny podtypy α_1-adrenergních receptorů.

b) V srdeční tkáni bude možné identifikovat minoritní podtyp muskarinových receptorů a na úrovni genové exprese i β_3-adrenergní receptory. Jednotlivé receptorové podtypy budou mít specifickou distribuci v srdečních oblastech, genová exprese bude odpovídat množství vazebných míst.

c) Stres bude měnit nejenom množství adrenergních receptorů, ale bude paralelně ovlivňovat i muskarinové receptory. Nitrobuněčná signalizace nebude těmito změnami významně ovlivněna.

d) CRH je nezbytný pro normální průběh stresové reakce.

3. VÝSLEDKOVÁ – EXPERIMENTÁLNÍ ČÁST

Publikované práce

Dále jsou zařazeny následující práce publikované v impaktovaných časopisech, které jsou náplní cílů disertace a ověřením hypotéz formulovaných v předchozí části.

1) **M. Nováková,** J. Mysliveček: Identification of all α1-adrenoceptor subtypes in rat lung. Gen. Physiol. Biophys. 24: 349-353, 2005 (IF_{2009}=0.741)

   Specifická otázka v části cíle pod písmenem a)

2) J.Mysliveček, **M. Nováková**, M. Palkovits, O. Križanová, R. Kvetnanský: Distribution of mRNA and binding sites of adrenoceptors and muscarinic receptors in the rat heart. Life Sci. 79: 112-120, 2006 (IF_{2009}=2.56)

   Specifická otázka v části cíle pod písmenem b)

3) Myslivecek J, Klein M, **Novakova M**, Ricny J: The detection of non-M2 muscarinic receptor subtype in the rat heart atria and ventricles. N-S Arch Pharmacol, 378(1):103-116, 2008 (IF_{2009}=2.631)

   Specifická otázka v části cíle pod písmenem b)

4) **Novakova M.**, Kvetnansky R., Myslivecek J.: Sexual dimorphism in stress-induced changes in adrenergic and muscarinic receptor densities in the lung of wild type and CRH-knockout mice. Stress 13(1): 22–35, 2010 (IF_{2009}=3.205)

   Specifická otázka v části cíle pod písmenem c), d)

5) Myslivecek J., Tillinger A., **Novakova M.**, Kvetnansky R.: Regulation of adrenoceptors and muscarinic receptors gene expression after single and repeated stress. Annals NY Acad Sci 1148: 367-376, 2008 (IF_{2009}=2.67)

   Specifická otázka v části cíle pod písmenem c)

6) Tillinger A., Myslivecek J., **Nováková M.**, Križanová O., Kvetnanský R.: Gene expression of adrenoceptors in the heart of cold acclimated rats exposed to a novel stressor. Annals NY Acad Sci 1148: 393-399, 2008 (IF_{2009}=2.67)

Specifická otázka v části cíle pod písmenem c)

Short Communication

Identification of All α_1-Adrenoceptor Subtypes in Rat Lung

M. Nováková and J. Mysliveček

Institute of Physiology, 1st Faculty of Medicine,
Charles University, Prague, Czech Republic. E-mail: noma@1fl.cuni.cz

Abstract. The function of lung tissue is regulated *via* a release of neurotransmitters from autonomic nerves. The neurotransmitters of sympathetic nervous system, adrenaline and noradrenaline, activate both α-adrenoceptors and β-adrenoceptors. Although the function and expression of β-adrenoceptors can be considered major, some doubts exist about the function and expression of α_1-adrenoceptor subtypes in the lung tissue. Therefore a set of competition binding experiments was employed in order to discriminate between the α_1-adrenoceptor binding site subtypes in the rat lung. We identified three subpopulations of α_1-adrenoceptor binding sites in the rat lung (α_{1A}, α_{1B} and α_{1D}).

Key words: α_1-adrenoceptor subtypes — Lung — Rat

α-adrenoceptors belong to the most numerous family of membrane receptors – the G-protein coupled receptor family. They can be divided into three subtypes: α_{1A}, α_{1B} and α_{1D} (Zhong and Minneman 1999). All these subtypes activate $G_{q/11}$ family of G-proteins and consequently phospholipase Cβ (PLC) that cleaves phosphatidilinositol to inositoltrisphosphate and diacylglycerol. Both molecules are able to act as second messengers. Tissue distribution of these receptor subtypes is now generally considered to be a mixture of all three subtypes (Zhong and Minneman 1999). Although the role of α_1-adrenoceptors in the lung function is assumed to be minor, some findings have revealed the role of low-affinity prazosine binding sites (previously assumed as α_{1L} receptors) in allergic bronchoconstriction (Nobata et al. 2002). This finding is also important as the ligand used (JTH-601) in this study has high affinity for α_{1A}- and α_{1B}-adrenoceptors but low affinity for α_{1D}-adrenoceptors (Takahashi et al. 2000). Surprisingly, some papers have described only two subtypes in the lung tissue (Yang et al. 1998). On the other hand, in the middle of 90's, Hiramatsu et al. (1994) have suggested that the α_1-adrenoceptors of rat lung are composed of three distinct subtypes. Similarly, all three mRNA subtypes have been detected in the lung tissue (Faure et al. 1994; Alonso-Llamazares

Correspondence to: Jaromír Mysliveček, Institute of Physiology, 1st Faculty of Medicine, Charles University, Albertov 5, 128 00 Prague, Czech Republic.
E-mail: jmys@lf1.cuni.cz

et al. 1995). Nevertheless, the relationship between mRNA and protein expression is not always obvious (Zhong and Minneman 1999). Therefore we have employed the set of competition binding experiments with selective antagonists for α_{1A} (RS 17053), α_{1B} (L-765,314) and α_{1D} (BMY 7378) aiming to determine the binding site subtype proportion in the lung tissue.

Experiments were performed on adult Wistar male rats (200–250 g weight, age 46–55 days). They were housed in standard conditions (12/12 light/dark cycle, feeding and drinking water *ad libitum*). Animals were sacrificed by cervical dislocation and decapitation. Lung tissue was isolated, adjacent tissue was carefully discarded, the lung tissue was weighed, cut to small pieces and homogenized with an UltraTurrax homogenizer (Janke and Kunkel, Staufen, Germany) in an ice-cold saline. The homogenates were stored frozen at −20 ℃ until the measurements of radioligand binding. Radioligand binding experiments were performed as described previously (Mysliveček et al. 2003) with minor modifications. The homogenates were re-homogenized just before the addition of tissue to the mixture of radioligad and antagonist. Preliminary saturation binding experiments with ^{3}H-prazosin (specific activity 2.77 TBq/mmol; Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) revealed the following binding parameters: B_{max} = 253 fmol/mg of protein (255.7 and 250.3) and K_D = 585 pmol/l (657.1 and 514.7). The K_D value is in good agreement with our previously published data on rat heart α_1-adrenoceptors (Mysliveček et al. 2003) and B_{max} is similar to the data of other authors on lung ^{3}H-prazosin binding (Latifpour and Bylund 1983). The competition binding experiments with ^{3}H-prazosin (560 pmol/l) and specific antagonists were performed in order to ascertain the proportion of α_1-adrenoceptor subtypes in the lung tissue. The following compounds were used as specific antagonists for α_{1A}, α_{1B} and α_{1D}: RS 17053 (Tocris-Coocson, Bristol, UK), L-765,314 and BMY 7378 (Sigma-Aldrich, Prague, Czech Republic). The antagonists' ranges were between 10^{-11} and 10^{-5} mol/l (RS 17053), between 10^{-10} and 10^{-4} mol/l (L-765,314), and between 10^{-10} and 10^{-4} mol/l (BMY 7378) using two concentrations *per* grade. The incubations were performed in triplicates in 25 ℃ for 90 min and were terminated by fast washing with ice-cold distilled water – the same washing method as published previously (Mysliveček et al. 2003) – through Whatman GF/B filter that were pre-soaked in distilled water in order to diminish the non-specific binding with the use of a Brandel cell harvester. Generally, the washing of filters using Brandel cell harvester is extremely fast and therefore the differences in the composition of washing fluid are not of great effect on the binding. Moreover, we have compared the binding on filters washed with buffer and ice-cold distilled water and no differences have been found, therefore we have employed this washing method. The filters were dried overnight and the radioactivity retained on them was measured by liquid scintillation spectrometry using Bray's solution. Proteins were determined using modified Lowry's method. Data were analysed using GraphPad Prism 3.0 program (GraphPad Software, San Diego, CA).

All three α_1-adrenoceptor binding site subtypes were detected in the rat tissue, i.e. all three antagonists competed with ^{3}H-prazosin in a biphasic manner (see

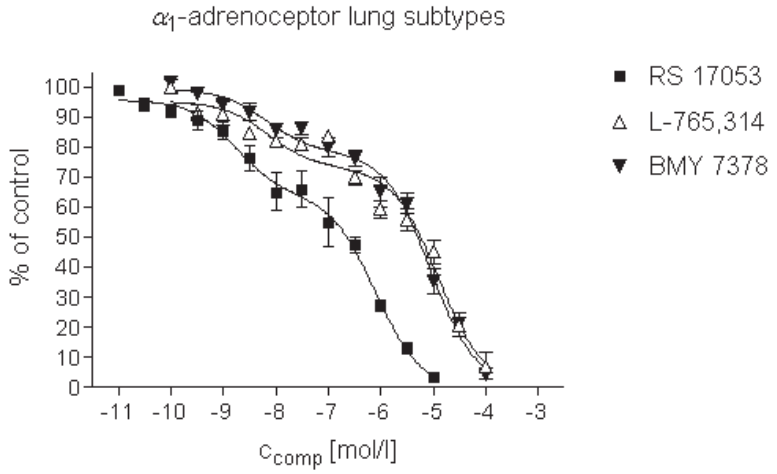


Figure 1. The competition binding of ^{3}H-prazosin and different antagonists in the lung. Data from three independent experiments are presented as means ± S.E.M. Abscissa: common logarithm of competitor concentration (c_{comp}) in mol/l. Ordinate: % of total bound. Explanation of symbols is inserted.

Table 1. The affinities and fractions revealed from competition binding experiments. Data from three independent experiments performed in triplicates are expressed as means ± S.E.M.

Competitor	Fraction 1 (%)	pKB1	pKB2
RS 17053	32.72 ± 3.49	9.76 ± 0.29	6.85 ± 0.16
L-765,314	23.06 ± 2.83	9.28 ± 0.29	5.79 ± 0.25
BMY 7378	21.07 ± 2.47	9.37 ± 0.25	5.90 ± 0.39

Figure 1). The respective values of high affinity fraction, pKB1 and pKB2, are given in Table 1. We can therefore conclude that the lung tissue contains all three binding site subtypes of α_1-adrenoceptors. As it can be seen from the Table 1, there is another proportion of ^{3}H-prazosin binding that is undistinguishable by α_1 antagonists.

It has been mentioned previously that the major attention was paid to the function of β-adrenoceptors and the consequences of α_1-adrenoceptor function are still not clear. The results concerning the role of α_1-adrenoceptors in asthma are difficult to interpret (for review, see Goldie et al. 1990). Although the role of α_1-adrenoceptors in the function of respiratory tract is not fully understood yet, some findings suggest the possibility that their role lies in the potentiation of β-adrenoceptor function (Nousiainen et al. 1977; Goldie et al. 1990). The role of α_1-adrenoceptor subtype has not been studied yet. There are some findings that

lung tissue also contains α_2-adrenoceptors (for review, see Calzada and de Artinano 2001). Our results show that all three α_1-adrenoceptor binding site subtypes are present in the lung tissue. All three competitors revealed high-affinity binding sites whose binding affinities (pKB_1, see Table 1) were comparable to that for cloned α_{1A}-, α_{1B}- and α_{1D}-adrenoceptor subtypes. Thus, pKB1 revealed by RS 17053 (α_{1A}-specific antagonist) was almost same (9.76 *vs.* 9.1–9.9) as that indicated by Ford et al. (1996) for cloned α_{1A}-adrenoceptors, pKB1 for L-765,314 (α_{1B}-specific antagonist) was similar (9.28 *vs.* 8.7) to that indicated by Patane et al. (1998) for cloned α_{1B}-adrenoceptors, and pKB1 for BMY 7378 (specific antagonist of α_{1D}-adrenoceptors) was virtually same (9.37 *vs.* 9.0–9.4) as that reviewed by Willems et al. (2003) for cloned α_{1D}-adrenoceptor. On the other hand, the low affinity binding sites (pKB2) have shown the values that implies to the presence of more binding sites with similar affinities. By other words, it shows the presence of one high-affinity binding site and the other (with low affinity) that is mixture of two other α_1-adrenoceptor binding site subtypes. Moreover, there is another proportion of ^{3}H-prazosin binding that is undistinguishable by α_1 antagonists (see Table 1). As ^{3}H-prazosin also binds to the α_2-adrenoceptors, it is possible to assign this binding to α_2-adrenoceptors. These receptors are also present in the lung tissue as have been demonstrated multiple times (for review, see Goldie et al. 1990).

Our results, describing three binding sites, are in good agreement with data from the studies that detected mRNAs for these receptor subtypes (Faure et al. 1994; Alonso-Llamazares et al. 1995). Also, Hiramatsu et al. (1994) have identified three subtypes in rat lung using radioligand binding studies. However, the authors could only concluded, in context with erstwhile knowledge, that in the rat lung α_{1A}, α_{1B} and unknown subtypes are present. On the other hand, Yang et al. (1998) have identified only α_{1A} and α_{1B} binding subtypes in the murine tissue. Similarly, Calzada and de Artinano (2001) have reviewed also the expression of two mRNAs (α_{1A} and α_{1B} only). It is not fully clear why our data vary from those obtained from papers by Yang et al. (1998) and Calzada and de Artinano (2001). Contrary to that, there is another reference (Hiramatsu et al. 1994) showing the binding to all three α_1-adrenoceptor subtypes. Our finding of three subtypes of α_1-adrenoceptor binding sites in lung seems to support opinion on the existence of all three (α_{1A}-, α_{1B}- and α_{1D}-adrenoceptor subtypes) in this tissue.

Acknowledgements. This work was supported by grant NATO LST.EAP.CLG.980745. The excellent technical assistance by Miss Eva Šimáková is greatly appreciated.

References

Alonso-Llamazares A., Zamanillo D., Tasanova E., Ovalle S., Calvo P., Chinchetru M. A. (1995): Molecular cloning of α_1-adrenergic receptor and tissue distribution of three α_1-adrenergic receptor subtypes in mouse. J. Neurochem. **65**, 2387—2392

Calzada B. C., de Artinano A. A. (2001): Alpha-adrenoceptor subtypes. Pharmacol. Res. **44**, 195—208

Faure C., Pimoule C., Arbilla S., Langer S. Z., Graham D. (1994): Expression of α_1-adrenoceptor subtypes in rat tissues: implications for α_1-adrenoceptor classification. Eur. J. Pharmacol. **268**, 141—149

Ford A. P., Arredondo N. F., Blue D. R. Jr., Bonhaus D. W., Jasper J., Kava M. S., Lesnick J., Pfister J. R., Shieh I. A., Vimont R. L., Williams T. J., McNeal J. E., Stamey T. A., Clarke D. E. (1996): RS-17053 (N-[2-(2-cyclopropylmethoxyphenoxy)ethyl]-5-chloro-α, α-dimethyl-1H-indole-3-ethanamine hydrochloride), a selective α_1-adrenoceptor antagonist, displays low affinity for functional α_1-adrenoceptors in human prostate: implications for adrenoceptor classification. Mol. Pharmacol. **49**, 209—215

Goldie R. G., Paterson J. W., Lulich K. M. (1990): Adrenoceptors in airway smooth muscle. Pharmacol. Ther. **48**, 295—322

Hiramatsu Y., Muraoka R., Kigoshi S., Muramatsu I. (1994): Identification of α_1-adrenoceptor subtypes in rat lung by binding of [3H]-prazosin and [3H]-WB4101. J Recept. Res. **14**, 75—98

Latifpour J., Bylund D. B. (1983): Characterization of adrenergic receptor binding in rat lung: physiological regulation. J. Pharmacol. Exp. Ther. **224**, 186—192

Mysliveček J., Říčný J., Kolář F., Tuček S. (2003): The effects of hydrocortisone on rat heart muscarinic and adrenergic α_1, β_1 and β_2 receptors, propranolol-resistant binding sites and on some consequent steps in intracellular signalling. Nuanyn Schmiedebergs Arch. Pharmacol. **368**, 366—376

Nobata K., Fujimura M., Ishiura Y., Hirose T., Furusyou S., Myou S., Kurashima K., Kasahara K., Nakao S. (2002): α_{1L}-, but not α_{1H}-, adrenoceptor antagonist prevents allergic bronchoconstriction in guinea pigs *in vivo*. Eur. J. Pharmacol. **452**, 97—104

Nousiainen O. T., Arnala I., Airaksinen M. M., Kokkola K. (1977): Potentiation of β-sympathomimetics with α-adrenoceptor blocking drugs in guinea pig trachea and human bronchus preparations. Pharmacology **15**, 469—77

Patane M. A., Scott A. L., Broten T. P., Chang R. S. L., Ransom R. W., DiSalvo J., Forray C., Bock M. G. (1998): 4-Amino-2-[4-[1-(benzyloxycarbonyl)-2(S)-[[(1,1-dimethylethyl)amino]carbonyl]-piperazinyl]-6,7-dimethoxyquinazoline (L-765,314): a potent and selective α_{1B}-adrenergic receptor antagonist. J. Med. Chem. **41**, 1205—1208

Takahashi M., Taniguchi T., Kanamaru H., Okada K., Muramatsu I. (2000): Pharmacological characterization of [3H]-JTH-601, a novel α_1-adrenoceptor antagonist. Binding to recombinant human α_1-adrenoceptors and human prostates. Life Sci. **67**, 2443—2451

Willems E. W., Valdivia L. F., Villalón C. M., Saxena P. R. (2003): Possible role of α-adrenoceptor subtypes in acute migraine therapy. Cephalalgia **23**, 245—257

Yang M., Reese J., Cotecchia S., Michel M. C. (1998): Murine α_1-adrenoceptor subtypes. I. Radioligand binding studies. J. Pharmacol. Exp. Ther. **286**, 841—847

Zhong H., Minneman K. P. (1999): α_1-adrenoceptor subtypes. Eur. J. Pharmacol. **375**, 261—276

Final version accepted: June 20, 2005

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Life Sciences 79 (2006) 112–120
Life Sciences
www.elsevier.com/locate/lifescie

Distribution of mRNA and binding sites of adrenoceptors and muscarinic receptors in the rat heart

Jaromír Mysliveček [a,d,*], Martina Nováková [a], Miklós Palkovits [b], Ol'ga Križanová [c,d], Richard Kvetňanský [d]

[a] Institute of Physiology, 1st Faculty of Medicine, Charles University, Prague, Czech Republic
[b] Laboratory of Neuromorphology, Hungarian Academy of Sciences and Semmelweis University, Budapest, Hungary
[c] Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Bratislava, Slovakia
[d] Institute of Experimental Endocrinology, Slovak Academy of Sciences, Bratislava, Slovakia

Received 14 July 2005; accepted 15 December 2005

Abstract

Since there exist some obscurities in the expression of mRNAs and their receptors in the heart, we have investigated the gene expression (mRNA levels) of adrenoceptors (α_{1A}-, α_{1B}-, β_1-, β_2-, β_3-) and muscarinic receptors (M_2) and the density of receptor binding sites (α_{1A}-, α_{1B}-, β_1-, β_2-adrenoceptors, muscarinic receptors). Moreover, the heart regions consist of tissue rich in ganglion cells (that are of importance in heart neural circuits) and those virtually free of them (myocytes). Therefore, we have examined the differences in the distribution of mRNAs/receptor binding sites in the atrial samples of the heart rich in ganglion cells vs. those are virtually free of them. Binding sites and mRNAs of muscarinic receptors and α_{1B}-adrenoceptors differ in their distribution in different heart regions. The mRNAs for β_1- and β_2-adrenoceptors were almost equally distributed herein, while the amount of β-adrenoceptors significantly differs in the heart regions. The α_{1A}- and β_3-adrenoceptors mRNAs were also found in all investigated heart regions, but at significantly lower level and have not shown region differences. This is a new finding, especially to β_3-adrenoceptors, as they were not regularly found in each heart regions. α_{1B}-adrenoceptors have similar distribution of their mRNAs and binding sites in some heart parts. Thus, we can conclude that there are noticeable differences in the presence of receptors in heart regions that contain ganglion cells in comparison to those are virtually free of them.

Keywords: Adrenoceptors; Muscarinic receptors; Heart; Binding; mRNA

Introduction

The force and rate of cardiac contractions adapt continually to the changing needs of the organism. Sympathetic nerves (releasing noradrenaline) and vagal nerves (releasing acetylcholine) are mainly responsible for the short-term regulation of the heart activity in mammals, together with the changing concentrations of adrenomedullary hormones (adrenaline and noradrenaline) in the blood plasma. Noradrenaline and adrenaline act via the adrenoceptors, while the action of acetylcholine is mediated by the muscarinic receptors (for review see Brodde and Michel, 1999). Myocardial cells express a broad spectrum of muscarinic receptors and adrenoceptors, which belong to a large family of seven transmembrane-spanning proteins, namely G-protein coupled receptors (GPCRs). M_2 muscarinic and β_1-adrenoceptors are the most important receptor subtypes expressed in cardiomyocytes (Brodde and Michel, 1999), but the heart cells express also the other subtypes, i.e. β_2, and α_1-adrenoceptors probably also other non-M_2 subtype of muscarinic receptors (Wang et al., 2004) and β_3 adrenoceptors (Gauthier et al., 2000). The mRNAs for M_2 muscarinic receptors and β_1- and β_2-adrenoceptors have been repeatedly found in the heart (for review see Brodde and Michel, 1999). The expression of minor subtypes is now under investigation and has been discussed in

* Corresponding author. Institute of Physiology, 1st Faculty of Medicine, Charles University, Albertov 5, 128 00 Prague, Czech Republic. Tel.: +420 2249684845; fax: +420 224918816.
E-mail address: jmys@lf1.cuni.cz (J. Mysliveček).

0024-3205/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2005.12.026

recent reviews (Brodde and Michel, 1999; Granneman, 2001; Zimmer, 1997; Mysliveček and Trojan, 2003; Wang et al., 2004) or original papers (Kaumann and Molenaar, 1997; Gautier et al., 1996; Hamilton et al., 2001; Colecraft et al., 1998; Brixius et al., 2004). Wang et al. (2001) have identified the muscarinic receptor subtypes and mRNAs in the human heart. mRNAs for all muscarinic receptor subtypes have been recently quantitated in the rat heart (Krejčí and Tuček, 2002). Similarly, the α_1-adrenoceptor subtype expression in the heart is still a matter of debate and the differences in species expression could exist (see Brodde and Michel, 1999; Michel et al., 1994; Autelitano and Woodcock, 1998). Some discrepancies still exist about the expression of β_3 adrenoceptor subtype in the heart. While Gautier et al. (1996), Gauthier et al. (2000) repeatedly described their role in the heart, the others (Kaumann and Molenaar, 1997) have challenged these findings (for review see Gauthier et al., 2000). Very recently, the data about the function of β_3-adrenoceptors in human heart dramatically increased (Brixius et al., 2004; Tavernier et al., 2003; Pott et al., 2003) but the data on rat myocardium are still controversial. Moreover, the data about the gene expression of this receptor subtype in cells that are almost of neuronal origin (neuronal ganglia) and that of mainly of myocyte origin (non-ganglionic heart parts) are still missing.

Cardiac ganglia are groups of cells that possess heterogenous population of preganglionic, ganglionic and interconnecting local circuit neurons. The neurotransmitter systems including receptor to these molecules are still the subject of continuing research. In the mammalian heart the intrinsic cardiac ganglia are situated within the subepicardial plexus. A majority of them were found in the left subepicardial plexus adjacent to the pulmonary veins. Complex interactions between cardiac and extracardiac ganglion cells play a crucial role in the regulation of cardiac function. According to our knowledge, the gene expression of receptor mRNA and the receptor binding in the heart regions rich in ganglion cells and that virtually free of them were not followed yet. Therefore, we have divided the atrial heart parts into regions rich in these cells and those virtually free of neuronal cells in order to discriminate the possible differences in the gene expression (using the RT-PCR identification of mRNA) and receptor binding site density (using ligand binding studies).

We tried to identify mRNAs of adrenoceptor (α_{1A}, α_{1B}, β_1, β_2, β_3) and muscarinic receptors (M_2) and their binding sites (α_{1A}, α_{1B}, β_1, β_2, muscarinic receptors) in ten different heart parts. We divided the rat heart into left and right atria rich in ganglion cells or virtually free of ganglion cells, left and right ventricles and septum. As there is no suitable binding technique for β_3-adrenoceptor detection, these receptors were identified on the mRNA level only.

Materials and methods

Tissue preparation

Adult male Sprague–Dawley rats (300–350 g b.w.) were used. Animals were treated in accordance with the Guide for Care and Use of Laboratory Animals and the experimental protocol was approved by the Ethic Committee of the Institute of Experimental Endocrinology. Animals were kept on standard laboratory conditions with 12/12 light/dark cycle (light on at 6.00 a.m.) and supplied by dry rat food and drinking water ad libitum. After decapitation, hearts were quickly removed and immediately separated into parts as stated thereinafter. Seven heart parts were dissected for radioligand binding experiments: right and left heart atrial part virtually free of ganglion cells, right and left atrial tissue with the majority of heart ganglia, left and right ventricles and septum. Three more parts were dissected for the RT-PCR experiments: atrial septum, apex and the ventricular septum that was divided into upper and lower part. Samples were collected in Eppendorf tubes, flash frozen in liquid nitrogen and kept at −70 °C until use.

RNA isolation and relative quantification of mRNA levels by RT-PCR

RNA was isolated by RNAzol. Concentration and purity of RNA was determined spectrophotometrically on a GeneQuant Pro (Amersham Bioscience). Reverse transcription was performed using Ready-To-Go You-Prime First-Strand beads and $pd(N)_6$ primer. PCR for specific receptors was carried out afterward using the primers originally designed or obtained from the references given in Table 1. The initial denaturation was performed at 95 °C for 5 min followed by the process described in detail in Table 1 with the exception of M_2 muscarinic receptors when the denaturation lasting 5 min was followed by 35 s annealing at 60 °C and 45 s polymerization at 72 °C (according to Krejčí and Tuček, 2002 and the process described in Table 1). Polymerization was performed at 72 °C in all cases. The final polymerization lasting 7 min was performed at 72 °C in all cases.

PCR products were analysed on 2% agarose gels and visualised using ethidium bromide. As a control, the housekeeper glyceraldehydes 3-phosphate dehydrogenase (GAPDH) was used. Intensity of the individual bands was evaluated by PCBase software. For semiquantitative evaluation the values were normalised to the signal obtained with GAPDH.

Radioligand binding experiments

Preliminary saturation binding

The tissue was weighted and samples from 2–4 animals was pooled and homogenised for 30–45 s in homogeniser (Ultra-Turrax® T25 basic IKA®-Werke 24,000 r.p.m.) in physiological saline keeping the tissue on ice.

Density of muscarinic binding sites (B_{max}) were computed by non-linear regression of data obtained in saturation experiments with the binding of 31.25–1000 pmol/l [^{3}H]QNB to cardiac tissue homogenates performed in duplicates. The non-specific binding was determined in the presence of 5 μmol/l atropine and was less than 10%.

Density of β-adrenergic binding sites (B_{max}) were computed from saturation experiments with the binding of 62.5–

Table 1
Primer sequences and protocols used in PCR reactions

Gene	Primer sequence	Fragment size	Denaturation [°C]/time [s]	Annealing [°C]/time [s]	Polymerization time [s]	Number of cycles	Reference
α_{1A}-adrenoceptors	Sense: 5′-CGA GTC TAC GTA GTA GCC-3′ antisense: 5′-GTC TTG GCA GCT TTC TTC-3′	204	95/60	65/30	60	31	(Salomonsson et al., 2001)
α_{1B}-adrenoceptors	Sense: 5′-ATC GTG GCC AAG AGG ACC-3′ antisense: 5′-TTT GGC TGC TTT CTT TTC-3′	201	95/60	62/30	60	36	(Salomonsson et al., 2001)
β_1-adrenoceptors	Sense: 5′-GCC GAT CTG GTC ATG GGA-3′ antisense: 5′-GTT GTA GCA GCG GCG CG-3′	326	94/60	65/60	60	36	
β_2-adrenoceptors	Sense: 5′-ACC TCC TTC TTG CCT ATC CA-3′ antisense: 5′-TAG GTT TTC GAA GAA GAC CG-3′	540	94/60	65/60	60	36	
β_3-adrenoceptors	Sense: 5′-GCA ACC TGC TGG TAA TCA CA-3′ antisense: GGA TTG GAG TGA CAC TCT TG-3′	418	94/60	60/30	60	36	
M_2 muscarinic receptors	Sense: 5′-TAC CCT CTA CAC TGT GAT TGG C-3′ antisense: 5′-ATG ATG ACA GGC AGA TAG-3′	363	95/45	62/35	40	34	(Krejčí and Tuček, 2002)
GAPDH	Sense: 5′-AGA TCC ACA ACG GAT ACA TT-3′ antisense: 5′-TCC CTC AAG ATT GTC AGC AA-3′	309	94/60	60/30	60	37	

All polymerizations were performed at 72 °C. The initial denaturation was performed at 95 °C for 5 min with the exception of M_2 muscarinic receptors when the denaturation lasting 5 min was followed by 35 s annealing at 60 °C and 45 s polymerization at 72 °C. The final polymerization lasting 7 min was performed at 72 °C in all cases.

2000 pmol/l [^{3}H]CGP 12177 to the tissue homogenates performed in duplicates. The non-specific binding was determined in the presence of 5 μmol/l propranolol and was less than 15%.

Density of α_1-adrenergic binding sites (B_{max}) were computed from saturation experiments with the binding of 62.5–2000 pmol/l [^{3}H]prazosin to the tissue homogenates performed in duplicates. The non-specific binding was determined in the presence of 100 μmol/l phentolamine and was less than 15%.

The incubation was performed at 38 °C and lasted 2 h with [^{3}H]QNB, 1 h with [^{3}H]CGP 12177 and 90 min at 25 °C with [^{3}H]prazosin. In all cases, the incubation was terminated by a filtration through Whatman GF/B glass fibre filters in a Brandel cell harvester when the filters were washed with ice-cold water. Radioactivity retained on the filters was measured by scintillation counting in Bray's solution after desiccation overnight. The dissociation constants (K_D) were computed and used for the "single-point" measurement in order to determine the number of receptors saving the amount of tissue needed, as described below.

Radioligand binding using "single-point" measurements

The densities of muscarinic, α- and β-adrenergic receptor binding sites were determined by using single point measurements of the binding. Homogenates were incubated in a single fully saturating concentration of the radioligand (1000 pmol/l [^{3}H]QNB for muscarinic receptors, 1000 pmol/l [^{3}H]prazosin for α-adrenergic receptors and 3200 pmol/l [^{3}H]CGP 12177 for β-adrenergic receptors), and the B_{max} values were computed according to the equation $B_{max}=B\times([L]+K_L)/[L]$ (1), where B=bound of radioligand [fmol/mg of protein], L=radioligand concentration [fmol/l], and $K_L=K_D$ [fmol/l] of the radioligand. All of the tissue samples were processed in triplicates.

Determination of the densities of α_{1A}- and α_{1B}-adrenoceptors

Homogenates were incubated for 90 min at 25 °C. The composition of the incubation medium corresponded to that of the medium used for homogenisation, with the addition of [^{3}H]prazosin as the specific radioligand (800 pmol/l), and of the subtype-selective antagonists where indicated. To ascertain the proportion of the α_{1A} and α_{1B} subtypes, the binding of [^{3}H]prazosin was determined in duplicates in the absence and the presence of 10^{-8} mol/l RS17053 (a selective α_{1A}-adrenoceptor antagonist) or 10^{-8} mol/l L-765,314 (a selective α_{1B}-adrenoceptor antagonist). The concentrations of RS17053 and L-765,314 suitable to distinguish the α_{1A}- and $\alpha_{?B}$-adrenoceptor subtypes had been determined in preliminary experiments using the full competition curve (data not shown) with three concentrations of antagonist per grade.

Determination of the densities of β_1- and β_2-adrenoceptors

Homogenates were incubated for 60 min at 38 °C. The composition of the incubation medium corresponded to that of the medium used for homogenisation, with the addition of [^{3}H]CGP 12177 as the specific radioligand (400 pmol/l), and of the subtype-selective antagonists where indicated. To ascertain the proportion of the β_1 and β_2 subtypes, the binding of [^{3}H]CGP 12177 was determined in duplicates in the absence and the presence of 10^{-7} mol/l CGP 20712A (a selective β_1-adrenoceptor antagonist) or 10^{-8} mol/l ICI 118.551 (a selective

β_2-adrenoceptor antagonist). The concentrations of CGP 20712A and ICI 118.551 suitable to distinguish the β_1- and β_2-adrenoceptor subtypes had been determined in preliminary experiments using the full competition curve with three concentrations of antagonist per grade. In all instances the proportion of total [^{3}H]CGP 12177 binding inhibited by 10^{-7} mol/l CGP 20712A was virtually identical with the proportion of binding not inhibited by 10^{-8} mol/l ICI 118.551, and the proportion of binding inhibited by 10^{-8} mol/l ICI 118.551 was virtually identical with the proportion of binding not inhibited by 10^{-7} mol/l CGP 20712A.

Data analysis

Radioligand binding data have been treated as described previously (Mysliveček et al., 2003), with the use GraphPad Prism 3.0 (GraphPad Software, San Diego, CA) program. Statistical differences among groups were determined by one-way analysis of variance ANOVA. For multiple comparison, an adjusted *t*-test with *P* values corrected by the Student–Newman–Keuls Method or Dunn's methods were used. For some comparisons the *t*-test was also used (see further text for details).

Source of reagents

[^{3}H]Quinuclidinyl benzilate ([^{3}H]QNB, 1.59 TBq/mmol), -(−)4-(3-*tert*-butylamino-2-hydroxypropoyx)-[5,7^{3}H]benzimidazol-2-one ([^{3}H]CGP 12177, 1.85 TBq/mmol), and [3H] prazosin (75 Ci/mmol) were purchased from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, England). Atropine and propranolol were from ICN Biomedicals Inc., Aurora, OH, USA; compounds CGP20712A ((1-[2-(3-carbamoyl-4-hydroxyphenoxy)-ethylamino]-3-[4-(1-methyl-4-trifluormethyl-2-imidazolyl)-phenoxy]-propanol-methansulfonate), ICI 118.551 (erythro[±]-1-

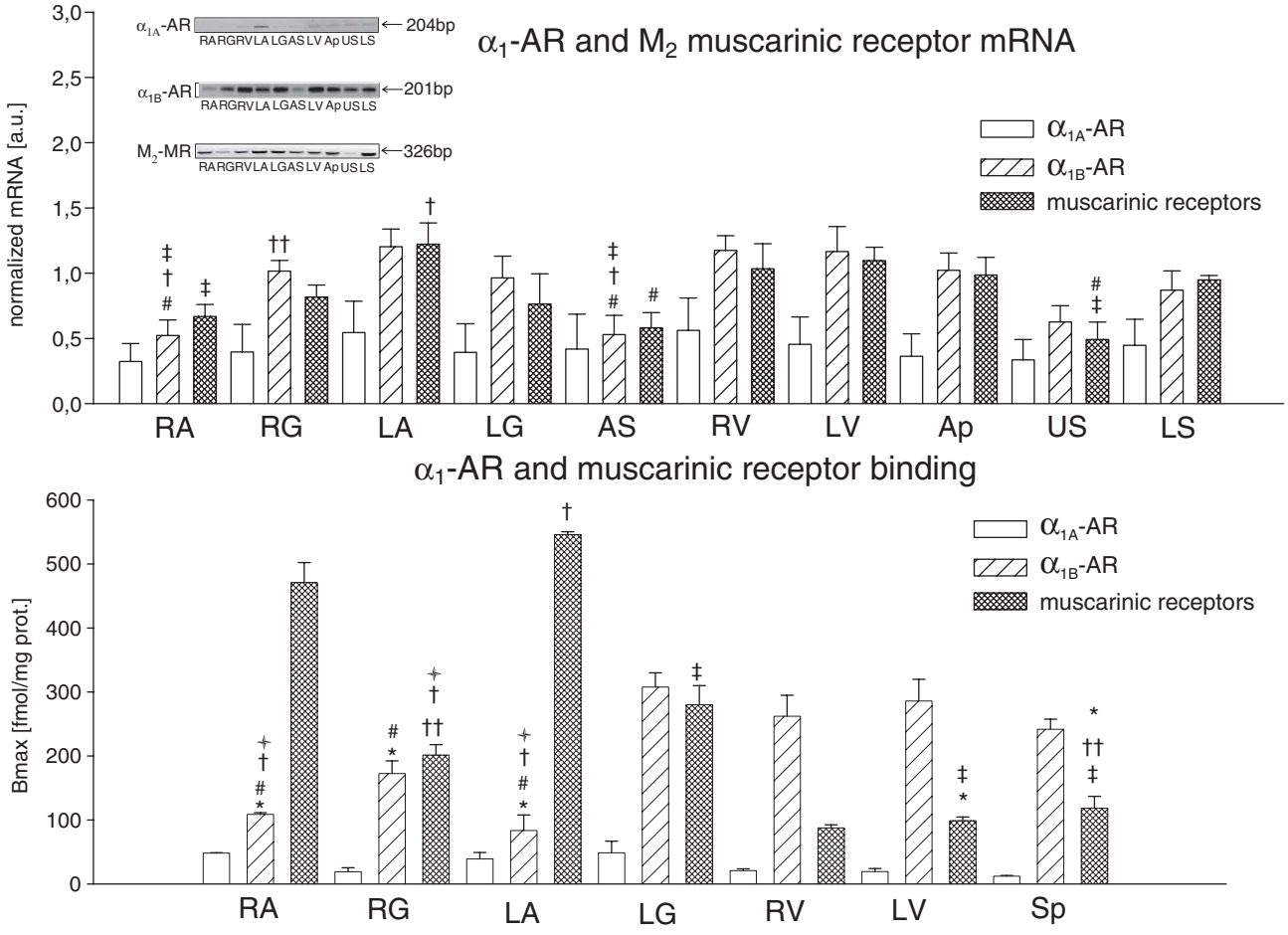


Fig. 1. Above: distribution of α_{1A}-adrenoceptor, α_{1B}-adrenoceptor and muscarinic receptor mRNA in different heart regions. RA, right atria virtually free of ganglion cells; RG, right atria rich in ganglion cells; RV, right ventricle; LA, left atria virtually free of ganglion cells; LG, left atria rich in ganglion cells; AS, atrial septum; LV, left ventricle; Ap, apex; US, upper septum; LS, lower septum. Data are expressed as means±S.E.M. and represent an average of at least 5 animals. An example of gel electrophoresis with α_{1A}-, α_{1B}-adrenoceptor and muscarinic receptor primers is inserted. Bottom: distribution of binding sites to α_{1A}-, α_{1B}-adrenoceptors and muscarinic receptors in different heart regions. Legend as above; Sp, ventricular septum. Data are expressed as means±S.E.M. and represent an average of 3–5 experiments where the tissues from 3 to 4 animals have been pooled. *Significantly different from LG. $^{\#}$Significantly different form LV. †Significantly different from RV. ††Significantly different from RA. ✦Significantly different from Sp. ‡Significantly different from LA.

[7-methylindan-4-yloxyl]-3-isopropylamino-butan-2-ol), L-765,314 ((2S)-4-(4-amino-6,7-dimethoxy-2-quinazolinyl)-2-[[(1,1-dimethylethyl)amino]carbonyl]-1-piperazinecarboxylic acid, phenylmethyl ester), HEPES (*N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonate) were purchased from Sigma Czech Republic. RS 17053 ((*N*-[2-(2-Cyclopropyl-methoxyphenoxy)ethyl]-5-chloro-a,a-dimethyl-1H-indole-3-ethanamine) hydrochloride) was from Tocris–Coocson (Bristol, UK). RNAzol was from Tel-Test, USA, Ready-To-Go You-Prime First-Strand beads and pd(N)$_6$ primer were from Amersham Biosciences (AP Biotech Czech Republic). Buffer F511 and polymerase (DyNAzyme II Polymerase) were from Finnzymes, USA.

Results

Levels of mRNA coding for adrenoceptors and muscarinic receptors in heart areas

Data obtained from these experiments are shown in Figs. 1 and 2 (upper part). One-way ANOVA have shown that the amount of mRNA of M_2 muscarinic receptors ($p=0.011$) and α_{1B}-adrenoceptors ($p<0.002$) significantly differs in the areas of our interest. In more detail, the amount of M_2 mRNA was significantly higher in the left atria than in the upper part of the septum (see Fig. 1, upper part). When comparing the areas with *t*-test, the significant difference can be found between left and right atrial part virtually free of ganglia, left atria atrial part virtually free of ganglia and upper part of septum, left ventricle and upper part of septum and also between left ventricle and atrial septum. The significant differences were also achieved in the amount of α_{1B}-adrenoceptor mRNA between the following heart areas: in the left atrial part virtually free of ganglia, there was higher expression of mRNA than in the right atrial part virtually free of ganglia and also than in the atrial septum, in the right ventricle there was higher amount of mRNA than in the right atrial part virtually free of ganglia and in the atrial septum, and, finally, in the left ventricle the expression of α_{1B}-adrenoceptor mRNA was significantly higher than in the right atrial part virtually free of ganglia and atrial septum (see Fig. 1, upper part). When comparing the areas with *t*-test, the significant difference can be found between the right atrial

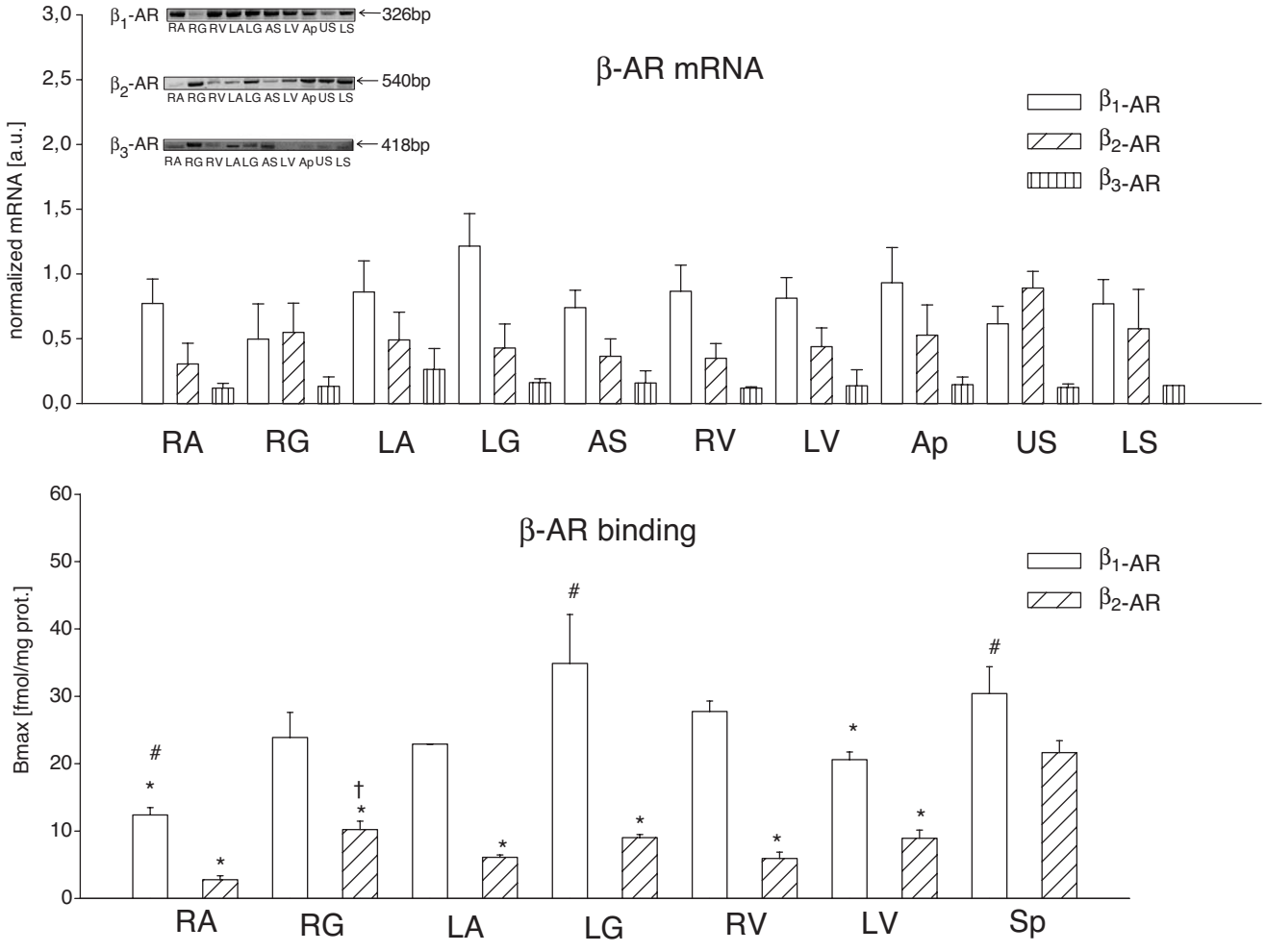


Fig. 2. Above: Distribution of β_1-, β_2-, β_3-adrenoceptor mRNA in different heart regions. For legend see Fig. 1. Data are expressed as means ± S.E.M. and represent an average of at least 5 animals. The example of gel electrophoresis with β_1-, β_2-, β_3-adrenoceptor primers is inserted. Bottom: distribution of binding sites to α_{1B}-adrenoceptors in different heart regions. For legend see Fig. 1. Data are expressed as means ± S.E.M. and represent an average of 3–5 experiments where the tissues from 3 to 4 animals have been pooled. *Significantly different from septum. #Significantly different from LV. †Significantly different from RA.

part virtually free of ganglia and the right atrial parts rich in ganglion cells.

Radioligand binding experiments

Preliminary saturation binding

Preliminary saturation binding experiments ($n=3$, tissue was pooled from 2 to 4 animals) have shown the $K_D=0.59\pm0.07$ nmol/l for α-adrenergic receptors, $K_D=0.69\pm0.08$ nmol/l for β-adrenoceptors, and $K_D=0.09\pm0.02$ nmol/l for muscarinic receptors. The respective B_{max} were as follows (fmol/mg prot.): α-adrenergic receptors: atria 234.74±36.59, ventricles 304.35±19.38; β-adrenoceptors: atria: 32.48±3.69, ventricles: 35.58±3.29; muscarinic receptors: atria: 281.70±58.68, ventricles: 99.87±5.64.

Radioligand binding using "single-point" measurements and determination of the densities of α_{1A}-, α_{1B}-, β_1- and β_2-adrenoceptors and M_2 muscarinic receptors

Data obtained from these experiments are shown in Figs. 1 and 2 (bottom part). One-way ANOVA have shown that there is significant difference in the density of receptor binding sites for α_{1B}- ($p<0.001$), β_1- ($p=0.025$), β_2-adrenoceptors ($p<0.001$), and muscarinic receptors ($p<0.001$) in the different heart regions.

In more detail, the densities of α_{1B}-adrenoceptor binding sites were higher in the left atrial part rich in ganglion cells than in the left atrial part virtually free of ganglion cells and in the right atrial part virtually free of ganglion cells and also in the right atrial part rich in ganglion cells Moreover, the receptor density is higher in the left ventricle than in the left atrial part virtually free of ganglion cells, right atrial part virtually free of ganglion cells and right atrial part rich in ganglion cells; the density is higher in the right ventricles than in the right atrial part virtually free of ganglion cells and left atrial part virtually free of ganglion cells; and, finally, the receptor density measured in the septum was higher than the density in the left and right atrial parts virtually free of ganglion cells. The β_1-adrenoceptor density (tested by *t*-test) was higher in the septum than in the right atrial part virtually free of ganglion cells and left ventricles, and the density in the right ventricles was higher than in the right atrial part virtually free of ganglion cells. On the contrary, the density of β_1-adrenoceptors in the left atrial part rich in ganglion cells was higher than in the left ventricles. The densities of β_2-adrenoceptors were lower in all regions than in the septum. When using *t*-test, there was also a significant difference between the density of binding sites in the right atrial parts containing ganglion cells (it was higher) than the density of these binding sites in the right atria virtually free of neuronal ganglionic cells. The amount of M_2 muscarinic receptors was higher in the left atrial part virtually free of ganglion cells than in the right ventricles. When the *t*-test for comparing two areas each other was applied, the following differences were revealed: the receptor density was higher in the right atrial part virtually free of ganglion cells than in the right atrial parts containing ganglion cells, in the left ventricles and in the septum. The density was higher in the left atrial part virtually free of ganglion cells than in the left atrial part rich in them, and in the septum. The density was also higher in the right atrial part rich in ganglion cells than in the right ventricle, and in the septum. Similarly, the density was higher in the left atrial part rich in ganglion cells than in the left ventricle, right ventricle and than in the septum.

Taken together, the data obtained can be summarized:

1) The α_{1A}-adrenoceptor mRNA levels and density of binding sites are equally distributed in the heart and do not reveal the differences between parts rich in ganglion cells and that free of them.
2) The highest levels of α_{1B}-adrenoceptor mRNA were observed in left ventricle, right ventricle and left atrial part virtually free of ganglion cells in which values were significantly higher than mRNA levels in the atrial septum and right atrial part virtually free of ganglion cells (see Fig. 1, upper part). Moreover, the amount of mRNA in the right atrial tissue rich in neuronal ganglia was higher than that in the right atrial part free of ganglia. The density of α_{1B}-adrenoceptor binding sites was higher in the ventricles than in the atrial parts without ganglia. The differences were also evident in left atrial parts virtually free of ganglion cells and that rich in ganglion cells. The highest densities of α_{1B}-adrenoceptor binding sites observed in the left ventricle and left atrial ganglionic part (LV, LG, Fig. 1, bottom part) correspond to the mRNA measurements (Fig. 1, upper part). The lowest receptor densities were observed in the right atria free of ganglion cells that is in agreement with the low amount of mRNA in that part.
3) The β_1-adrenoceptor mRNA is equally distributed in the heart and do not reveal the differences between parts rich of neuronal ganglia and that free of them. On the other hand there were some differences in the density of receptor binding sites between different regions (septum vs. right atrial part virtually free of ganglion cells and left ventricles, right ventricles vs. right atrial part virtually free of ganglion cells, left atrial part rich in ganglion cells vs. left ventricles).
4) Although the β_2-adrenoceptor mRNA was equally distributed in the heart parts, the densities of binding sites were significantly higher in the septum than in the other parts. Also, the density of binding sites in the right atrial part rich in ganglion cells was higher than the density of these binding sites in the right atrial part virtually free of ganglion cells.
5) We were able to detect β_3-adrenoceptor mRNA in all heart regions.
6) The highest amount of M_2 muscarinic receptor mRNA was found in the left atrial part virtually free of ganglion cells what correspond to the highest amount of receptor binding sites in this region. Moreover, there was higher density of receptor binding sites in that region than in the left atrial part virtually free of ganglion cells. Similarly, the density of muscarinic receptor binding sites was higher in the right atrial tissue without ganglia than in the right atrial part rich in ganglion cells.
7) It is evident, that the amount of α_{1B}-adrenoceptor mRNA (see Fig. 1, upper part; significant differences not marked) as

well as the density of α_{1B}-adrenoceptor binding sites (see Fig. 1, bottom part; significant differences not marked) is higher than the amount of α_{1A}-adrenoceptor mRNA and α_{1A}-adrenoceptor binding sites in following heart areas: right atrial part rich of ganglion cells, left atrial part virtually free of ganglion cells, left ventricle and apex.

8) The amount of mRNA of β_1- and β_2-adrenoceptors did not differed in right atrial part rich of ganglion cells and that virtually free of them, and in the left atrial part virtually free of ganglion cells (Fig. 2, upper part), but was different in the left atrial part rich of ganglion cells. The difference was also apparent in the right ventricle. On the contrary, the density of receptor binding sites in these areas was different (see Fig. 2, bottom part).

Discussion

Generally, the amount of appropriate mRNA coding adrenergic and muscarinic receptors in the respective heart region corresponds to the density of receptor binding sites in that area. Some exceptions can be found in the atria when compared to the amount of α_{1B} mRNA and binding sites in parts rich in ganglion cells and that virtually free of them. Similarly, we have found that the highest amount of mRNA for M_2 muscarinic receptor is expressed in the left atrial part virtually free of ganglion cells that corresponds to the highest density of receptor binding sites in that heart part and also that the lesser amount of mRNA is expressed in the left atrial part rich in ganglion cells, that is in agreement with the density of binding sites in that area. On the other hand, the amount of M_2 muscarinic receptor mRNA did not differ in the ventricular heart parts and in the atrial regions, but the density of binding sites was higher in the atrial parts without neuronal ganglia. These results suggest that the muscarinic receptor reserve could exist. One could speculate that this reserve might allow the receptor protein in the ventricles to be rapidly transcribed in the case of an acute need. These results vary from that of Krejčí and Tuček, 2002 that have found the amount of M_2 mRNA higher in the atria than in the ventricles. Although we do not know the reason for this discrepancy, the difference could lie in the fact that these authors did not divide the atria into parts virtually free of ganglion cells and those regions rich in ganglion cells. Moreover, these authors used another strain of rats. This can be evident from the fact that the left atrial tissue free of ganglia has the similar mRNA expression as the density of binding sites. It is also possible to mention that even if there was no difference in the mRNA expression of β_1- and β_2-adrenoceptors, the receptors, determined using radioligand binding experiments, were differently expressed in the heart regions. This is mainly the case of β_2-adrenoceptors: mRNA was almost equally distributed in all the heart regions but the transcript was especially found in the ventricular septum.

Our results have shown that mRNAs for muscarinic receptors and α_{1B}-adrenoceptors are differently distributed in the ten heart parts. Also, the densities of binding sites of muscarinic receptors and α_{1B}-adrenoceptors have been significantly different in heart parts analysed in our study. On the contrary, mRNAs of β_1- and β_2-adrenoceptors were not differently distributed in the heart regions while the respective receptors, determined using radioligand binding experiments, were. α_{1A}-adrenoceptor and β_3-adrenoceptor mRNAs have a similar distribution in the heart regions we have divided. According to our knowledge this is the first observation about the distribution of mRNAs and their transcripts in ten heart regions in which atria were divided into parts virtually free/rich of ganglion cells.

In brief, our data agree with that reviewed by Brodde and Michel (1999) for human heart in the following conclusions:

1) in the heart, the amount of β_1-adrenoceptors is much higher than that of β_2-adrenoceptors,
2) the amount of α_{1B}-adrenoceptors is much higher than that of α_{1A}-adrenoceptors,
3) the amount of α_1-adrenoceptors was much higher in the ventricles than in the atria,
4) the density of muscarinic binding sites is much higher in the atria than in the ventricles,
5) the amount of β_3-adrenoceptor mRNA is very low.

But, as it has been mentioned before, our data are new in the findings about the distribution of all receptor subtypes in the heart regions rich in ganglion cells. This fact can be of great importance in receptor regulation and in mutual interconnection between different receptor subtypes both in myocyte and neuronal cells.

In general, our data also agree with those of Wolff et al. (1998) about the distribution of α_1-adrenoceptor mRNA in different parts of the heart although we do not find the differences in the distribution of α_{1A}-adrenoceptor mRNA. Moreover, we were able to identify not only the differences in the distribution of mRNA, but also in the density of binding sites for α_{1B}-adrenergic receptors.

We have also obtained similar result as Hardouin et al. (1998) about the distribution of β_2-adrenoceptor mRNA in the different heart chambers, despite the fact that these authors did not discriminate between heart parts rich in ganglion cells and that virtually free of ganglion cells.

Even though the expression of mRNAs for M_2 muscarinic receptors, β_1- and β_2-adrenoceptors, α_{1B}-adrenoceptors was repeatedly noticed in the rat heart (for review see Brodde and Michel, 1999), there still exist doubts about the appearance of β_3-adrenoceptor mRNA in the heart. Also, the expression of α_{1A}-adrenoceptor mRNA was not regularly recorded and it is conceivable that species differences may exist (for review see Brodde and Michel, 1999). In respect to β_3-adrenoceptors, while majority of authors (for review see Gauthier et al., 2000) have determined the β_3-adrenoceptors functionally and also found their mRNA, the others (Kaumann and Molenaar, 1997; Oostendorp and Kaumann, 2000) have found only marginal effects of β_3-adrenoceptor specific agonist on heart function. Moreover, Evans et al. (1996), Berkowitz et al. (1995) and Krief et al. (1993) were not able to identify β_3-adrenoceptor mRNA in the heart. We have identified the mRNA for β_3-adrenoceptors in all heart regions. On the other hand, it is important to note that

the average expression (in all heart regions) of β_3-adrenoceptor mRNA was about 5 times lower than those of β_1-adrenoceptors and 2–3 times lower than those of β_2-adrenoceptors.

Similarly, we have confirmed that from the α-adrenoceptor subtype mRNAs, the α_{1B}-adrenoceptor mRNA is more abundant in the rat heart than that of α_{1A}-adrenoceptors. Of course, it is necessary to mention that in the human heart the amount of α_1-adrenoceptors is much smaller than in the rat heart (Brodde and Michel, 1999).

According to our knowledge this is the first study describing the differences in the density of receptor binding sites and their mRNAs in heart regions divided into parts virtually free/rich in autonomic ganglion cells.

In brief, we have found that heart receptors to transmitters that are released by autonomic nerves have different distribution in heart regions. The mRNAs for these receptors have shown less differences in the distribution throughout the heart regions assuming that there could exist the other mechanisms (i.e. transcriptional, posttranscriptional and posttranslational modification) that are able to determine the receptor binding sites density on the membranes.

Conclusions

The main findings concerning the differences in receptor mRNA/binding sites distribution between the heart parts virtually free/rich in ganglion cells are as follows:

1) The amount of mRNA of muscarinic receptors and α_{1B}-adrenoceptors differ between heart regions,
2) The density of binding sites for muscarinic receptors, α_{1B}-adrenoceptors, β_1-adrenoceptors and β_2-adrenoceptors differ between heart regions.

Expressly, the heart parts virtually free/rich of ganglion cells, differ in following:

3) We have found significant differences in the amount of α_{1B}-adrenoceptor mRNA in heart regions comprising neuronal ganglia and the other heart areas.
4) We have found significant differences in the α_{1B}-adrenoceptor and β_2-adrenoceptor density in heart regions comprising neuronal ganglia and the other heart areas.
5) The levels of minor subtypes (α_{1A}- and β_3-adrenoceptors) mRNA is some heart parts (in RA in case of α_{1A}-adrenoceptor and in LA and RG of β_3-adrenoceptor) similar to the levels of major subtypes.

These results indicate the different roles of receptors in the heart areas virtually free/rich in ganglion cells. This could be seen on the level of mRNAs as well as on the density of receptor binding sites. Also, the finding that β_3-adrenoceptor mRNA can be found in all heart areas including that virtually free/rich in ganglion cells, is new. We were able to identify the heart regions rich in ganglion cells (right atrial part rich in ganglion cells) that express relatively high levels of minor receptor subtypes mRNA. These findings could indicate that not only myocytes but also possibly other cells present in the myocardial tissue could express this minor adrenoceptor subtype. In summary, the differences in receptor expression in heart parts virtually free/rich in ganglion cells could indicate the role of appropriate heart part in the receptor mutual interconnections targeting in normal heart function.

Acknowledgement

This work was supported by Grant of SP 51/028 08 00/028 08 02, by Grant NATO LST.EAP.CLG.980745, by Grants GAUK 36/04 and 39/05 and by Grant VEGA 2/5125. The excellent technical assistance of Miss Eva Šimáková is greatly acknowledged.

References

Autelitano, D.J., Woodcock, E.A., 1998. Selective activation of alpha1A-adrenergic receptors in neonatal cardiac myocytes is sufficient to cause hypertrophy and differential regulation of alpha1-adrenergic receptor subtype mRNAs. Journal of Molecular and Cellular Cardiology 30 (8), 1515–1523.

Berkowitz, D.E., Nardone, N.A., Smiley, R.M., Price, D.T., Kreutter, D.K., Fremeau, R.T., Schwinn, D.A., 1995. Distribution of β3-adrenoceptor mRNA in human tissues. European Journal of Pharmacology 289, 223–228.

Brixius, K., Bloch, W., Pott, C., Napp, A., Krahwinkel, A., Ziskoven, C., Koriller, M., Mehlhorn, U., Hescheler, J., Fleischmann, B., Schwinger, R.H., 2004. Mechanisms of beta3-adrenoceptor-induced eNOS activation in right atrial and left ventricular human myocardium. British Journal of Pharmacology 143 (8), 1014–1022.

Brodde, O.E., Michel, M.C., 1999. Adrenergic and muscarinic receptors in the human heart. Pharmacological Review 51, 651–688.

Colecraft, H.M., Egamino, J.P., Sharma, V.K., Sheu, S.S., 1998. Signaling mechanisms underlying muscarinic receptor-mediated increase in contraction rate in cultured heart cells. Journal of Biological Chemistry 273, 32158–32166.

Evans, B.A., Papaioannou, M., Bonazzi, V.R., Summers, R.J., 1996. Expression of beta 3-adrenoceptor mRNA in rat tissues. British Journal of Pharmacology 117, 1210–1216.

Gautier, C., Tavernier, G., Charpentier, F., Langin, D., Marec, H., 1996. Functional β_3-adrenoceptors in human heart. Journal of Clinical Investigation 98, 556–562.

Gauthier, C., Langin, D., Balligand, J.L., 2000. β_3-adrenoceptors in the cardiovascular system. Trends in Pharmacological Sciences 21, 426–431.

Granneman, J., 2001. The putative β_4-adrenergic receptor is a novel state of the β_1-adrenergic receptor. American Journal of Physiology: Endocrinology and Metabolism 43, E199–E202.

Hamilton, S.E., Hardouin, S.N., Anagnostaras, S.G., Murphy, V.C.G., Richmond, K.N., Silva, A.J., Feigl, E.O., Nathanson, N.M., 2001. Alteration of cardiovascular and neuronal function in M1 knockout mice. Life Sciences 68, 2489–2493.

Hardouin, S., Bourgeois, F., Toraasson, M., Oubenaissa, A., Elalouf, J.M., Fellmann, D., Dakhli, T., Swynghedauw, B., Moalic, J.M., 1998. Beta-adrenergic and muscarinic receptor mRNA accumulation in the sinoatrial node area of adult and senescent rat hearts. Mechanisms of Ageing and Development 100, 277–297.

Kaumann, A.J., Molenaar, P., 1997. Modulation of human cardiac function through 4 β-adrenoceptor populations. Naunyn-Schmiedeberg's Archive of Pharmacology 355, 667–681.

Krejčí, A., Tuček, S., 2002. Quantitation of mRNAs for M_1 to M_5 subtypes of muscarinic receptors in rat heart and brain cortex. Molecular Pharmacology 61, 1267–1272.

Krief, S., Lönnqvist, F., Raimbault, S., Baude, B., Van Spronsen, A., Arner, P., Strosberg, D., Ricquier, D., Emorine, L.J., 1993. Tissue distribution of β3-

adrenergic receptor mRNA in man. Journal of Clinical Investigation 91, 344–349.

Michel, M.C., Hanft, G., Gross, G., 1994. Radioligand binding studies of alpha 1-adrenoceptor subtypes in rat heart. British Journal of Pharmacology 111, 533–538.

Mysliveček, J., Trojan, S., 2003. Regulation of α-adrenoceptors and muscarinic receptors in the heart. General Physiology and Biohysics 22 (1), 3–14.

Mysliveček, J., Říčný, J., Kolář, F., Tuček, S., 2003. The effects of hydrocortisone treatment on the rat heart muscarinic and adrenergic α1, α1 and α2 receptors, propranolol-resistant binding sites and on some consequent steps in intracellular signaling. Naunyn-Schmiedeberg's Archive of Pharmacology 368, 366–376.

Oostendorp, J., Kaumann, A.J., 2000. Pertussis toxin suppresses carbachol-evoked cardiodepression but does not modify cardiostimulation mediated through beta(1)- and putative beta(4)-adrenoceptors in mouse left atria: no evidence for beta(2)- and beta(3)-adrenoceptor function. Naunyn-Schmiedeberg's Archive of Pharmacology 361, 134–145.

Pott, C., Brixius, K., Bundkirchen, A., Bolck, B., Bloch, W., Steinritz, D., Mehlhorn, U., Schwinger, R.H., 2003. The preferential beta3-adrenoceptor agonist BRL 37344 increases force via beta1-/beta2-adrenoceptors and induces endothelial nitric oxide synthase via beta3-adrenoceptors in human atrial myocardium. British Journal of Pharmacology 138 (3), 521–529.

Salomonsson, M., Oker, M., Kim, S., Zhang, H., Faber, J.E., Arendshorst, W.J., 2001. Alpha1-adrenoceptor subtypes on rat afferent arterioles assessed by radioligand binding and RT-PCR. American Journal of Physiology. Renal Physiology 281 (1), F172–F178.

Tavernier, G., Toumaniantz, G., Erfanian, M., Heymann, M.F., Laurent, K., Langin, D., Gauthier, C., 2003. Beta3-Adrenergic stimulation produces a decrease of cardiac contractility ex vivo in mice overexpressing the human beta3-adrenergic receptor. Cardiovascular Research 59 (2), 288–296.

Wang, H.Z., Han, H., Zhang, L.M., Si, H., Schram, G., Nattel, S., Wang, Z.G., 2001. Expression of multiple subtypes of muscarinic receptors and cellular distribution in the human heart. Molecular Pharmacology 59, 1029–1036.

Wang, Y., Shi, H., Wang, H., 2004. Functional M3 muscarinic acetylcholine receptors in mammalian hearts. British Journal of Pharmacology 142, 395–408.

Wolff, D.W., Dang, H.K., Liu, M.F., Jeffries, W.B., Scofiled, M.A., 1998. Distribution of α1-adrenergic receptor mRNA species in rat heart. Journal of Cardiovascular Pharmacology 32, 117–122.

Zimmer, H.G., 1997. Catecholamine-induced cardiac hypertrophy: significance of proto-oncogene expression. Journal of Molecular Medicine 75, 849–859.

Naunyn-Schmiedeberg's Arch Pharmacol (2008) 378:103–116
DOI 10.1007/s00210-008-0285-8

ORIGINAL ARTICLE

The detection of the non-M_2 muscarinic receptor subtype in the rat heart atria and ventricles

Jaromir Myslivecek · Martin Klein · Martina Novakova · Jan Ricny

Received: 27 July 2004 / Accepted: 17 March 2008 / Published online: 29 April 2008

Abstract Mammal heart tissue has long been assumed to be the exclusive domain of the M_2 subtype of muscarinic receptor, but data supporting the presence of other subtypes also exist. We have tested the hypothesis that muscarinic receptors other than the M_2 subtype are present in the heart as minor populations. We used several approaches: a set of competition binding experiments with pirenzepine, AFDX-116, 4-DAMP, PD 102807, p-F-HHSiD, AQ-RA 741, DAU 5884, methoctramine and tripinamide, blockage of M_1 muscarinic receptors using MT7 toxin, subtype-specific immunoprecipitation experiments and determination of phospholipase C activity. We also attempted to block M_1–M_4 receptors using co-treatment with MT7 and AQ-RA 741. Our results show that only the M_2 subtype is present in the atria. In the ventricles, however, we were able to determine that 20% (on average) of the muscarinic receptors were subtypes other than M_2, with the majority of these belonging to the M_1 subtype. We were also able to detect a marginal fraction (6±2%) of receptors that, based on other findings, belong mainly to the M_5 muscarinic receptors. Co-treatment with MT7 and AQ-RA 741 was not a suitable tool for blocking of M_1–M_4 receptors and can not therefore be used as a method for M_5 muscarinic receptor detection in substitution to crude venom. These results provide further evidence of the expression of the M_1 muscarinic receptor subtype in the rat heart and also show that the heart contains at least one other, albeit minor, muscarinic receptor population, which most likely belongs to the M_5 muscarinic receptors but not to that of the M_3 receptors.

Keywords Heart · Immunoprecipitation · MT7 mamba toxin · Muscarinic receptors · Non-M2 muscarinic receptor · PLC activity

J. Myslivecek (✉) · M. Klein · M. Novakova
Institute of Physiology, 1st Faculty of Medicine,
Charles University,
Albertov 5,
12800 Prague, Czech Republic
e-mail: jmys@lf1.cuni.cz

J. Ricny
Prague Psychiatric Centre,
Ustavni 91,
181 03 Prague, Czech Republic

J. Ricny
Institute of Physiology,
Academy of Sciences of the Czech Republic,
Videnska 1083,
14220 Prague, Czech Republic

Introduction

Muscarinic receptors belong to the G protein-coupled receptor family, and they transduce signals of the parasympathetic nervous system, i.e. they can reduce the heart rate. To date, five muscarinic receptor subtypes have been described and cloned. The odd-numbered subtypes (M_1, M_3, M_5) stimulate phospholipase C (PLC; via pertussis toxin-insensitive G_q protein), which cleaves phosphatidilinositolbisphosphate (PIP_2) to inositoltrisphosphate (IP_3) and diacylglycerol (DAG). The even-numbered subtypes (M_2, M_4) inhibit adenylyl cyclase (AC; via pertussis toxin-sensitive G_I protein), i.e. they decrease the amount of cyclic adenosine monophosphate (Moscona-Amir et al. 1989) and decrease the activity of protein kinase A (PKA).

It has long been believed that only one subtype is present in the mammalian heart—the M_2 receptor. This assumption was experimentally supported by a study with M_2 knockout

mice (Gomeza et al. 1999). However, the concept of the heart possessing a fully homogenous muscarinic receptor population has been challenged during the last decade (reviewed concisely by Dhein et al. 2001).

Studies based upon mRNA expression

There are many papers dealing with M_1–M_5 gene expression in the heart. Gallo et al. (1993) were first to report the presence of a non-M_2 mRNA (namely, M_1) in the mammalian heart, and the presence of mRNA for the other muscarinic acetylcholine receptor (mAChR) subtypes has been reported as well (Hassal et al. 1993; Hoower et al. 1994; Sharma et al. 1996; Colecraft et al. 1998; Shi et al. 1999; Hellgren et al. 2000; Oberhauser et al. 2001; Wang et al. 2001). Quantitative determination of mRNAs for all five mAChR subtypes has been recently reported by Krejčí and Tuček (2002).

Studies based upon radioligand binding/methods to measure receptor protein

One of the pioneering studies on muscarinic receptor subtype in the heart was reported in the strictly pharmacological paper of Yang et al. (1993). These researchers concluded that the second population of muscarinic receptors in the heart cells of rats is of the M_3 subtype. Shi et al. (1999) found (by electrophysiological and pharmacological methods) not only M_3, but also M_4 receptors in canine heart tissue, and Wang et al. (2001) demonstrated the presence of multiple subtypes (M_1, M_2, M_3 and M_5) in the human heart by immunochemistry and analysis of ligand binding. The minor population of heart muscarinic receptors and, in particular, the functional heart M_3 muscarinic receptors was discussed in review of Wang et al. 2004). In agreement with these results, Pérez et al. (2006) confirmed the presence of mRNA of the M_1–M_5 mAChR subtypes in both human cardiac atria and ventricles. Using immunoblotting methods, multiple antigen blot assay (MABA) and enyzme-linked immunosorbent assay (ELISA) with subtype-specific antibodies they also confirmed the presence of M_1, M_2, M_3, M_4 and M_5 proteins in membrane preparations from both atria and ventricles. These authors also suggest that muscarinic receptor subtypes undergo discrete transitions from a non-cooperative kinetics of non-interacting monomers to a cooperative kinetics of interacting oligomers. Luthin et al. (1988) found a small population of receptors in rat heart with a high affinity for pirenzepine. However, they showed that these [^{3}H]pirenzepine-labeled cardiac receptors were not precipitated by anti-M_1 antibody but by anti-M_2 antibody. They therefore concluded that rat heart contains pirenzepine-sensitive M_2 receptors. Luthin et al. (1988) also found that anti-M_1 antibody did not precipitate QNB-labeled receptor from rat heart.

Functional studies

As mentioned, the odd-numbered muscarinic receptors have been characterized primarily as stimulators of inositol metabolism. Consequently, evidence of increased phosphoinositide metabolism following cholinergic stimulation is one of the experimental findings supporting the conclusion that heart cells express more than one muscarinic receptor subtype (see Brown and Brown 1983; Moscona-Amir et al. 1989; Nadler et al. 1993; Sterin-Borda et al. 1995; Sun et al. 1996). Wang et al. (2004, 2007) reviewed the ever-increasing body of data indicating the presence of the M_3 muscarinic receptor and the functional consequences of the presence of this receptor in human heart. As these reviews were focused mainly on the human heart and the M_3 muscarinic receptor, the question of the existence of multiple subtypes existing in the heart tissue and of species differences arises. Ford et al. (1992) only found M_2 mRNA in the guinea pig heart, although they did observe stimulation of phosphoinositide metabolism that was not explainable by the properties of the M_2 receptors. In sheep, IP_3 synthesis was found to decrease with aging (Birk and Riemer 1992), and there was also a substantial decrease in M_2 mRNA. Pharmacological characterization revealed that the muscarinic population in sheep heart is consistent with the properties of M_2 and of M_3 or M_5 receptors. Data from functional studies of phosphoinositide metabolism changes in heart tissue and the data from the studies following ontogenetic aspects of muscarinic subtypes expression are still controversial: heart atria of rats express M_1 and M_2 receptors in newborn rats, while only M_2 are expressed in adult ones (Camusso et al. 1995; Borda et al. 1997). Nadler et al. (1993) described the stimulation of IP_3 synthesis in rat ventricles. Sterin-Borda et al. (1995) proposed that positive inotropy caused by carbachol in isolated atria is secondary, mediated via the PLC-NO pathway. Sun et al. (1996) concluded that the positive inotropic effect of carbachol in myocytes from rat heart ventricles is M_3 mediated. To support their conclusion, they reported the following experimental findings: (1) the increase in the force of contraction cannot be blocked by M_1 and M_2 antagonists but it can be blocked by the M_3 antagonist; (2) IP_3 accumulation is stimulated by carbachol and blocked by the M_3 antagonist; (3) stimulation of phosphoinositide hydrolysis is pertussis toxin-insensitive. In agreement with these findings, Trendelenburg et al. (2003, 2005) demonstrated, using M_2 and M_3 knockout mice, a heterogenous population of muscarinic receptors mediating the inhibition of sympathetic transmitter release (both M_2 and M_3 receptors affect the release). Similarly, Ponicke et al. (2003) demonstrated that the G_q coupled muscarinic receptor in the heart is of the M_3 subtype. They also found an increase in the heart rate after selective stimulation of these receptors. In contrast, Colecraft et al.

(1998) described the coupling of neonatal heart (ventricular) M_1 receptors to G_q, coupling to PLC.

In summary

To date, the nature of the minor heart muscarinic subtype remains unclear. The studies dealing with gene expression are limited by the fact that they are not able to detect the receptor protein expressed in the tissue, while the functional studies are typically limited by the fact that there is no specific ligand (agonist, antagonist) that is able to affect the specific receptor subtypes.

Moreover, there are a number of questionable points that need to be taken into consideration:

1) minor subtypes can differ in different species,
2) receptor expression and/or second messenger activation can change during ontogenesis,
3) the expression of the total number of muscarinic receptors differs in the heart atria and ventricles (a fact not taken into account in all studies).

Here we attempt to quantify muscarinic receptor subtypes—other than the M_2 subtype(s)— in the atria and ventricles of rat heart using different approaches:

1) set of competition binding experiments using three concentrations of antagonist per grade with pirenzepine, AFDX-116, 4-DAMP, PD 102807, *p*-F-HHSiD, DAU 5884, AQ-RA 741, methoctramine and tripinamide;
2) blockage of M_1 muscarinic receptors using MT7 toxin;
3) subtype-specific immunoprecipitation experiments; immunoprecipitation experiments are more sensitive than the Western blots because the former do not show protein expression as there is no binding to the appropriate ligand.
4) activation of PLC and using specific competitors (pirenzepine, DAU 5884 and AFDX 384) to block the effects of carbachol;
5) attempted blocking of M_1–M_4 receptors using incubation with AQ-RA 741 and MT7.

Material and methods

Reagents

[^{3}H]NMS ([methlyl-^{3}H]-*N*-scopolamine methyl chloride; specific activity 84 Ci/mmol) and [3H]QNB (L-quinuclidinyl [L-quinuclidinyl [phenyl-4-^{3}H]benzilate; specific activity 48 Ci/mmol) were obtained from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). AQ-RA 741 ((11-({4-[4-(diethylamino)butyl]-1-piperidinyl}acetyl)-5,11-dihydro-6H-pyrido(2,3-b)) was a generous gift from Boehringer Ingelheim (Biberach, Germany), p-F-HHSiD (para-fluoro analog hexahydro-sila-difenidol hydrocholride) was obtained from RBI (Natick, MA), pirenzepine (5,11-dihydro-11-([4-methyl-1-piperazinyl]acetyl)-6H-pyrido(2,3-b) benzodiazepin-6-one), methoctramine (methoctramine tetrahydrochloride *N*,*N*′-bis [6-[[(2-methoxyphenyl)-methyl] amino]hexyl]-1,8-octane diamiline tetrahydrochloride) and 4-DAMP (4-diphenylacetoxy-*N*-methylpiperidine methiodide) were from Sigma (St. Louis, MO), compounds DAU 5884 (8-methyl-8-azabicyclo-3-endo[3.2.1]oct-3-yl-1,4-dihydro -2-oxo-3(2H)-quinazolinecarboxylic acid ester hydrochloride) and AFDX 384 (*N*-[2-[2-[(Dipropylamino) methyl]-1-piperidinyl]ethyl]-5,6-dihydro-6-oxo-11H-pyrido [2,3b] [1,4]benzodiazepine-11 -carboxamide) were from Tocris (Bristol, UK), compound PD 102807 ([carboxyethyl, methyl, 9-O-methyl)]benzoxalazine isoquinoline) was a generous gift from Parke-Davis Pharmaceutical Research, tripinamide (tripinamide dihydrochloride) was a generous gift from Prof. C. Melchiorre, MT7 (muscarinic toxin 7, green mamba toxin) was from the Peptide Institute, (Osaka, Japan), AFDX-116 ([11-[12-diethylamino-methyl]-1-piperidinil]acetyl]-5-11-dihydro-6H-pyrido-2-3-b][1,4] benzo-diazepie-6-one]) was generous gift from Prof. H. Ladinsky. Antibodies to muscarinic receptors were purchased as follows: anti-M_2 (M9558) from Sigma-Aldrich (Czech Republic), anti-M_3 (sc-9108), anti-M_4 (sc-9109) and anti-M_5 (sc-9110) from Santa Cruz Biotechnology (Santa Cruz, CA), anti-M_1 from Alomone Labs (Jerusalem, Israel) (AMR-001, lot AN-01), Santa Cruz Biotechnology (sc-9106, lot A140) or Sigma-Aldrich (M9808, lot 90 K1372). All anti-receptor antibodies used were from rabbit. Goat anti-rabbit Ig (R5506) and donkey anti-goat Ig (G7767) were obtained from Sigma-Aldrich.

Animals and preparation of homogenates

Experiments were performed on juvenile male Wistar rats aged 46–55 days. The animals were handled in accordance with the legislature of the Czech Republic and EU Guidelines, and the experimental protocol was approved by the Committee for the Protection of Experimental Animals of the Institute of Physiology. The animals were sacrificed by cervical dislocation and decapitation. The cardiac atria and left and right ventricles were isolated, connective tissue was carefully discarded and the heart tissue was weighed, cut into small pieces and homogenized with an UltraTurrax homogenizer (Janke and Kunkel, Staufen, Germany) in ice-cold medium consisting of 20 mmol l^{-1} Na-HEPES (pH 7.4) and 1 mmol l^{-1} $MgCl_2$. The homogenates were filtered through medical gaze and centrifuged for 10 min at 600 *g* at 4°C. The sediments were resuspended in homogenization medium (the same as before) and washed twice by centrifugation (10 min at 600 *g*). The supernatants were

pooled and re-homogenized. The homogenates were stored frozen at −60°C until the measurements of radioligand binding.

Cell lines

Chinese hamster ovary (CHO) cells stably transfected with the human genes for muscarinic m_1–m_5 receptors were provided by Dr. M. Brann, University of Vermont Medical School, Burlington, VT. The cells were grown in plastic dishes in Dulbecco's modified Eagle's medium with 10% calf serum and 0.005% Geneticin. They were harvested by mild trypsinization 7 days after subculturing and washed twice by centrifugation (3 min at 300 *g*).

Competition binding studies on muscarinic receptors

Competition binding studies were performed using [^{3}H]NMS as a ligand in a modified procedure of Mysliveček et al. (2003). Homogenates corresponding to 2 or 5 mg fresh tissue per tube were incubated for 120 min at 25°C in a total volume of 1 ml. The incubation medium contained NaCl (136 mmol l^{-1}), KCl (5 mmol l^{-1}), $MgCl_2$ (1 mmol l^{-1}), Na-phosphate (pH 7.4, 1 mmol l^{-1}), Na-HEPES (pH 7.4; 10 mmol l^{-1}), phenylmethylsulfonyl fluoride (0.1 mmol l^{-1}) and [^{3}H]NMS (1800 pmol l^{-1}). The non-specific binding of the radioligand was measured in tubes to which atropine (5 μmol l^{-1} final concentration) had been added before the start of the incubation. The incubation was arrested by dilution with ice-cold distilled water, followed by rapid filtration through Whatman GF/B glass fibre filters in a Brandel cell harvester. The filters were washed with distilled water, and the retained radioactivity was measured by liquid scintillation spectrometry. The range of the concentrations of antagonists used is given in the Results. In general, we used three different concentrations of antagonist per grade.

Radioligand binding studies with MT7

M_1-receptor-selective toxin (MT7, m1-Toxin1) from green mamba (*Dendroaspis angusticeps*) venom has an extremely high selectivity for the M_1 subtype (Adem et al. 1988; Max et al. 1993; Olinas et al. 2000; Carsi and Potter 2000). Two different approaches were employed:

1) the binding of [^{3}H]NMS (2 nmol l^{-1}) to homogenates preincubated 30 min with MT7 (concentration range: 10^{-11}–10^{-7} mol l^{-1});
2) saturation binding experiments with increasing concentrations of [^{3}H]NMS (0.125–4 mol l^{-1}) in tissue preincubated 30 min with MT7 (10^{-8} mol l^{-1}).

Homogenates corresponding to 2 or 5 mg fresh tissue per tube were preincubated for 60 min at 38°C in a total volume of 1 ml. Incubation with [^{3}H]NMS followed for 60 min. The incubation medium contained NaCl (136 mmol l^{-1}), KCl (5 mmol l^{-1}), $MgCl_2$ (1 mmol l^{-1}), Na-phosphate (pH 7.4; 1 mmol l^{-1}), Na-HEPES (pH 7.4; 10 mmol l^{-1}), phenylmethylsulfonyl fluoride (0.1 mmol l^{-1}). The non-specific binding, termination of incubation and radioactivity measurements were made as stated before.

Determination of M_5 muscarinic receptors

The determination of the M_5 muscarinic receptor was performed using a modified method of Reever et al. (1997). These authors pre-treated tissue in 30 μg ml^{-1} crude green mamba toxin and 1 μmol l^{-1}AQ-RA741 to block all muscarinic receptors except for than M_5. This procedure blocked 99% of the M_1, M_2, and M_4 receptors and 85% of the M_3 receptors, while sparing the majority of M_5 receptors. The remaining receptors were then measured by binding with [^{3}H]NMS. We adopted a similar procedure using MT7 toxin instead of crude venom. First, we have evaluated the binding of 0.5 nmol l^{-1} [^{3}H]NMS in CHO cells individually expressing the human muscarinic receptor subtypes (M_1–M_5). The tissue was preincubated for 60 min with MT7 toxin (10^{-6} mol l^{-1}) and AQ-RA 741 (10^{-6} mol l^{-1}) and then incubated for 60 min with [^{3}H]NMS. For comparison, the binding of [^{3}H]NMS to specific subtypes pre-treated with AQ-RA 741 (10^{-6} mol l^{-1}) only was also performed.

Membrane preparation, receptor labeling, solubilization and immunoprecipitation

Membranes from stably transfected CHO cells individually expressing the human muscarinic receptor subtypes (M_1–M_5) and from rat atria and ventricles were prepared by homogenization (Ultra-Turrax homogenizer, 2×20 s, 20,500 rpm, on ice in 10 mmol l^{-1} Hepes pH 7.4+ 250 mmol l^{-1} sucrose+2 mmol l^{-1} EDTA+1 mmol l^{-1} PMSF) followed by slow centrifugation (Hettich centrifuge, 5 min/800 rpm/4°C). Pellets from the first centrifugation were rehomogenized and spun again under the same conditions. Combined supernatants were centrifuged at a higher speed (Sorvall centrifuge, SS34 rotor, 30 min/ 15,000 rpm/4°C). The resulting sediments were resuspended in glass-teflon homogenizer in 10 mmol l^{-1} Hepes pH 7.4+1 mmol l^{-1}EDTA+1 mmol l^{-1} EGTA+0.2 mmol l^{-1}PMSF (protein concentration: 1–2 mg ml^{-1} for cell lines and 3–4 mg ml^{-1} for the tissue). Muscarinic receptors present in the membranes were radiolabeled with [^{3}H]QNB (a subtype-nonselective antagonist with a high affinity and very slow dissociation; 2 nmol l^{-1} for 2 h at 30°C). The samples were then cooled on ice and centrifuged (Sorvall centrifuge, SS34 rotor, 15,000 rpm/30 min/4°C). Receptors

from the pellet were solubilized with digitonin and sodium cholate (extraction in 10 mmol l^{-1} Hepes pH 7.4/1 mmol l^{-1} EDTA+1 mmol l^{-1}EGTA+1% digitonin+0.2% sodium cholate; 1 h on ice, centrifugation 15,000 rpm/30 min/4°C). The mAChR content in the membranes was quantified by incubation with [^{3}H]QNB (0.2 ml; saturation binding: 0.1–2 nmol l^{-1} QNB for 2 h at 30°C) followed by filtration through Whatman GF/B glass fiber filters. The concentration of solubilized receptors was determined by gel filtration on a G-25 Sephadex column (1×18 cm, equilibrated with 10 mmol l^{-1} Hepes pH 7.4+1 mmol l^{-1} EDTA+1 mmol l^{-1} EGTA+0.2% Triton X-100; 0.3 ml solubilized receptors applied; 0.5-ml fractions of the eluate measured by liquid scintillation spectrometry). Incubation in the presence of 5 μmol l^{-1} atropin served as a control for non-specific binding. Soluble receptors were immunoprecipitated by subtype-selective antisera; all manipulations were carried out in the cold room (4°C) or on ice: 1 ml extract (containing 0.2–0.8 pmol l^{-1} mAChR) was incubated overnight with 5 μl (1 μg) of subtype-selective antisera (rabbit non-immune serum served as the negative control). After 12–16 h of incubation, 5 μl of goat anti-rabbit immunoglobulin (Ig)G was added to the extract-antisera solution; this was followed 4 h later with 10 μl of donkey anti-goat IgG. The solution was allowed to co-precipitate overnight. The samples were then centrifuged (Hettich centrifuge, 12,000 rpm/10 min/4°C), the immunoprecipitate were washed 3×1 ml 10 mmol l^{-1} Hepes pH 7.4+0.1% digitonin+0.02% sodium cholate, dissolved in 1% sodium dodecyl sulfate (SDS) (0.2+0.2+0.1 ml) and measured by liquid scintillation spectrometry.

Phospholipase C activity

Phospholipase C activity was measured using the enzymatic assay described by Dwivedy and Pandey (1999). Briefly, the tissue was homogenized on ice (10 mg of tissue per 100 μl of buffer) in 20 mmol l^{-1} Tris-HCl (pH 7.4), 2 mmol l^{-1} EGTA, 5 mmol l^{-1} EDTA, 1.5 mmol l^{-1} pepstatin, 2 mmol l^{-1} leupeptin, 0.5 mmol l^{-1} phenylmethylsulfonylfluoride, 0.2 U ml^{-1} aprotinin, and 2 mmol l^{-1} dithiothreitol using two short pulses of 10 s with a 30 s pause between pulses. For the assay, 5 μg of protein per tube was used. The tissue was incubated in incubation buffer (20 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} $CaCl_2$ and 100 mmol l^{-1} KCl, pH 7.4) containing 10 mmol l^{-1} LiCl, PIP_2 substrate (50 μmol l^{-1} unlabeled PIP_2), 2.0 mCi ml^{-1} [^{3}H]PIP_2 and 0.5 mg ml^{-1} cetrimide) in a total volume of 100 μl at 37°C (Thermoblock Biometra T1) for 40 min with buffer (basal activity) or with the addition of carbachol (1 mmol l^{-1}), carbachol + pirenzepine (1 μmol l^{-1}), carbachol + DAU 5884 (1 μmol l^{-1}) or carbachol + AFDX 384 (10 μmol l^{-1}). The reaction was terminated by the addition of 500 μl of 1 *M* HCl and 500 μl of a mixture of chloroform/methanol (1:1 vol/vol). The tubes were vigorously mixed and centrifuged at 1000 *g* for 10 min. The aqueous (upper) phase was transferred to a scintillation vial containing scintillation fluid, and the radioactivity counted. Each experiment had its blank in which the protein suspension was added after the reaction had been stopped with chloroform/methanol.

Data treatment

Radioligand binding data were treated as described previously (Mysliveček et al. 2003) with the use of GRAPHPAD PRISM ver. 5.01 (GraphPad Software, San Diego, CA) program. In detail, the curves were fitted using non-linear regression. The goodness of fit was determined using R^2, that was determined using the equation $R^2=1-SS_{reg}/SS_{tot}$, where SS_{reg} is sum of the squares of the distances of the points from the best-fit curve determined by nonlinear regression, and SS_{tot} is sum of the square of the distances of the points from a horizontal line through the mean of all *Y* values. The decision of preferred model was based on Akaike's information criterion (AIC; Akaike 1974) comparing one-site competition and two-site competition. Protein determination was performed using Peterson's modification of Lowry's method. Statistical significance of differences between means was evaluated with an unpaired two-tailed Student's *t*-test.

Results

Competition binding with muscarinic antagonists

Atria

Preliminary saturation binding experiments ($n=3$) with [^{3}H]NMS revealed the density of receptors to be 778.2±25.63 fmol mg^{-1} protein and the affinity (K_D) to be 1.10±0.10 nmol l^{-1}. The competition binding experiments with pirenzepine (10^{-10}–10^{-3} *M*) revealed one binding site in the heart atria [p*KI*=6.14±0.04, *nH* (Hill coefficient for binding of the radioligand) = 0.97±0.1]. Similarly, the competition with AFDX-116 (concentration range: 10^{-9}–10^{-4} *M*) showed a p*KI*=7.50±0.09 (*nH*=0.94±0.08). Finally, the competition with 4-DAMP (10^{-11}–10^{-5}) revealed only one binding site (p*KI*=7.81±0.03; n*H*=0.93±0.06). These results correspond to a uniform (M_2) muscarinic receptor population. The curves are shown in Fig. 1.

Ventricles

Preliminary saturation binding experiments ($n=3$) with [^{3}H]NMS revealed that the densities of receptors in the left and right ventricles were 290.2±4.4 and 367.9±8.9 fmol mg^{-1}

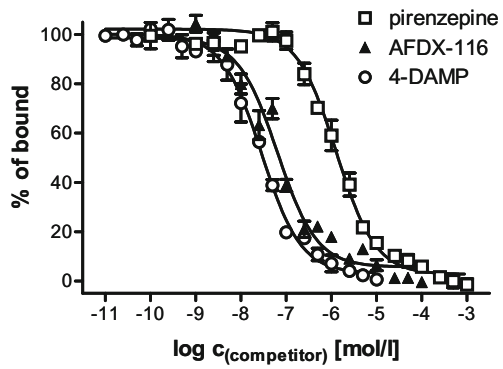


Fig. 1 The competition binding of [^{3}H]NMS and different antagonists in heart atria. Data are from three independent experiments in which tissue from three to four animals was pooled. Data are presented as means ± standard error of the mean (SEM). *Abscissa* Common logarithm of competitor concentration in mol/l, *ordinate* percentage of total bound

protein, respectively. The K_D was 1.43±0.06 and 1.01± 0.15 nmol l^{-1}, respectively. The competitions were carried on whole ventricles only, without differentiating the left and right ventricles. These competition-binding experiments with pirenzepine (10^{-10}–10^{-3} *M*) revealed two binding sites. Similarly, the competition with AFDX-116 (concentration range: 10^{-9}–10^{-4} *M*), 4-DAMP (10^{-11}–10^{-5} *M*), DAU 5884 (10^{-11}–10^{-6} *M*) and AQ-RA 741 (10^{-10}–10^{-5} *M*) showed two binding sites. In contrast, competitions with PD 102807 (10^{-9}–10^{-4} *M*), p-F-HHSiD (10^{-9}–10^{-4} *M*), tripinamide (10^{-10}–10^{-4} *M*) and methoctramine (10^{-10}–10^{-5} *M*) revealed one binding site. The data (pKI_1 or p*KI*, pKI_2 and fraction 1) are summarized in Table 1. The curves are shown in Fig. 2.

Subtype-specific immunoprecipitation experiments

Labeling of mAChR with [^{3}H]QNB was highly efficient (approaching complete saturation) and stable. The yield of mAChR solubilized from membranes was 40–55%, depending on the concentration of the protein. Dissociation of the label and/or degradation of receptors in solubilized receptor preparations were assessed by gel filtration. We found that 80–90% of the initial activity of the solubilized preparation (as measured immediately after solubilization) was still present in the extracts after 90 h under the conditions used for immunoprecipitation. The efficiencies of the receptor-specific antisera to immunoprecipitate the respective receptor protein were quantitated using receptors prepared from appropriate CHO cell lines. The results are shown at Fig. 3. At the antibody concentration used for the experiments (5 μl ml^{-1}), the efficiency of immunoprecipitation was 41% for anti-M_2 serum, 32% for anti-M_3, 45% for anti-M_4 and 50% for anti-M_5. We also tested anti-m_1 antisera from Alomone, Santa Cruz Biotechnology and Sigma (lot numbers given in Reagents), but all showed precipitation efficiencies that were too low to be applicable in our experiments. The specificities of antisera were confirmed by measuring the precipitation of comparable amounts of cloned mAChR subtypes. For heart tissue, the potential cross-reactivity of antibodies against M_2 receptors is of particular importance. The selectivities of individual antisera, as established by the immunoprecipitating receptor extract from m_2-transfected CHO cells, are illustrated in Fig. 4a: immunoprecipitation by anti-M_3, M_4 and M_5 was at the level of non-specific binding (control nonspecific trapping with non-immune serum). In a number of other cases, our antisera did not show cross-reactivity. It is also important to note that CHO cells themselves express different M receptor subtypes. Our assessment of the sensitivity of the immunoprecipitation is based on the following parameters and criteria. We were using 18,000–60,000 dpm specifically bound in tissue extracts. Non-specific precipitation represents 1.4–1.6% of total labeled receptors. Standard deviations (SD) of blanks were 30–70 dpm (for triplicates, depending on total radioactivity;

Table 1 Affinity (p*KI*) and the Hill coefficient for binding of the radioligand (*nH*) from binding experiments in the ventricles

Competitor[a]	Fraction 1	pKI_1 or p*KI*	pKI_2	*nH*
Pirenzepine	0.13±0.05	8.76±0.26	6.28±0.09	0.63±0.04
AFDX-116	0.86±0.004	7.78±0.02	5.65±0.11	0.78±0.04
4-DAMP	0.20±0.01	9.23±0.65	7.92±0.16	0.73±0.07
DAU 5884	0.35±0.02	9.81±0.11	7.23±0.07	0.47±0.02
AQ-RA 741	0.81±0.11	8.24±0.16	6.78±0.88	0.79±0.05
PD 102807	–	6.19±0.29	–	0.90±0.08
Tripinamide	–	7.73±0.09	–	1.15±0.12
Methoctramine	–	7.82±0.11	–	1.48±0.13
p-F-HHSiD	–	5.92±0.07	–	1.11±0.08

Data are means from three to five independent experiments in which tissue from three to four animals was pooled. The data are expressed as means ± standard error of the mean (SEM). These data are averaged values form the above-mentioned number of independent experiments. Note, that fraction 1 does not mean the major population in all cases

[a] See Reagents for the definitions of the competitors

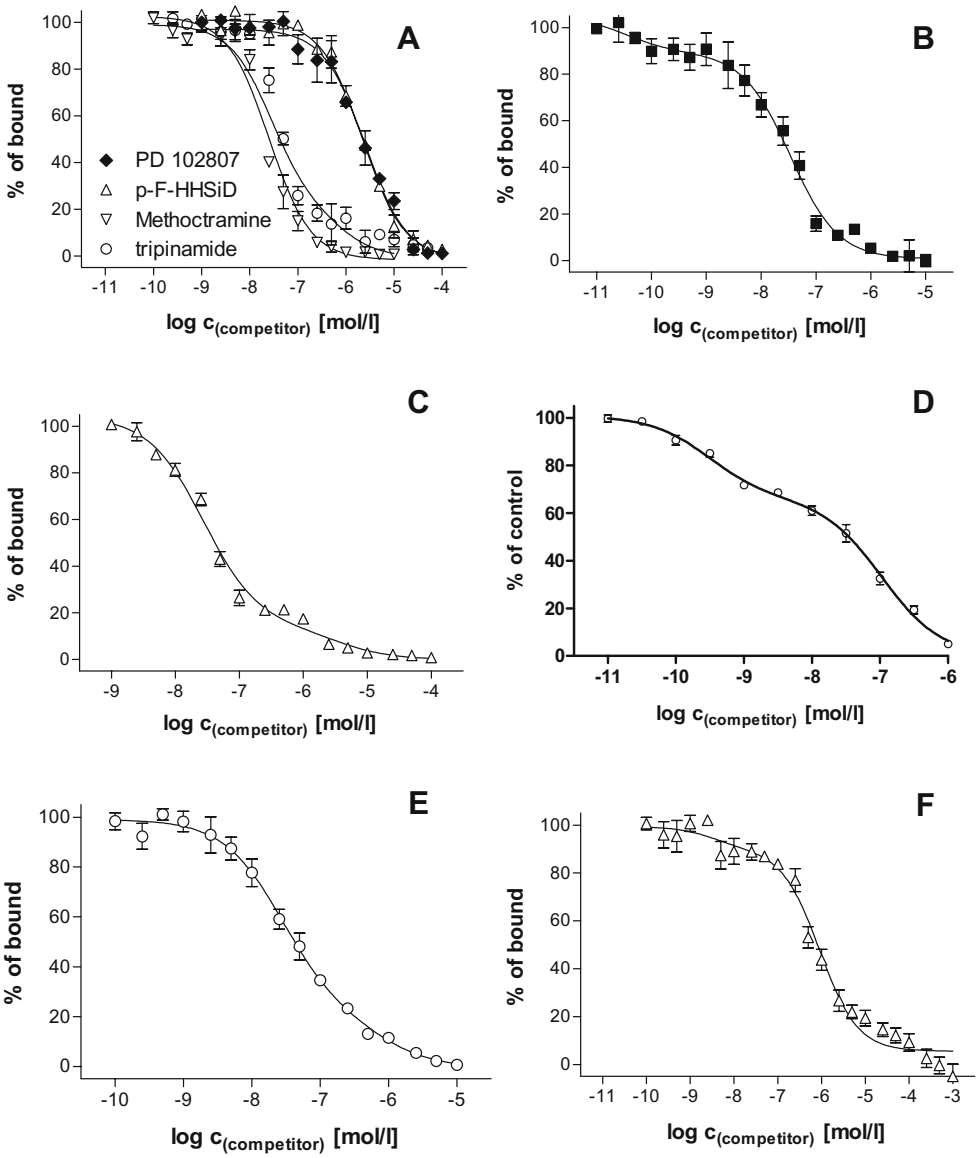


Fig. 2 The competition binding curves [^{3}H]NMS and different antagonists in heart ventricles. Data are from three to five independent experiments in which tissue from three to four animals was pooled. Data are presented as the means ± SEM. **a** The competition binding curves of antagonists which revealed one binding site only, **b–f** The competition binding curves of antagonists that revealed two binding sites: **b** competition with 4-DAMP, **c** competition with AFDX-116, **d** competition with DAU 5884, **e** competition with AQ-RA 741, **f** competition with pirenzepine. *Abscissa* Common logarithm of competitor concentration in mol/l, *ordinate* percentage of total bound

coefficient of variation 0.07–0.15). Specific signals representing threefold SD was considered to be the statistically correct limit of detection. We would be able to detect 90 dpm specifically precipitated from 18,000 dpm and 210 dpm precipitated from 60,000 dpm. Taking into account a precipitation efficiency of 32% (anti-M_3 antibody), we arrive at 0.4–1.6% of total receptors as the detection limit of our procedure (1.6% being the worst-case scenario). The preparations used in our experiments contained 0.35–0.5 pmol mAChR mg^{-1} protein for atria and 0.15–0.2 pmol mg^{-1} protein for ventricles. Illustrative results with immunoprecipitations from the left ventricular extract are shown at Fig. 4b. Combined estimations, expressed as the percentage of receptor subtypes precipitated by subtype-specific antibody (corrected for precipitation efficiency) from the three parts of the rat heart are listed in Table 2. Our anti-M_2 antibody precipitated 96±8%

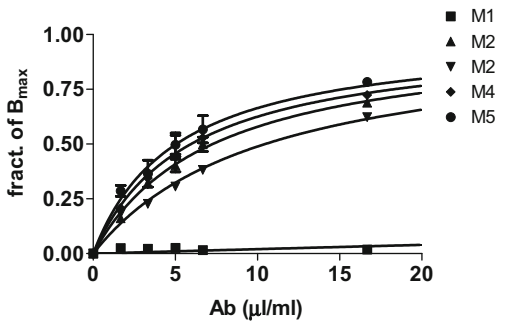


Fig. 3 Efficiency of immunoprecipitation (determined with receptors isolated from Chinese hamster ovary (CHO) cells transfected with the appropriate mAChR subtype). At concentration 5 µl ml^{-1} (concentration used for heart receptor immunoprecipitation), anti-M_2 antibody precipitated 41%, anti-M_3 32%, anti-M_4 45% and anti-M_5 50% of the receptors. This was only case in which [^{3}H]QNB was used as the radioligand

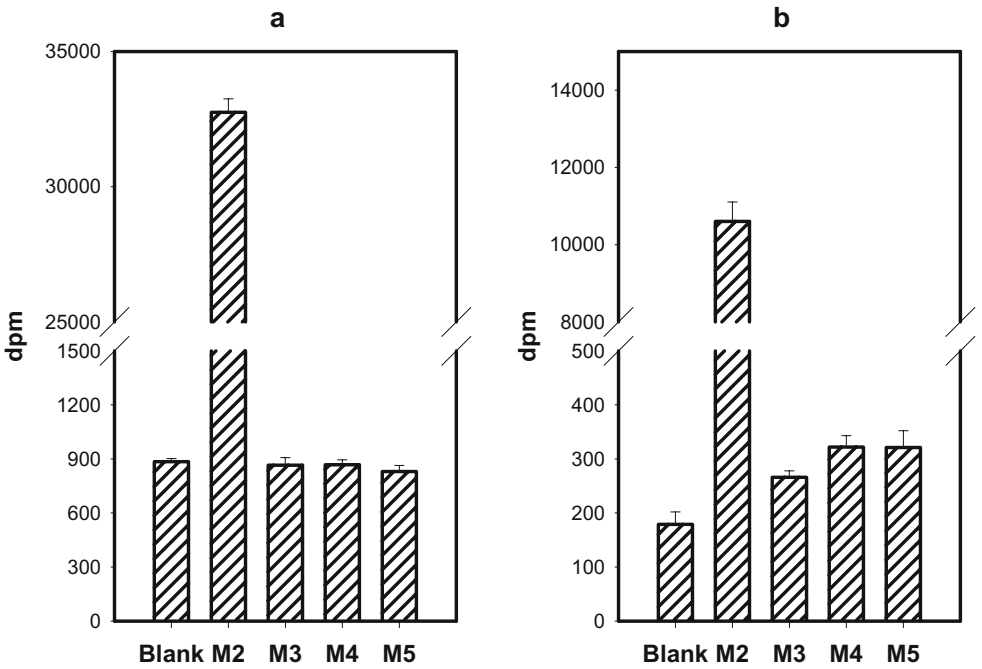


Fig. 4 **a** Selectivities of individual antisera were examined by using receptor extract from m_2-transfected CHO cells. Non-immune rabbit serum was used as to determine non-specific binding. The precipitation of M_2 receptors by anti-M_3, M_4 and M_5 was at the level of non-specific binding. **b** Representative experiment with immunoprecipitation of individual receptor subtype from the left ventricle. ^{3}H-QNB was used as radioligand (as this experiments served as control to immunoprecipitation experiments)

of labeled receptors, anti-M_3, M_4 and M_5 antisera showed low but detectable specific precipitation of their respective receptors which was close to the detection limit of our procedure. The anti-m_1 antisera available to us (from Alomone, Sigma and Santa Cruz Biotechnology, respectively) were not suitable for quantitative estimation of the M_1 subtype.

Binding to homogenates preincubated with MT7

Atria

The preincubation of homogenates with MT7 caused no substantial decrease in the binding of [^{3}H]NMS at all concentrations (data not shown). These results correspond to the presence of a uniform (M_2) muscarinic receptor population.

Ventricles

The preincubation of homogenates with MT7 (10^{-11}–10^{-7} *M*) caused a concentration-dependent decrease in which the data were best fitted with the model containing two binding sites: one comprising 9±1%, with apparent pK_I=9.7 and the other with apparent pKI_2=6.84, consistent with a major M_2 muscarinic receptor population (Fig. 5). These results can indicate that the minor population of muscarinic receptors in heart ventricles belongs mainly to the M_1 subtype.

Saturation binding experiments in tissue preincubated with MT7

The comparison of the saturation experiments in presence and absence of MT7 are shown in Table 3. These experiments confirmed that in the heart atria there is no M_1 subtype. On the other hand both left and right ventricles showed decrease in [^{3}H]NMS binding after MT7 pre-treatment.

Competition binding experiments in tissue preincubated with MT7

The competition binding experiments with pirenzepine showed that there are two binding sites. Pre-treatment of

Table 2 Data on the immunoprecipitation of the M_1–M_5 subtypes (expressed as percentage of receptor subtype precipitated by subtype-specific antibody and corrected for differences in the efficiencies of individual antisera)

	M_1	M_2	M_3	M_4	M_5
Atria (%)	ND	103±5	0.4±0.14	0.7±0.24	0.5±0.31
Left ventricle (V)	ND	93±8	1.5±0.21	2.5±0.37	2.5±0.53
Right ventricle (V)	ND	95±7	1.1±0.2	0.6±0.12	0.6±0.18

ND, Not determined

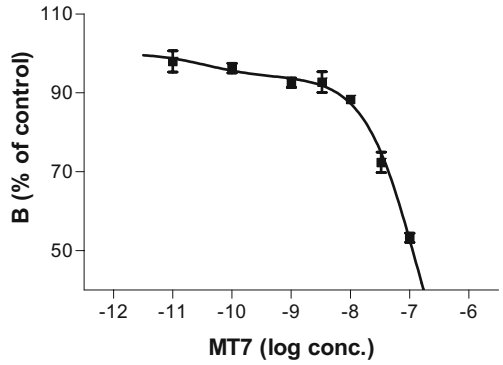


Fig. 5 Binding of [^{3}H]NMS to ventricular membranes preincubated with MT7 toxin. Data obtained from the fitted curve gave best fit with the two-binding site model: high-affinity (accounting for 9±1.1%) with pKI_{B1}=9.7 and low-affinity with pKI_2=6.84

the tissue with MT7 toxin abolished this biphasic binding in both the left (pKI=6.11±0.22; n=3) and right ventricles (pKI=6.37±0.06; n=3), clearly indicating a pKI for M_2 muscarinic receptors (see Table 4).

Determination of M_5 muscarinic receptors using the modified method of Reever et al. (1997) in CHO cells stably expressing M_1-M_5 muscarinic receptors

Co-incubation with both AQ-RA741 and MT7 toxin has been supposed to be a useful modification of Reever et al.'s method (1997) for M_5 muscarinic receptor determination. Therefore, we attempted to replace crude venom by MT7 toxin. In CHO cells stably transfected with human M_1–M_5 muscarinic receptor subtype, this procedure (MT7 toxin + AQ-RA 741) resulted in an 81% inhibition of binding in M_1 muscarinic receptors, 79% inhibition of binding in M_2 muscarinic receptors, 49% inhibition of binding in M_3 muscarinic receptors, 47% inhibition of binding in M_4 muscarinic receptors and 4% inhibition of binding in M_5 muscarinic (Fig. 6). Moreover, the comparison of binding in the presence of AQ-RA 741 only and the combination of drugs showed that the purified toxin behaves differently from crude the venom used by Reever et al. (1997): in the latter case the venom decreased the binding to all muscarinic receptor subtypes, while here MT7 decreased the binding to M_1 subtype only (see Fig. 6). Moreover, the addition of MT7 toxin also increased the binding to the M_3 and M_4 subtypes. Therefore, this method can not routinely replace the method of Reever for M_5 receptor determination.

Determination of M_5 muscarinic receptors in rat ventricles

Despite the fact that the combination of AQ-RA 741 and MT7 toxin can not be used for determining the M_5 muscarinic receptor subtype, we attempted to use this procedure to determine the "sub-minor" muscarinic receptor population that is probably masked in the amount of minor population based on the results of the competition binding experiments. Co-incubation of rat ventricles with both AQ-RA741 and MT7 toxin showed that there is 5.5±1.6% of muscarinic receptor subtypes that can not be blocked using this combination of drugs.

PLC activity

Carbachol was able to increase PLC activity (to 152% of control). This increase was not diminished by addition of the M_3 selective antagonist DAU 5884, but was inhibited by pirenzepine (M_1 antagonist) and AFDX 384 (M_5 antagonist). These data show that M_1 and M_5 receptors are able to affect PLC activity in the heart ventricles. All data are shown in Fig. 7.

Table 3 The densities and affinities of muscarinic receptors in heart atria and left and right ventricles pre-treated with MT7 or without pre-treatment (control)

Control		MT7	
Atria			
Bmax (fmol mg protein^{-1})	778.25±25.63	Bmax (fmol mg protein^{-1})	772.80±59.20
K_D (nmol l^{-1})	1.10±0.10	K_D (nmol l^{-1})	0,97±0,02
Left ventricles			
Bmax (fmol mg protein^{-1})	290.25±4.35	Bmax (fmol mg protein^{-1})	258.40±3.10 (89%)*
K_D (nmol l^{-1})	1.43±0.059	K_D (nmol l^{-1})	1.41±0.14
Right ventricles			
Bmax (fmol mg protein^{-1})	367.92±8.88	Bmax (fmol mg protein^{-1})	304.55±15.95 (83%)*
K_D (nmol l^{-1})	1.01±0.15	K_D (nmol l^{-1})	0.760±0.02

n=3–4
Percentage of control value is given in parenthesis; the asterisk (*) indicates when it is significantly different from control at $p<0.05$)

Table 4 Antagonist affinity constants (log affinity or p*KI* values) for muscarinic receptor subtypes

Antagonist	Receptor subtype				
	M_1	M_2	M_3	M_4	M_5
Pirenzepine	7.8–8.5	6.3–6.7	6.7–7.1	7.1–8.1	6.2–7.1
Methoctramine	7.1–7.8	7.8–8.3	6.3–6.9	7.4–8.1	6.2–7.2
4-DAMP	8.6–9.2	7.8–8.4	8.9–9.3	8.4–9.4	8.9–9.0
AF-DX 116	5.8–6.9	7.1–7.3	5.5–6.6	6.2–7.0	5.4–6.6
AF-DX 384	7.3–7.5	8.2–9.0	7.2–7.8	8.0–8.7	6.3
DAU 5884	9.40±0.04	7.40±0.05	8.80±0.03	8.50±0.02	Not known
Tripinamide	7.2–7.4	7.9–9.3	5.15–5.33	6.68–6.92	Not known
PD 102807	5.3–5.5	5.7–5.9	6.2–6.7	7.3–7.4	5.2–5.5
p-F-HHSiD	6.68–7.3	6.01–6.6	7.5–7.84	7.2	6.6–7.0
AQ-RA 741	7.6–7.8	8.21–8.9	7.4–7.5	7.9–8.2	5.8–6.1
MT7 toxin	9.8	<6	<6	<6	<6

Data were obtained from Caulfield and Birdsal 1998; Dhein et al. 2001; Doods et al. 1993, 1994; Eglen and Nahorski 2000; Choppin et al. 1999; Lazareno et al. 1998; Buckley et al. 1990; Bolognesi et al. 1998; Wang et al. 2004.

Discussion

Binding experiments

In general, the pharmacological characterization of the minor muscarinic receptor subtype presents some difficulties. There is no specific antagonist to one receptor subtype, except for mamba MT7 toxin (more than three orders of selectivity towards M_1 receptors). Therefore, we employed several approaches using MT7 toxin: (1) competition of heart tissue with MT7 toxin (decrease in binding means that there are M_1 receptors), (2) saturation binding after pre-incubation with MT7 toxin (if there is decrease in the Bmax, the minor M_1 population should be present) and (3) competition with pirenzepine after pre-incubation of tissue with MT7 toxin (if the M_1 receptor was present, then there should not be two population as all M_1 muscarinic receptors would be blocked, i.e. it would also prove that pirenzepine inhibits M_1 muscarinic receptors). It has also been shown previously that co-incubation of crude mamba toxin with AQ-RA 741 resulted in inhibition of all muscarinic subtypes except for M_5 (see Material and methods). Therefore, we tried to replace crude venom by MT7 toxin.

Our data shows that in the atria there is no other subtype than M_2. In the ventricles, we were able to identify certain amounts (13–35%, 19.6% on average) of non-M_2 muscarinic receptor subtype, which is the most probably the M_1 subtype. Also, a marginal fraction (about 5.5%) can be detected using a combination treatment consisting of MT7

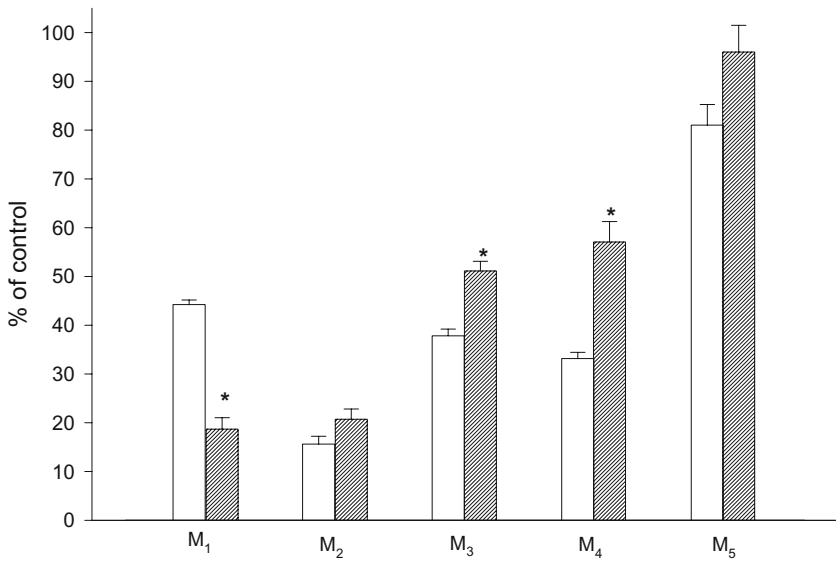


Fig. 6 Binding of [^{3}H]NMS to CHO cells transfected with appropriate human mAChR subtype. Comparison of preserved binding sites when incubated with AQ-RA 741 (10^{-6} mol l^{-1}) only [*left (empty) columns*] and with pre-treatment with AQ-RA 741 and MT7 toxin, as described in the Methods section [*right (shaded) columns*]. *$p<0.05$ difference between groups (AQ-RA 741 alone and combination of AQ-RA 741 and MT7 toxin)

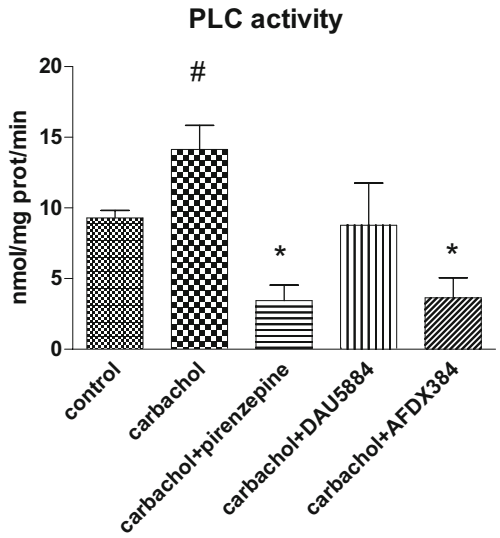


Fig. 7 Phospholipase C (*PLC*) activity in rat heart ventricles. *Ordinate* PLC activity expressed as nmol of IP_3 mg protein^{-1} min^{-1}. *$p<0.05$ indicates a difference from carbachol, #$p<0.05$ indicates a difference from control. The tissue was incubated for 40 min with buffer (basal activity) or with the addition of carbachol (1 mmol l^{-1}), carbachol + pirenzepine (1 μmol l^{-1}), carbachol + DAU 5884 (1 μmol. l^{-1}) or carbachol + AFDX 384(10 μmol l^{-1})

toxin and AQ-RA 741. It is possible to exclude the possibility that the minor subtypes are M_4 receptors (PD 102 807 did not show a biphasic curve). In addition, the possibility that the minor population belongs to M_3 receptors is rather improbable. There are more reasons why we draw this conclusion: (1) the competition with p-F-HHSiD showed a monophasic curve with p*KI*=5.92 that corresponds to the p*KI* in M_2 cloned receptors (6.01–6.6) but is inconsistent with the p*KI* for the M_3 subtype (7.5–7.84); (2) methoctramine and tripinamide, which have a low affinity for the M_3 muscarinic receptors (see Table 4) did not show biphasic curves; (3) DAU 5884, which is a highly functionally potent M_3 muscarinic antagonist, revealed pKI_1=9.81, which corresponds to the M_1 muscarinic receptors (p*K*=9.4, see Table 4) but not to M_3 (p*K*=8.8) and pKI_2=7.23, which correspond to the M_2 muscarinic receptors; (4) the competition and saturation binding experiments with almost the most specific drug for M_1 muscarinic receptors (MT7 toxin) clearly showed that the majority portion (9–17%) of the second muscarinic population belongs to the M_1 muscarinic receptors; (5) competitions with pirenzepine, AFDX-116 and 4-DAMP and competition with pirenzepine in tissue pre-treated with MT7 toxin showed that the minor population belongs to the M_1 subtype, but it is not possible to absolutely exclude any proportion of the M_5 subtype as the pKI_2 in competitions with AFDX-116 can correspond both to the M_1 and the M_5 subtype; (6) pKI_2 computed from AQ-RA 741 - [^{3}H]NMS competition lies between the estimated values of M_1, M_3 and M_5 receptors; therefore, we employed the method described in the Material and methods for identifying the M_5 receptors in order to be able to discriminate between the subtypes. Taken together, these data show that minor population (if it is up to 20%) represents binding to more receptor subtypes that can (as we show by functional experiments) contribute to cardiostimulating effects (increase in PLC activity) and, therefore, they protect the heart (like α_1-adrenoceptors; see Brodde et al. 2001).

Immunoprecipitations

Our data on immunoprecipitation confirmed that the population of M_2 receptors is not the only one in the heart ventricles, but their proportion in the total amount of receptors is very small (about 2.5% of M_5, 2.5% of M_4 and 1.5% of M_3 in the left ventricles and less than 1.1% of M_3 in the right ventricles). Also, these data confirmed our findings on the uniform muscarinic population in the atria. Unfortunately, the antibodies available to us were not able to detect the M_1 receptors. On the other hand, the immunoprecipitations also revealed that on the level of protein, there is another minor population of muscarinic receptor subtypes other than M_1. These data show that on the protein level the amount of M_5 and M_4 in the left ventricle is slightly higher than the amount of M_3 muscarinic receptor protein. Therefore, we cannot exclude the possibility that the heart minor muscarinic population is heterogeneous and consists of more than one muscarinic receptor subtype. This finding can be also supported by data from the competition binding studies with MT7 and AQ-RA 741.

Subtype-specific antibodies to muscarinic acetylcholine receptors (targeted to specific peptide sequences of all five subtypes) have been developed (Luthin et al. 1988; Levey et al. 1990, 1991; Wall et al. 1991a, b; Li et al. 1991; Mayanil et al. 1991; Yasuda et al. 1992) and used for quantifying muscarinic acetylcholine receptors by immunoprecipitation in a number of tissues in various species. In general, immunoprecipitation experiments are more sensitive than Western blots as they do not show protein expression despite the fact it does not bind to the appropriate ligand. Therefore, we employed this method instead of blotting. No cross-reactivity of the anti-M_3, anti-M_4 or anti-M_5 sera with the M_2 subtype was detected. Similarly to our data, Luthin et al. (1988) found that there was a small population of receptors in the rat heart with a high affinity for pirenzepine. However, these researchers showed that these [^{3}H]pirenzepine-labeled and also [^{3}H]QNB-labeled cardiac receptors were not precipitated by an anti-M_1 antibody but by an anti-M_2 antibody. They concluded that rat heart contains pirenzepine-sensitive M_2 receptors. These results

appear to negate our conclusion. On the other hand, it is improbable that other M_1-specific irreversible antagonist (MT7 toxin) can also detect pirenzepine-sensitive M_2 receptors. Therefore, these findings are more likely able to explain why these sites were not precipitated by an anti-M_1 antibody, i.e. it is more likely that these sites are immunoprecipitation pirenzepine-insensitive M_1 receptors. Hardouin et al. (2002), in contrast, did not detect QNB-labeled receptors in mouse heart and reported unchanged mAChR-mediated activation of PLC in the atria and ventricles of M_1-deficient mice, while Perez et al. (2006) detected all muscarinic receptor subtypes in the human heart using ELISA. However, the data from the latter study opens the question of whether this method should be used as it may lead to misinterpretation of the results given that in the atrium, septum and left and right ventricles these authors obtained a higher optical density for M_1 than for M_2 muscarinic receptors. Nevertheless, this study presumes a very important fact—that muscarinic receptors can co-operate with each other to form hetero- and homooligomers. Taken together with the conclusions of Luthin et al. (1988) and Colecraft et al. (1998), the data reviewed by Wang et al. (2004, 2007) and our data, the concept of specific binding properties of minor muscarinic receptor population is one of the most probable explanations of the different results obtained in the heart tissue.

Co-incubation of tissue with AQ-RA 741and MT7

Although, as is stated elsewhere, the co-incubation of AQ-RA 741 with MT7 toxin did not result in blockage of M_1–M_4 muscarinic receptors (as was in the case of crude venom toxin), these results indicate that the majority of this "sub-minor" population belongs to M_5 muscarinic receptors. This population, which is resist to co-incubation of tissue with MT7 and AQ-RA 741, can not belong to the M_3 and M_4 muscarinic receptors (as revealed by the competition experiments). The remaining subtypes (M_1, M_2 and M_5) should have the ratio (see Fig. 6) 19:21:96. Therefore, it is possible to suggest when 5.5% of the binding sites are resistant to combination with MT7 and AQ-RA 741, 0.9% belong to the M_1 muscarinic receptor subtype, 0.8% to the M_2 subtype and 3.9% to the M_5subtype.

PLC activity assay

The results of our experiments show that carbachol increased the production of IP_3, which could be inhibited most of all by pirenzepine, suggesting the primary importance of M_1 muscarinic receptors in phosphoinositide metabolism. On the contrary, DAU 5884, which is considered to be a functionally highly selective M_3 muscarinic antagonist, failed to inhibit the carbachol action. Inhibition of the carbachol effect was also recorded with 10^{-5} mol l^{-1} AFDX 384, i.e. the concentration that is able to block M_5 muscarinic receptors. These data are in part in agreement with the results of Dobrev et al. (2002) who were able to functionally characterize both M_1 and M_3 muscarinic receptors in human heart atria.

General discussion

Our data are in good agreement with that of Colecraft et al. (1998) who identified the M_1 receptors in functional and single-cell RT-PCR mRNA assays on neonatal rat ventricular myocytes. Yang et al. (1993), however, found the M_3 muscarinic receptors as the minor heart subtype, and Wang et al. (2004, 2007) have also reviewed this subtype as a probable minor subtype. The findings that favor the presence of M_3 muscarinic receptors are those of Ponicke et al. (2003). On the other hand, some findings have shown that human atria appear to possess both M_1, M_3 and M_5 receptors (Wang et al. 2001; Willmy-Matthes et al. 2003). There is no mRNA for M_3-receptors in rat atrial myocytes (single-cell PCR; Meyer et al. 2001). Moreover, Fisher et al. (2004) clearly demonstrated that heart rate responses remained unchanged in M_3 receptor-deficient mice, whereas bronchoconstrictor responses were totally abolished in these animals. These findings also question the role of the M_3 muscarinic receptor subtype in the regulation of the heart rate. On the other hand, Krejčí and Tuček (2002) found about 100-fold less mRNA for M_1 muscarinic receptors than for the other minor subtype. These results are in antinomy to that of Colecraft et al. (1998) who identified no other minor mRNA than M_1 in rat heart ventricular myocytes. The explanation of this disparity could lie in the fact that Colecraft et al. employed myocytes, but Krejčí and Tuček used a mixture of both myocyte and non-myocyte cells. We have also recently identified differences in receptor expression in cardiac tissue with neuronal ganglia and tissue that is virtually free of neuronal cells (Mysliveček et al. 2004, 2006).

It is also necessary to state that species differences and disease state may be the key in resolving the controversy surrounding the existence of non-M_2 receptors in the heart. For example, data available for canine atria clearly show the absence of M_1 and the presence of M_2 through M_4, but there is uncertainty regarding M_5 (Wang et al. 2001, 2004, 2007). Data on the human atrium clearly show that M_1 is present at the protein level and is functional in regulating potassium channels (Dobrev et al. 2002). Although it cannot be excluded that M_1 receptors are expressed in a diseased atrium only, it appears that their existence is a general phenomenon, although M_1 is not present in all species (i.e. dog; see data of Wang's group.).

In addition to data on the minor muscarinic population in the rat heart, we have also demonstrated that MT7 toxin can not be used instead of crude mamba venom (Reever et al. 1997) in simple routine M_5 muscarinic receptor detection. The increase in receptor binding (see Fig. 6) in comparison to [^{3}H]NMS binding when following a pre-treatment with AQ-RA 741 only suggests positive allosteric binding or positive co-operative binding with MT7 toxin.

Taken together, all these data provide further evidence that there are minor muscarinic receptor subtypes in the heart. Also, based on the results using different approaches, it is possible to suggest that the expression and function of minor muscarinic receptor subtypes is subject to very complicated interrelationships.

Acknowledgements We would like to thank Prof. Lincoln Potter from University of Miami for his advice concerning MT7 and M_5 muscarinic receptor detection, Dr. Maria-Laura Bolognesi from the University of Bologna for her advice with tripinamide properties, Prof. C. Melchiorre from the same University for his generous gift of this compound and Prof. K. Ladinsky from the Istituto De Angeli, S.p.A for his generous gift of compound AFDX-116. We appreciate the gift of AQ-RA 741 from Boehringer Ingelheim, Biberach, Germany (Dr. Doods and Dr. Mueller). The gift of PD 102807 from Parke-Davis is greatly appreciated. The work was supported mainly by grant GAUK 519/07 from Grant Agency of Charles University and in part by grant GAUK 289/07 from the same Grant Agency. Another support was obtained from grants MSMT CR LC554, MSM0021620849, GACR 304/08/0256 and by the Research project AV0Z 50110509. We gratefully acknowledge the technical assistance by Mrs. Dana Ungerová, Romana Ondřejová, Olga Martínková and Eva Šimáková.

References

Adem A, Asblom A, Johnasson G, Mbuga PM, Karlsson E (1988) Toxins from the venom of the green mamba dendroaspis angusticeps that inhibit the binding of quinuclinidil benzilate to muscarinic acetylcholine receptors. Biochim Biophys Acta 968:340–345

Akaike H (1974) A new look at the statistical model identification. IEEE Trans Autom Control 19:716–723

Birk E, Riemer RK (1992) Myocardial cholinergic signaling changes with age. Pediatr Res 31:601–605

Bolognesi ML, Minarini A, Budriesi R, Cacciaguerra S, Chiarini A, Spampinato S, Tumiatti V, Melchiorre C (1998) Universal template approach to drug design: polyamines as selective muscarinic receptor Antagonists. J Med Chem 41:4150–4160

Borda ES, Perez Leiros C, Camusso JJ, Bacman S, Sterin-Borda L (1997) Differential cholinoceptor subtype-dependent activation of signal transduction pathways in neonatal versus adult rat atria. Biochem Pharmacol 53:959–967

Brodde O-E, Bruck H, Leineweber K, Seyfarth T (2001) Presence, distribution and physiological function of adrenergic and muscarinic receptor subtypes in the human heart. Basic Res Cardiol 96:528–538

Brown SL, Brown JH (1983) Muscarinic stimulation of phosphatidylinositol metabolism in atria. Mol Pharmacol 24:351–356

Buckley NJ, Hulme EC, Birdsall NJ (1990) Use of clonal cell lines in the analysis of neurotransmitter receptor mechanisms and function. Biochim Biophys Acta 1055:43–53

Camusso JJ, Sterin-Borda L, Rodriguez M, Bacman S, Borda E (1995) Pharmacological evidence for the existence of different subtypes of muscarinic acetylcholine receptors for phosphoinositide hydrolysis in neonatal versus adult rat atria. J Lipid Mediat Cell Signal 12:1–10

Carsi J, Potter LT (2000) M1-toxin isotoxins from the green mamba (Dendroaspis Angusticeps) that selectively block M1 muscarinic receptors. Toxicon 38:187–198

Caulfield MP, Birdsal NJM (1998) International union of pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Pharmacol Rev 50:279–290

Choppin A, Stepan GJ, Loury DN, Watson N, Eglen RM (1999) Characterization of the muscarinic receptor in isolated uterus of sham operated and ovariectomized rats. Br J Pharmacol 127:1551–1558

Colecraft HM, Egamino JP, Sharma VK, Sheu S-S (1998) Signaling mechanisms underlying muscarinic receptor-mediated increase in contraction rate in cultured heart cells. J Biol Chem 73:32158–32166

Dhein S, van Koppen CJ, Brodde OE (2001) Muscarinic receptors in the mammalian heart. Pharmacol Res 44:161–182

Dobrev D, Knuschke D, Richter F, Wettwer E, Christ T, Knaut M, Ravens U (2002) Functional identification of m1 and m3 muscarinic acetylcholine receptors in human atrial myocytes: influence of chronic atrial fibrillation. Circulation 106(Suppl):11–154

Doods HN, Willim KD, Boddeke HW, Entzeroth M (1993) Characterization of muscarinic receptors in guinea-pig uterus. Eur J Pharmacol 250:223–230

Doods HN, Entzeroth M, Ziegler H, Mayer N, Holzer P (1994) Pharmacological profile of selective muscarinic receptor antagonists on guinea-pig ileal smooth muscle. Eur J Pharmacol 253:275–281

Dwivedi Y, Pandey GN (1999) Repeated administration of dexamethasone increases phosphoinositide-specific phospholipase C activity and mRNA and protein expression of the phospholipase C beta 1 isozyme in rat brain. J Neurochem 73:780–790

Eglen RM, Nahorski SR (2000) The muscarinic M(5) receptor: a silent or emerging subtype? Br J Pharmacol 130:13–21

Fisher JT, Vincent SG, Gomeza J, Yamada M, Wess J (2004) Loss of vagally mediated bradycardia and bronchoconstriction in mice lacking M2 or M3 muscarinic acetylcholine receptors. FASEB J 18:711–713

Ford APDW, Eglen RM, Whiting RL (1992) Analysis of muscarinic cholinoceptors mediating phosphoinositide hydrolysis in Guinea pig cardiac muscle. Eur J Pharmacol Mol Pharmacol Section 225:105–112

Gallo MP, Alloatti G, Carola E, Oberto A, Cesare Levi R (1993) —1 muscarinic receptors increase calcium current and phosphoinositide turnover in Guinea-pig ventricular cardiocytes. J Physiol (London) 471:41–60

Gomeza J, Shannon H, Konstenis E, Felder C, Zhang L, Brodkin J, Grinberg A, Sheng H, Wess J (1999) Pronounced pharmacologic deficits in M_2 muscarinic acetylcholine receptor knockout mice. Proc Natl Acad Sci USA 96:1692–1697

Hardouin SN, Richmond KN, Zimmerman A, Hamilton SE, Feigl EO, Nathanson NM (2002) Altered cardiovascular responses in mice lacking the M1 muscarinic acetylcholine receptor. J Pharmacol Exp Ther 301:129–137

Hassal CJS, Stanford SC, Burnstok G, Buckley NJ (1993) Co-expression of four muscarinic receptor genes by the intrinsic neurons of the rat and Guinea-pig heart. Neuroscience 56:1041–1048

Hellgren I, Mustafa A, Riazi M, Sulliman I, Sylvén C, Adem A (2000) Muscarinic M_3 receptor subtype gene expression in the human heart. Cell Mol Life Sci 57:175–180

Hoower DB, Baisden RH, Ximoy SX (1994) Localization of muscarinic receptor MRNAs in rat heart and intrinsic cardiac ganglia by in situ hybridization. Circ Res 75:813–820

Krejčí A, Tuček S (2002) Quantitation of MRNAs for M_1 to M_5 subtypes of muscarinic receptors in rat heart and brain cortex. Mol Pharmacol 61:1267–1272

Lazareno S, Gharagozloo P, Kuonen D, Popham A, Birdsall NJ (1998) Subtype-selective positive cooperative interactions between brucine analogues and acetylcholine at muscarinic receptors: radioligand binding studies. Mol Pharmacol 53:573–589

Levey AI, Stormann TM, Brann MR (1990) Bacterial expression of human muscarinic receptor fusion proteins and generation of subtype-specific antisera. FEBS Lett 275:65–69

Levey AI, Kitt CA, Simonds WF, Price DL, Brann MR (1991) Identification and localization of muscarinic acetylcholine receptor proteins in brain with subtype-specific antibodies. J Neurosci 11:3218–3226

Li M, Yasuda RP, Wall SJ, Wellstein A, Wolfe BB (1991) Distribution of M2 muscarinic receptors in rat brain using antisera selective for M3 receptors. Mol Pharmacol 40:28–35

Luthin GR, Harkness J, Artymyshyn RP, Wolfe BR (1988) Antibodies to a synthetic peptide can be used to distinguish between muscarinic acetylcholine receptor binding sites in brain and heart. Mol Pharmacol 34:327–333

Max SI, Liang JS, Potter LT (1993) Stable allosteric binding of m1-toxin to m1 muscarinic receptors. Mol Pharmacol 44:1171–1175

Mayanil CSK, Richardson RM, Hosey MM (1991) Subtype-specific antibodies for muscarinic receptors. I. Characterization using transfected cells and avian and mammalian cardiac membranes. Mol Pharmacol 40:900–907

Meyer T, Wellner-Kienitz MC, Biewald A, Bender K, Eickel A, Pott L (2001) Depletion of phosphatidylinositol 4,5-bisphosphate by activation of phospholipase C-coupled receptors causes slow inhibition but not desensitization of G protein-gated inward rectifier K+ current in atrial myocytes. J Biol Chem 276:5650–5658

Moscona-Amir E, Henis YI, Sokolovsky M (1989) Aging of rat heart myocytes disrupts muscarinic receptor coupling that leads to inhibition of CAMP accumulation and alters the pathway of muscarinic-stimulated phosphoinositide hydrolysis. Biochemistry 28:7130–7137

Mysliveček J, Říčný J, Kolář F, Tuček S (2003) The effects of hydrocortisone treatment on the rat heart muscarinic and adrenergic a1, b1 and b2 receptors, propranolol-resistant binding sites and on some consequent steps in intracellular signaling. NS Arch Pharmacol 368:366–376

Mysliveček J, Říčný J, Palkovits M, Kvetňanský R (2004) The effects of short-term immobilization stress on muscarinic receptors, beta-adrenoceptors and adenylyl cyclase In different heart regions. Ann NY Acad Sci 1018:315–322

Myslivecek J, Novakova M, Palkovits M, Krizanova O, Kvetnansky R (2006) Distribution of mRNA and binding sites of adrenoceptors and muscarinic receptors in the rat heart. Life Sci 79:112–120

Nadler E, Barnea O, Vidne B, Isakov A, Shavit G (1993) Positive inotropic effect in the heart produced by acetylcholine. J Basic Clin Physiol Pharmacol 4:229–248

Oberhauser V, Schwertfeger E, Rutz T, Beyersdorf F, Rump LC (2001) Acetylcholine release in human heart atrium. Influence of muscarinic acetylcholine autoreceptors, diabetes, and age. Circulation 103:1638–1643

Olinas MC, Maullu C, Adem A, Mulugeta E, Karlsson E, Onali P (2000) Inhibition of acetylcholine muscarinic M_1 receptor function by the M_1-selective ligand muscarinic toxin 7 (MT-7). Br J Pharmacol 131:447–452

Perez CC, Tobar ID, Jimenez E, Castaneda D, Rivero MB, Concepcion JL, Chiurillo MA, Bonfante-Cabarcas R (2006) Kinetic and molecular evidences that human cardiac muscle express non-M2 muscarinic receptor subtypes that are able to interact themselves. Pharmacol Res 54:345–355

Ponicke K, Heinroth-Hoffmann I, Brodde OE (2003) Demonstration of functional M3-muscarinic receptors in ventricular cardiomyocytes of adult rats. Br J Pharmacol 138:156–160

Reever CM, Ferrari-DiLeo G, Flynn DD (1997) The M5 (m5) receptor subtype: fact or fiction? Life Sci 60:1105–1112

Sharma VK, Colecraft HM, Wang DX, Levey AI, Grigorenko EV, Yeh HH, Sheu SS (1996) Molecular and functional identification of M_1 muscarinic acetylcholine receptors in rat ventricular myocytes. Circ Res 79:86–93

Shi H, Wang H, Wang Z (1999) Identification and characterization of multiple subtypes of muscarinic acetylcholine receptors and their physiological functions in canine hearts. Mol Pharmacol 55:497–507

Sterin-Borda L, Echague AV, Leiros CP, Genaro A, Borda E (1995) Endogenous nitric oxide signalling system and the cardiac muscarinic acetylcholine receptor-inotropic response. Br J Pharmacol 115:1525–1531

Sun LS, Huber F, Robinson RB, Bilezikian JP, Steinberg SF, Vulliemoz Y (1996) Muscarinic receptor heterogeneity in neonatal rat ventricular myocytes in culture. J Cardiovasc Pharmacol 27:455–461

Trendelenburg AU, Gomeza J, Klebroff W, Zhou H, Wess J (2003) Heterogeneity of presynaptic muscarinic receptors mediating inhibition of sympathetic transmitter release: a study with M2- and M4-receptor-deficient mice. Br J Pharmacol 138:469–480

Trendelenburg AU, Meyer A, Wess J, Starke K (2005) Distinct mixtures of muscarinic receptor subtypes mediate inhibition of noradrenaline release in different mouse peripheral tissues, as studied with receptor knockout mice. Br J Pharmacol 145:1153–1159

Wall SJ, Yasuda RP, Hory F, Flagg S, Martin BM, Ginnis EI, Wolfe BB (1991a) Production of antisera selective for M1 muscarinic receptors using fusion proteins: distribution of M1 receptors in rat brain. Mol Pharmacol 39:643–649

Wall SJ, Yasuda RP, Li M, Wolfe BB (1991b) Development of an antiserum against M3 muscarinic receptors: distribution of M3 receptors in rat tissues and clonal cell lines. Mol Pharmacol 40:783–789

Wang HZ, Han H, Zhang LM, Si H, Schram G, Nattel S, Wang ZG (2001) Expression of multiple subtypes of muscarinic receptors and cellular distribution in the human geart. Mol Pharmacol 59:1029–1036

Wang Z, Shi H, Wang H (2004) Functional M_3 muscarinic acetylcholine receptors in mammalian hearts. Br J Pharmacol 142:395–408

Wang H, Lu Y, Wan Z (2007) Function of cardiac M3 receptors. Autonom Autacoid Pharmacol 27:1–11

Willmy-Matthes P, Leineweber K, Wangemann T, Silber RE, Brodde OE (2003) Existence of functional M3-muscarinic receptors in the human heart. Naunyn Schmiedebergs Arch Pharmacol 368:316–319

Yang CM, Chen F-F, Sung T-C, Hsu H-F, Wu D (1993) Pharmacological characterization of muscarinic receptors in neonatal rat cardiomyocytes. Am J Physiol 265:C666–C673

Yasuda RP, Ciesla W, Flores LR, Wall SJ, Li M, Satkus SA, Weisstein JS, Spagnola BV, Wolfe BB (1992) Development of antisera selective for M4 and M5 muscarinic cholinergic receptors: distribution of M4 and M5 receptors in rat brain. Mol Pharmacol 43:149–157

Stress, January 2010; 13(1): 22–35

ISSN 1025-3890 print/ISSN 1607-8888 online
DOI: 10.3109/10253890902849840

informa
healthcare

Sexual dimorphism in stress-induced changes in adrenergic and muscarinic receptor densities in the lung of wild type and corticotropin-releasing hormone-knockout mice

MARTINA NOVAKOVA[1], RICHARD KVETNANSKY[2], & JAROMIR MYSLIVECEK[1]

[1]*1st Faculty of Medicine, Institute of Physiology, Charles University, Prague, Czech Republic, and* [2]*Institute of Experimental Endocrinology, Slovak Academy of Sciences, Bratislava, Slovakia*

(*Received 24 September 2008; revised 25 February 2009; accepted 25 February 2009*)

Abstract

We tested the hypothesis that single and repeated immobilization stress affect densities of α_1-adrenoceptor (α_1-AR) and β-AR subtypes, muscarinic receptors (MR), adenylyl cyclase activity (AC) and phospholipase C activity (PLC) in lungs of male and female wild type (WT) and corticotropin-releasing hormone gene (CRH-knockout (KO)) disrupted mice. We found sex differences in the basal levels of α_1-AR subtypes (females had 2–3 times higher density of receptors than males) and MR (males had twice the density found in females). In marked contrast, β-AR subtype densities did not differ between sexes. CRH gene disruption decreased all three studied receptors in intact mice (to 20–50% of WT) in both sexes (except β_1-AR in females). Stress induced sexually dimorphic responses, while all α_1-AR subtypes decreased in females (to 30% of control approximately), only α_{1A}-AR level diminished (about 50%) in males. β_1-AR decreased in males (to about 40%) but remained stable in females. β_2-AR diminished in females (to about 20–60%) and also in males (to about 30–60%). MR decreased in both sexes (approximately to 50%). AC activity diminished in males (to $<$50%) while PLC activity was not changed. In CRH-KO mice, the stress response was severely diminished. Paradoxically, the receptor response to stress was less affected by CRH-KO in males than in females. AC activity did not change in CRH-KO mice. In conclusion, in mice the stress reaction is sexually dimorphic and an intact hypothalamo-pituitary–adrenocortical system is required for the normal reaction of pulmonary adrenergic and MR to stress.

Keywords: *stress, males, females, CRH knockout, adrenoceptors, muscarinic receptor*

Introduction

Lung tissue expresses several subtypes of adrenoceptors (ARs). β_2-ARs are the most abundant subtype and of major importance in lung physiology. They are localized in the smooth muscles and they are responsible for bronchodilatation. However, they are also expressed in alveoli. β_2-AR are also found on epithelial cells of the respiratory tract (Aksoy et al. 2002). Moreover, another β-AR subtype—β_1-AR are expressed in lung tissue in humans (Mak et al. 1996) and mice (Ota et al. 1993). Gene expression of β_3-AR has been shown in porcine lung, while in rat β_3-AR mRNA was not detected (McNeel and Mersmann 1999). β-AR are mainly located in the lung parenchyma (Carswell and Nahorski 1983; Hislop et al. 2002; Abraham et al. 2003), which has higher β-AR density than bronchial and tracheal tissue. The β_1-AR/β_2-AR ratio is similar in all parts of the lung and is about 20–25/80–75. Besides the β-AR, lung tissue also expresses α-AR (subtype α_{1A}, α_{1B}, α_{1D}; (Faure et al. 1994), which have also been detected at the mRNA level in mouse tissue (Alonso-Llamazares et al. 1995). The data on lung α_1-AR distribution are sparse: there are only reports about α_1-AR density in rat lung vessels (Nozik-Grayck et al. 2006) and murine lung parenchyma (Yang et al. 1998). Although the role of α_1-AR in lung function is assumed to be minor, some findings have revealed a role of low-affinity prazosine-binding sites (α_{1L} receptors) in allergic bronchoconstriction (Nobata et al. 2002). Moreover, it has been shown that in both

Correspondence: J. Myslivecek, 1st Faculty of Medicine, Institute of Physiology, Charles University, Albertov 5, 12 800 Prague, Czech Republic. Tel: 420 2 24968485. Fax: 420 2 24918816. E-mail: jmys@lf1.cuni.cz

guinea-pig and rat isolated tracheal tissue, α-AR-mediated contraction appeared to involve the activation of α_1-AR (Preuss et al. 1998). Surprisingly, some papers described only two subtypes of α_1-AR in lung tissue (Yang et al. 1998). Recently, we identified all α_1-AR subtypes in rat lung using radioligand-binding competition studies (Novakova and Myslivecek 2005). This is supported by finding that all three subtypes mRNAs are expressed in the lung tissue (Faure et al. 1994). However, the relationship between mRNA and protein expression is not consistent (Zhong and Minneman 1999).

Multiple subtypes of muscarinic receptors (MR) are expressed in the lung tissue (M_1, M_2, and M3). The balanced ratio between M_2 and M_3 MR in the respiratory tract is essential for normal function of the airways (Fryer and Jacoby 1998; Kadota et al. 2001). In tracheal smooth muscle, the amount of M_2 and M_3 mRNA is comparable but in the alveoli M_3 MR mRNA dominates (Kadota et al. 2001). While M_2 MR are responsible for inhibition of β-AR mediated relaxation of bronchial muscles, M_3 receptor activation directly contracts the smooth bronchial muscle (Fryer and Jacoby 1998). Likewise β-AR, MR (M_1 subtype) is also found on epithelial cells (Fryer and Jacoby 1998), indicating that in these cells functional antagonism may exist. In contrast to β-AR, MR is mainly found in the trachea and bronchi, with a more than 20 times higher receptor density than in the lung parenchyma (Abraham et al. 2007).

There are sex differences in anatomy and physiology of the lung in mice (Chang and Mitzner 2007). In detail, females have smaller alveoli and greater surface area, males have greater total lung capacity. Sex differences also exist for dead space volume, vital capacity, static compliance, inspiratory reserve capacity. There are also strain differences in this regard: in some strains there are gender differences while in the others the lung parameters are similar between the sexes. In humans, the large airways grow faster than parenchymal tissue in young females, while the growth of the large airways in young males tends to reveal so called dysanaptic growth that leads to relatively narrower airways in young males (Carey et al. 2007). With respect to development of asthma, it is important to note gender differences in airway hyperresponsiveness in C57Bl/6 mice that cannot be seen in the hyperresponsive A/J strain (Chang and Mitzner 2007). Moreover, it seems that there is no difference in baseline resistance between male and female mice, but significant differences exist in airway responsiveness to cholinergic challenges—females are less responsive (Chang and Mitzner 2007).

Many reports have shown the effects of stress on lung immune functions (Kanemi et al. 2005) but the changes in AR subtypes and MR under stress have not been studied in depth. With respect to the function of AR, stress can provide a useful investigative approach. Stress can increase norepinephrine (NE) and epinephrine (EPI) release (Pacak and Palkovits 2001). The main source of circulating EPI in stress is the adrenal medulla, however, about 70% of circulating NE is from the peripheral sympathoneural system (Kvetnansky et al. 1979). Moreover, the peripheral cholinergic system (via MR) is affected by stressful stimuli as well (Myslivecek and Kvetnansky 2006). Stress may also involve increased glucocorticoid hormone secretion (with peak circulating levels within 30 min for most stressors), with effects on AR and MR. These effects can be direct, i.e. via glucocorticoid response elements (GRE), which are present in the promoter region of the β_2-AR (Cornett et al. 1998) and β_1-AR genes (Tseng et al. 2001). However, the effects of glucocorticoid hormones on muscarinic M_2 receptors are rather indirect (Zhou et al. 2001). GRE sequences are also found on promoter regions of α_{1B}-AR (Gao and Kunos 1993), but the binding and functional consequences of glucocorticoid action are unknown. The α_{1A}-AR gene contains six GREs (Scanga and Schwinn 1998). Such GRE sequences can differ between species (Tseng et al. 2001).

Glucocorticoids are essential for activation of phenylethanolamine *N*-methyltransferase (PNMT) gene transcription in stress (Kvetnansky et al. 2006). This enzyme catalyzes the biosynthesis of EPI. In transgenic mice lacking the gene for corticotropin-releasing hormone (CRH-KO) the amount of circulating glucocorticoid under stress is dramatically decreased (Jeong et al. 2000; Kvetnansky et al. 2006). Moreover, CRH-KO mice fail to increase glucocorticoid levels during the circadian peak (Muglia et al. 1997). In these mice, PNMT expression is reduced, and NE is not efficiently methylated to EPI. Hence, plasma EPI levels are reduced and NE levels increased (Jeong et al. 2000).

CRH-KO ($^{-/-}$) mice born of mating between homozygous CRH-KO males and females have impaired lung function shortly after delivery that leads to death on the first postnatal day (Venihaki and Majzoub 1999). This fatal respiratory failure occurs because CRH is essential for glucocorticoid secretion and hence the stimulation of surfactant synthesis. In contrast, there is no postnatal mortality in CRH-KO mice from heterozygote mating ($CRH^{+/-}$ male/ $CRH^{+/-}$ female), indicating that the heterozygote mothers produce sufficient glucocorticoid for lung maturation in the homozygous offspring. Accordingly, glucocorticoid addition to drinking water for $CRH^{-/-}$ pregnant mice prevents the mortality of pups.

We have shown that acute immobilization stress down-regulates MR, and β_1- and β_2-AR in the heart (Myslivecek et al. 2004). Here, we investigated if α_1-AR and β-AR subtypes, and MR are altered by stress in lung tissue. We also assessed effects of glucocorticoid status on α-AR and β-AR subtypes. We sought sex differences because sex differences have

an important role in the development of asthma (Almqvist et al. 2008), and airway diameter is a result of AR/MR action. In humans, stress is implicated in the development and manifestations of asthma (Vig et al. 2006), while the role of AR and MR in the lung parenchyma under stress has not been studied before.

The aim of this study was to test the hypothesis that lung parenchyma levels of α_{1A}-AR, α_{1B}-AR, α_{1D}-AR, β_1-AR, β_2-AR, and MR, and consequently second messenger synthesizing enzyme activity, are altered in $CRH^{-/-}$ mice, with sex differences. We also expected responses to stress would be different in $CRH^{-/-}$ mice, with sex differences.

Methods

Animals

Mice were treated in accordance with the legislature of the Czech Republic, Slovakia, and the European Union legislature, and the experimental protocol was approved by the Committees for the Protection of Experimental Animals of the 1st Medical Faculty, Charles University, Prague, and of the Institute of Experimental Endocrinology in Bratislava. CRH-KO mice were originally obtained from Harvard Medical School, Department of Endocrinology, Boston and then bred in our own animal facility. The wild type (WT) line was a mixed 129SVJ/C57Bl6 line. Mice were maintained under controlled environmental conditions (12/12 h light/dark cycle, 22 ± 1°C, lights on at 6 a.m.). Food and water were available *ad libitum*. For the study 81 males, of which 41 were WT and 40 were CRH-KO, and 42 females, of which 21 were WT and 21 CRH-KO were used. The females were housed separately from males and showed the Lee–Boot effect (i.e. synchronized oestrous cycles; Ma et al. 1998). Moreover, stress can suppress oestrous cycling (Jeong et al. 1999), so similar levels of sex hormones were expected in the females. The CRH-KO mice were obtained by mating of homozygous mice and the $CRH^{-/-}$ genotype was confirmed by DNA isolation from tail samples with subsequent PCR. CRH-KO pregnant mice were given glucocorticoid in the drinking water (corticosterone 10μg/ml) to enable the pups to survive (Venihaki and Majzoub 1999).

Experimental protocol and preparation of tissue

The mice (male and female $CRH^{-/-}$, body weight 20–25 g, 11–13 weeks old and their WT ($CRH^{+/+}$) age matched controls) were exposed to immobilization stress for 120 min (first immobilization) or repeatedly for 120 min across seven consecutive days (seventh immobilization). Immobilization was done by taping (with adhesive tape) all four legs, in the prone position, to a fixed board. The mice endured the stress procedure well and all survived the sevenfold exposure.

Mice were killed without anaesthetic by decapitation 3 h after the end of the last 2 h immobilization (i.e. after one or seven immobilizations). Lungs were collected, the main airways were discarded and the remaining lung parenchyma with adjacent bronchioli was flash frozen and stored at −80°C until analysis. The tissue of each mouse was used separately in each investigation.

EPI and NE determination

Trunk blood for catecholamine quantification was collected into heparinized Eppendorf tubes, centrifuged at 10,000*g* for 20 min, and separated plasma was stored at −70°C. The plasma concentrations of and NE were determined in 50 μl aliquots of plasma using a radioenzymatic method with subsequent thin layer chromatography of methylated products of EPI and NE, and detection by scintillation counting (Peuler and Johnson 1977).

Radioligand-binding experiments

Saturation binding. The tissue was weighed and homogenized for two or three pulses of 20–30 s in a homogenizer [Ultra-Turrax® T25 basic IKA®–Werke (Staufen, Germany) 24,000 rpm] in ice cold Tris–EDTA buffer (Tris–HCl 50 mmol l^{-1}, EDTA 2 mmol l^{-1}, pH adjusted to 7.4). The tubes were cooled on ice throughout.

In pilot experiments, we compared binding to membranes and to whole homogenates. Membranes were prepared as follows: the homogenate was centrifuged at 600*g* for 10 min (Hettich Micro 22R, Tuttlingen, Germany), the supernatant was collected and the sediment was re-suspended in buffer and centrifuged again. The second supernatant was collected, mixed with the first and centrifuged for 25 min at 31,990*g*. The supernatant was discarded; the sediment was re-suspended in buffer and centrifuged as before (25 min, 31,990*g*). We found similar ligand binding to whole homogenates and to membranes, but the disintegrations per minute per mg of tissue was greater in homogenates, so we used whole homogenates for the measurements.

Measurements were made as described previously (Myslivecek et al. 2003, 2004, 2006). Briefly, the amount of MR-binding sites (B_{max}) were computed by non-linear regression of data obtained in saturation experiments from binding of 65–2000 pmol/l [^{3}H]QNB to homogenates, in duplicates. Non-specific binding was determined in the presence of 5 μmol l^{-1} atropine. The amount of β-AR-binding sites (B_{max}) was computed from saturation experiments with binding of 65–2000 pmol/l [^{3}H]CGP 12177 to the tissue homogenates, in duplicates. Non-specific

binding was determined in the presence of 5 μmol l^{-1} propranolol. The amount of α_1-AR-binding sites (B_{max}) was computed from saturation experiments with binding of 93.75–3000 pmol/l [^{3}H]prazosin to the tissue homogenates, in duplicates. Non-specific binding was determined in the presence of 100 μmol l^{-1} phentolamine. B_{max} and the affinity constant (K_D) were computed by non-linear regression using the GraphPad Prism 5.01 program (GraphPad Software, San Diego, CA, USA). Incubations were at 38°C for 2 h with [^{3}H]QNB and 1 h with [^{3}H]CGP 12177. For α_1-AR, the incubation was for 90 min at 25°C; incubation times were optimized previously (Myslivecek et al. 2003, 2004, 2006; Novakova and Myslivecek 2005). The incubations were terminated by filtration through Whatman GF/B glass fibre filters pre-soaked with distilled water in a Brandel cell harvester (Brandel, Inc., Gaithersburg, MD, USA); the filters were washed three times with ice cold water. Radioactivity retained on the filters was measured by scintillation counting in Bray's solution after desiccation overnight. The affinity constants (K_D) were computed and used for the 'single-point' measurement in order to determine the number of receptors, reducing the amount of tissue needed, as described below.

Radioligand binding using 'single-point' measurements. The numbers of muscarinic, α- and β-AR-binding sites were determined by using single point measurements of binding. Homogenates were incubated in a single saturating concentration of the radioligand (2000 pmol/l [^{3}H]prazosin for α-AR, 2000 pmol/l [^{3}H]CGP 12177 for β-AR, and 2000 pmol/l [^{3}H]QNB for MR), and the B_{max} values were computed from

$$B_{max} = B \times ([L] + K_L)/[L] \quad (1)$$

where B = bound radioligand (fmol/mg of protein), L = radioligand concentration (fmol/l), and $K_L = K_D$ (fmol/l) of the radioligand. All tissue samples were processed in triplicates.

Determination of α_{1A}-, α_{1B}- and α_{1D}-AR densities. Homogenates were incubated for 90 min at 25°C. The incubation medium was as used for homogenization, with the addition of [^{3}H]prazosin as the specific radioligand (400 pmol/l), and subtype-selective antagonists where indicated. To ascertain the proportion of the α_{1A} and α_{1B}-AR subtypes, the binding of [^{3}H]prazosin was determined in triplicates in the absence and the presence of 10^{-8} mol/l RS 17053 (a selective α_{1A}-AR antagonist), or 10^{-8} mol/l L-765314 (a selective α_{1B}-AR antagonist), or 10^{-7} mol/l BMY 7378 (a selective α_{1D}-AR antagonist). The concentrations of RS 17053, L-765314 and BMY 7378 suitable to distinguish the α_{1A}-, α_{1B}- and α_{1B}-AR subtypes were determined in preliminary experiments using the full competition curve with three concentrations of antagonist per grade.

Determination of β_1- and β_2-AR densities. Homogenates were incubated for 60 min at 38°C. The incubation medium was as used for homogenization, with the addition of [^{3}H]CGP 12177 as the specific radioligand (400 pmol/l), and subtype-selective antagonists where indicated. To ascertain the proportion of the β_1 and β_2-AR subtypes, the binding of [^{3}H]CGP 12177 (300 pmol/l) was determined in triplicates in the absence and the presence of 10^{-7} mol/l CGP 20712A (a selective β_1-AR antagonist) or 10^{-8} mol/l ICI 118.552 (a selective β_2-AR antagonist). The concentrations of CGP 20712A and ICI 118.552 suitable to distinguish the β_1- and β_2-AR subtypes were determined in preliminary experiments using the full competition curve with three concentrations of antagonist per grade.

Cyclic adenosine-3′,5′-monophosphate cAMP assay

We measured the basal level of cAMP and forskolin-stimulated activity of adenylyl cyclase (AC) as previously described (Hoffert et al. 2005). Briefly, membranes were prepared as above (using Hank's stock solutions (HBSS) as a buffer) and incubated with buffer (HBSS; basal cAMP level) or in the presence of 40 μmol l^{-1} forskolin (to stimulate AC activity) for 10 min at 37°C. The reaction was stopped by adding 0.2 N HCl and incubating for 20 min at RT, followed by centrifugation at >10,000*g* for 10 min. Supernatants were saved for measurement of cAMP and pellets were used to measure protein content (bicinchoninic acid assay, Sigma, Prague, Czech Republic). cAMP content was measured using a non-radioactive enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA) based on competitive binding between endogenous cAMP and an exogenous cAMP tagged acetylcholinesterase tracer. Samples were run in 96-well microtiter plate format and measured at $\lambda = 413$ nm on a plate reader (Sunrise, Tecan, Männedorf, Switzerland). Absorbance data were analyzed using a spreadsheet program provided by Cayman Chemical which calculated cAMP content in pmol/ml.

Phosphatidylinositol-specific phospholipase C (PI-PLC) activity

PI-PLC activity was measured by the enzymatic assay described (Dwivedi and Pandey 1999). Briefly, the tissue was homogenized (10 mg of tissue per 100 μl of buffer) in 20 mmol l^{-1} Tris–HCl (pH 7.4),

2 mmol l^{-1} EGTA, 5 mmol l^{-1} EDTA, 1.5 mmol l^{-1} pepstatin, 2 mmol l^{-1} leupeptin, 0.5 mmol l^{-1} phenylmethylsulfonylfluoride, 0.2 U/ml aprotinin, and 2 mmol l^{-1} dithiothreitol using two short pulses of 10 s with a 30 s pause on ice. For the assay, 5 μg of protein per tube was used. The tissue was incubated in incubation buffer (20 mmol l^{-1} Tris–HCl, 1 mmol l^{-1} $CaCl_2$, and 100 mmol l^{-1} KCl, pH 7.4) containing 10 mmol l^{-1} lithium chloride, PIP_2 substrate (50 μmol l^{-1} unlabeled PIP_2), 2.0 mCi ml^{-1} [^{3}H]PIP_2, and 0.5 mg ml^{-1} cetrimide in a total volume of 100 μl at 37°C for 10 min (Thermoblock Biometra T1; Göttingen, Germany). The reaction was terminated by the addition of 500 μl 1M HCl and 500 μl chloroform/methanol (1:1 vol/vol). The tubes were vigorously mixed and centrifuged at 1000*g* for 10 min. The aqueous (upper) phase was transferred to a scintillation vial containing scintillation fluid, and the radioactivity counted. Blanks were included, in which the protein suspension was added after stopping the reaction with chloroform/methanol.

Statistical analysis

Radioligand-binding data were evaluated as described previously (Myslivecek et al. 2004) using non-linear regression. Statistical significance of differences between means was evaluated with one-way, two-way and three-way ANOVA. For multiple comparisons an adjusted *t*-test modified by Student–Newman–Keuls correction was used. Values of $p < 0.05$ were considered to be significant.

Sources of reagents

^{3}H-prazosine [7-metoxy-] (3.21 TBq/mmol), -(−)4-(3-tert-butylamino-2-hydroxypropoyx)-[5,7^{3}H]benzimidazol-2-one (^{3}H-CGP 12177, 1.22 TBq/mmol), ^{3}H-(±)-quinuclidinyl α-hydroxydiphenylacetate, L-[benzilic-4,4′-^{3}H]- (^{3}H-QNB, 1.35 TBq/mmol), were purchased from Perkin-Elmer, Boston, MA, USA. Atropine and propranolol were from ICN Biomedicals, Inc. (MP Biomedicals), Aurora, OH, USA. Compounds CGP 20712A (1-[2-(3-carbamoyl-4-hydroxyphenoxy)-ethylamino]-3-[4-(1-methyl-4-trifluormethyl-2-imidazolyl)-phenoxy]-propanol-methansulphonate), ICI 118.551(*erythro*[±]-1-[7-methylindan-4-yloxyl]-3-isopropylamino-butan-2-ol), L-765314 ((2*S*)-4-(4-amino-6,7-dimethoxy-2-quinazolinyl)-2-[[(1,1-dimethylethyl)amino]carbonyl]-1-piperazinecarboxylic acid, phenylmethyl ester), BMY 7873 (8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride), HEPES (*N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonate), EDTA (ethylenediaminetetraacetic acid), Tris–HCl (Tris(hydroxymethyl(aminoethane hydrochloride), phentolamine were purchased from Sigma. RS 17053 (*N*-[2-(2-cyclopropylmethoxyphenoxy)ethyl]-5-chloro-a,a-dimethyl-1H-indole-3-ethanamine hydrochloride) was from Tocris-Coocson (Bristol, UK).

Results

Wild-type (WT) mice

EPI and NE plasma levels. Plasma EPI concentration was significantly greater in the WT control female group compared to the WT male controls (164%, $p < 0.05$; Figure 1). After acute or repeated immobilization stress exposure, plasma EPI concentrations were reduced (one-way ANOVA, $p = 0.012$; $F = 3.517$, degrees of freedom (df) = 37), especially in females (one immobilization, reduction to 47%, $p < 0.05$; seven immobilizations, reduction to 58%, $p < 0.05$, Figure 1, WT females). It is important to note that plasma catecholamine levels were not elevated because they were measured 3 h after the 2 h immobilization. Plasma concentrations of NE in WT control males and females were not different from each other, and values and were not altered after immobilization (Figure 1, WT mice, two-way ANOVA, $p = 0.113$, $F = 2.325$, df = 40).

Receptor-binding characteristics

Saturation binding. Saturation-binding experiments for α_1-AR, β-AR, and MR did not reveal any significant differences between male and female WT mice in K_D (Table I), indicating that any changes in receptor densities are not the effects of changes in receptor affinity. There were some sex differences in K_D (i.e. for α_1-AR, 0.165 nmol l^{-1} vs. 0.109 nmol l^{-1} for males and females, respectively). The B_{max} values obtained in saturation-binding experiments do not differ from 'single-point' measurements. The β-AR receptor densities were similar to those reported previously (Abraham et al. 2003). The MR density values were greater than reported for another species (horse; Abraham et al. 2007). The α_1-AR densities in lung parenchyma for male mice were similar to those we found previously in male rats (Novakova and Myslivecek 2005). There were sex differences in WT control receptor levels (Figures 2, 4 and 5; left): females had 2–3.5-fold higher density of α_1-AR subtypes than males (α_{1A}-AR: 3.37-fold higher, $p < 0.001$; α_{1B}-AR: 2.83-fold, $p < 0.001$; α_{1D}-AR: 2.23-fold, $p = 0.004$), but males had almost twofold greater MR density than females (1.84-fold, $p < 0.001$). In contrast, β-AR subtype densities did not differ between sexes.

Densities of α_{1A}-, α_{1B}- and α_{1D}-AR. Overall, the sum of the densities of proportions of the subtypes was indistinguishable from unity. Hence, the antagonists acted selectively to block the α-AR subtypes. The B_{max}

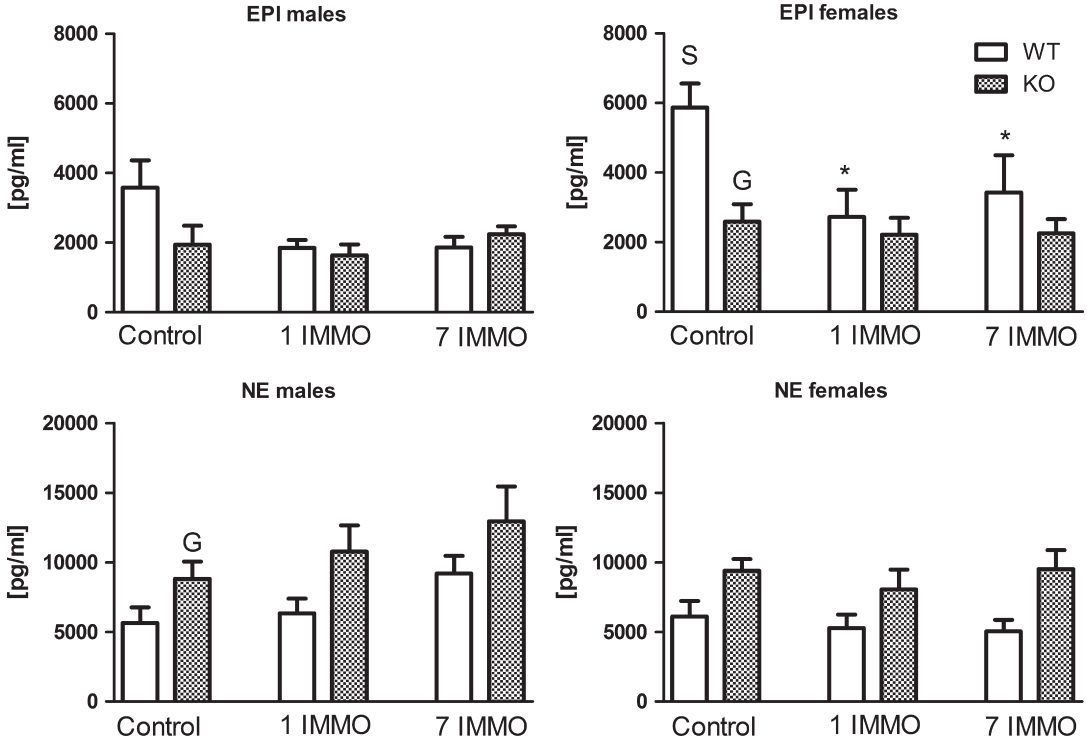


Figure 1. Plasma concentrations of EPI and NE in WT and CRH-KO male and female mice in control conditions and after exposure to a single or seven-times repeated immobilization stress (IMMO). See inset for explanation of symbols. Left: plasma EPI and NE levels in males. Right: plasma EPI and NE levels in females. *$p < 0.05$ versus WT control; S, $p < 0.05$ versus the other sex (the difference is indicated only in females); G, $p < 0.05$ versus the other genotype (i.e. from WT mice); $n = 6–8$ per group.

values were different for the are completely different for the three α_1-AR subtypes: α_{1A}-AR B_{max} in control male mice was ca 200 fmol/mg protein, α_{1B}-AR was ca 60 fmol/mg protein, and α_{1D}-AR was ca 20 fmol/mg protein.

Densities of β_1- and β_2-AR. In all instances, the proportion of total [^{3}H]CGP 12177 binding inhibited by 10^{-7} mol/l CGP 20712A was indistinguishable from the proportion of binding not inhibited by 10^{-8} mol/l ICI 118.552, and vice versa. Hence, the antagonists acted selectively to block the β-AR subtypes.

Sexual dimorphism in responses to stress

Immobilization decreased differently the densities of receptor subtypes in the male and female lung tissue. While in females all α_1-adrenergnic receptor subtype densities (Figure 2) were diminished (α_{1A}-AR: one-way ANOVA, $p = 0.0003$, $F = 18.79$, df = 13; α_{1B}-AR: one-way ANOVA, $p = 0.0005$, $F = 16.63$, df = 13; α_{1D}-AR: one-way ANOVA, $p = 0.0001$, $F = 28.63$, df = 11) by acute and repeated stress (to 32% of control, $p < 0.001$ and to 23% of control, $p < 0.001$ by acute and repeated stress, respectively, in α_{1A}-AR; to 28 and 21%, respectively, $p < 0.001$ in α_{1B}-AR, and to 29 and 21%, respectively, $p < 0.001$ in α_{1A}-AR), in males we found a decrease by acute stress only of α_{1A}-AR density (to 48% of control, $p = 0.047$). Other α_1-adrenergnic receptor subtypes were not affected by stress in males. Similarly, the β_1-AR and β_2-AR stress reaction differed between males and females (Figure 4). In males, β_1-AR density decreased (one-way ANOVA, $p = 0.0005$, $F = 12.65$, df = 18) both after acute (to 31%, $p < 0.001$) and repeated stress (to 59%, $p < 0.05$). In marked contrast, in females β_1-AR density was stable during the

Table I. Dissociation constants for ^{3}H-prazosine (α_1-AR), ^{3}H-CGP 12177A (β-AR) and ^{3}H-QNB (MR).

α_1-AR	K_D (nmol l^{-1})	*n*	β-AR	K_D (nmol l^{-1})	*n*	MR	K_D (nmol l^{-1})	*n*
Females WT	0.165 ± 0.023	6	Females WT	0.341 ± 0.035	6	Females WT	0.136 ± 0.031	6
Females KO	0.172 ± 0.016	6	Females KO	0.266 ± 0.055	6	Females KO	0.169 ± 0.026	6
Males WT	0.109 ± 0.022	6	Males WT	0.246 ± 0.044	6	Males WT	0.102 ± 0.016	6
Males KO	0.127 ± 0.017	6	Males KO	0.187 ± 0.020	6	Males KO	0.149 ± 0.035	6

Data are expressed as mean ± SEM. No significant differences (*t*-test) in K_D between WT and CRH$^{-/-}$ (KO) mice.

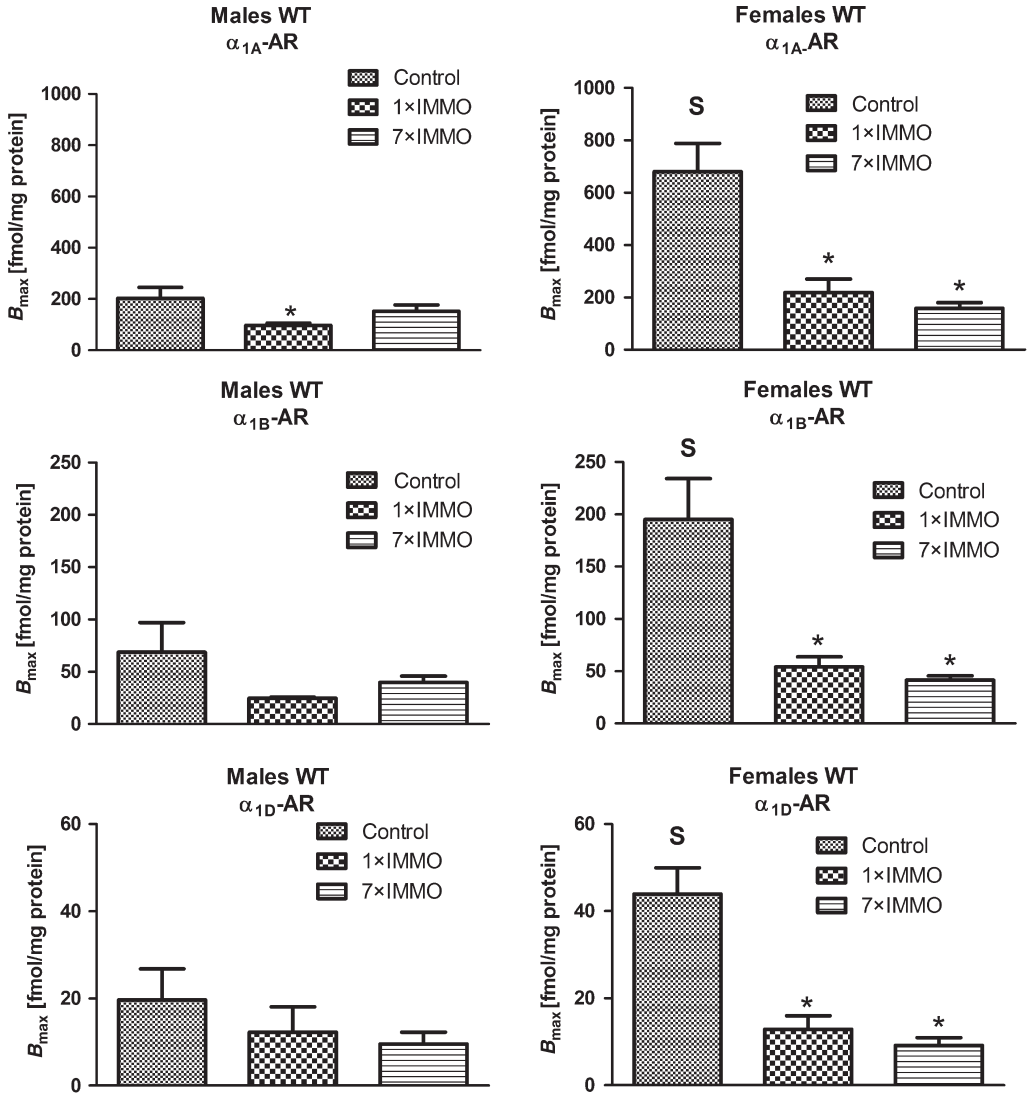


Figure 2. Changes in lung α_1-AR subtype (α_{1A}-AR, α_{1B}-AR, α_{1D}-AR) densities in WT mice. Left: males, right: females. See inset for explanation of symbols. Control, intact mice, 1 × IMMO mice subjected to one stress session, 7 × IMMO mice subjected to repeated stress session (seven immobilizations). *$p < 0.05$ versus control. S, $p < 0.05$ versus the other sex (the difference is indicated only in females); $n = 5$ per group.

stress (one-way ANOVA, $p = 0.9548$, $F = 0.04645$, df = 13). However, β_2-AR density was decreased by stress in females (one-way ANOVA, $p < 0.0001$, $F = 34.31$, df = 13), as for subtypes of α_1-AR: to 61%, $p < 0.01$ in acutely stressed mice and to 18%, $p < 0.001$ in repeatedly stressed mice. In males, both acute (to 33%, $p < 0.001$) and repeated stress (to 64%, $p < 0.01$) diminished the density of β_2-AR (one-way ANOVA, $p < 0.0001$, $F = 19.94$, df = 18). The changes in lung MR density (Figure 5) were similar in males (one-way ANOVA, $p = 0.0007$, $F = 11.08$, df = 20) and females (one-way ANOVA, $p = 0.0247$, $F = 5.481$, df = 12). In both sexes, MR density decreased both after acute (to 51%, $p < 0.01$ in males and to 37% in females, $p < 0.05$, respectively) and repeated stress (to 42%, $p < 0.01$ in males and to 67% females, $p < 0.05$, respectively).

The effects of immobilization on PLC activity

There was no difference in PLC activity after immobilization between groups (Table II; one-way ANOVA, $p = 0.334$, $F = 1.310$, df = 15).

The effects of immobilization on AC activity

Basal levels of cAMP decreased (one-way ANOVA, $p < 0.0001$, $F = 19.86$, df = 20) when the male mice were stressed both acutely (to 40% of control, $p < 0.001$) and repeatedly (to 24% of control, $p < 0.001$). Similarly, forskolin-stimulated AC activity was decreased after stress (to 49%, $p < 0.01$ in acutely stressed mice and to 70%, $p < 0.05$ in repeatedly stressed mice, respectively). Moreover, after acute immobilization AC did not respond to forskolin (Figure 6).

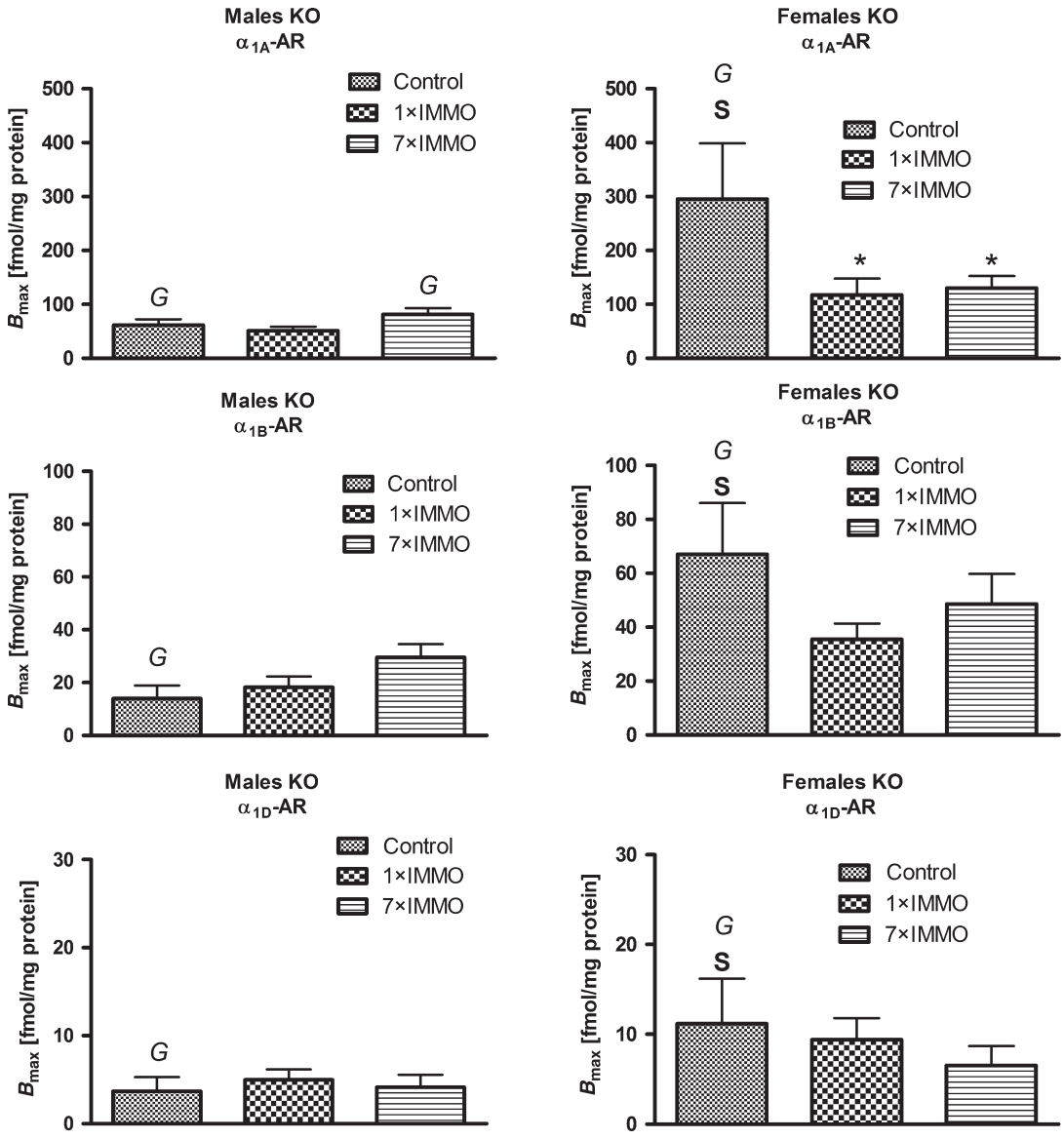


Figure 3. Changes in lung α_1-AR subtype (α_{1A}-AR, α_{1B}-AR, α_{1D}-AR) densities in KO mice. Left: males, right: females. Symbols and groups are as in Figure 2. $^{\star}p < 0.05$ versus control; S, $p < 0.05$ versus the other sex (the difference is indicated only in females); G, $p < 0.05$ versus the other genotype (i.e. WT mice); $n = 5$ per group.

CRH-knockout (CRH-KO) mice

Plasma EPI and NE concentrations. The basal plasma EPI concentrations in CRH-KO female and male mice were less than in WT mice. There were no effects of immobilization (3 h before sampling; Figure 1, two-way ANOVA, $p = 0.753$, $F = 0.286$, df = 38). The basal concentrations of NE were greater (increase to 156%, $p < 0.05$) in male control CRH-KO mice than in control WT mice (Figure 1). Immobilization stress did not alter plasma NE concentrations 3 h later (Figure 1).

Receptor-binding characteristics

Saturation binding. Saturation-binding experiments for α_1-AR, β-AR and MR did not reveal any differences between WT and KO mice in K_D (Table I), indicating that any changes in receptor densities (B_{max}) are not a consequence of changes in receptor affinity. Notably, B_{max} values of control KO mice were similar to the values for WT immobilized mice (Figures 2–5).

Changes in receptor binding. CRH gene disruption decreased the density of AR and MR-binding sites in intact male and female lungs. B_{max} values for α_1-AR sub-types (α_{1A}, α_{1B}, α_{1D}) were diminished to 34% (on average) of the levels in WT mice (Figures 2 and 3), and B_{max} values for β-AR (β_1, β_2) were diminished to 70% (on average; Figure 4) and MR were diminished to 48% of WT (Figure 5). In detail: α_{1A}-AR density in CRH-KO mice (two-way ANOVA, $p < 0.027$, $F = 4.251$, df = 28) was 43% of WT ($p < 0.001$); α_{1B}-AR density in CRH-KO mice (two-way ANOVA,

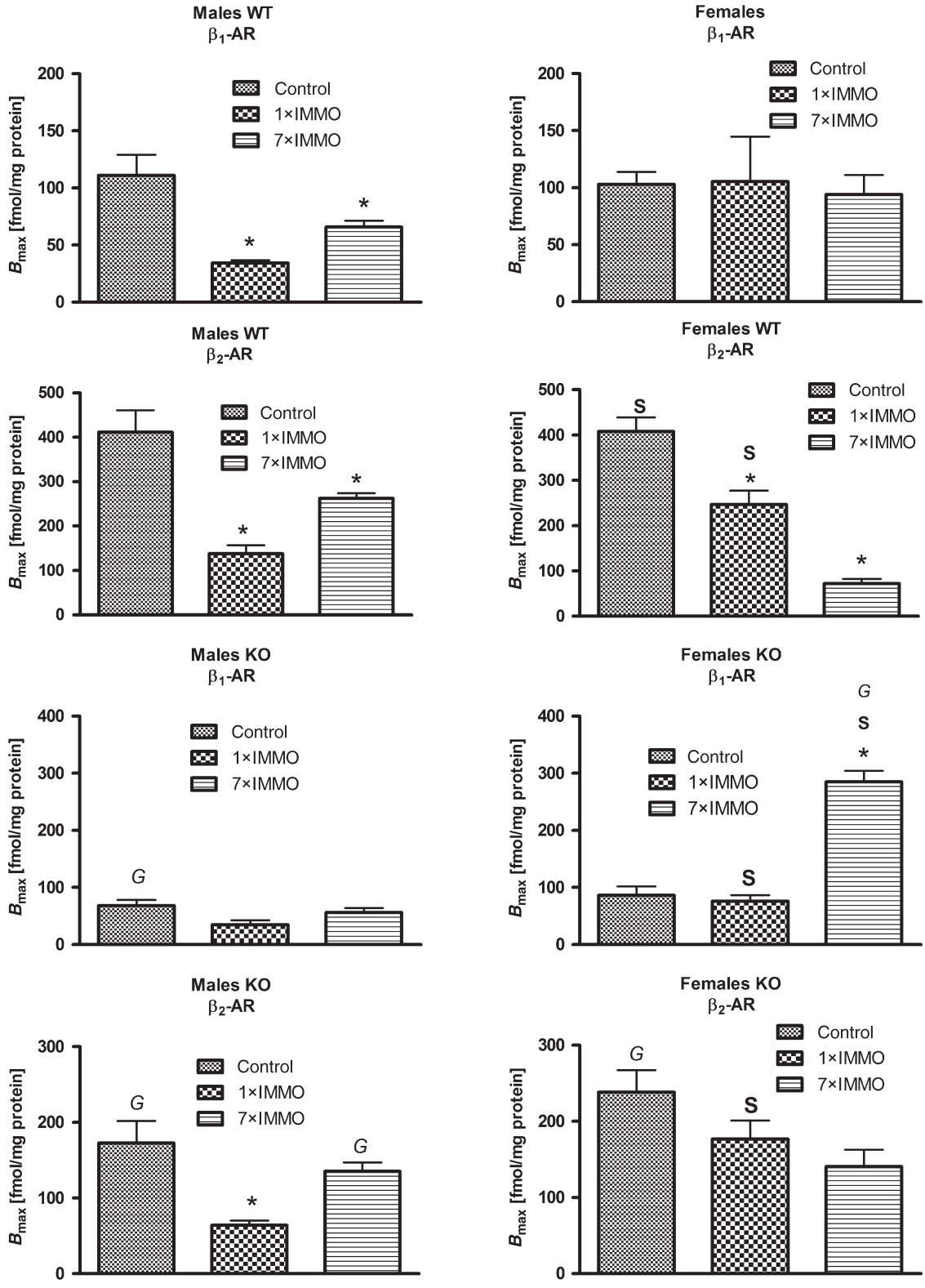


Figure 4. Changes in lung β_1-AR and β_2-AR densities in WT and KO mice. Left: males, right: females. Symbols and groups are as in Figure 2. *$p < 0.05$ versus control; S, $p < 0.05$ versus the other sex (the difference is indicated only in females); G, $p < 0.05$ versus the other genotype (i.e. WT mice); $n = 5–7$ per group.

$p = 0.001$, 8.851, df = 28) was 34% of WT ($p < 0.001$); α_{1D}-AR density in CRH-KO mice (two-way ANOVA, $p < 0.001$, 11.945, df = 24) was 25% of WT ($p < 0.001$); β_1-AR density in CRH-KO mice (two-way ANOVA, $p < 0.001$, $F = 14.558$, df = 26) was 84% of WT ($p < 0.05$); β_2-AR density in CRH-KO mice (two-way ANOVA, $p < 0.001$, $F = 9.197$, df = 26) was 58% of WT

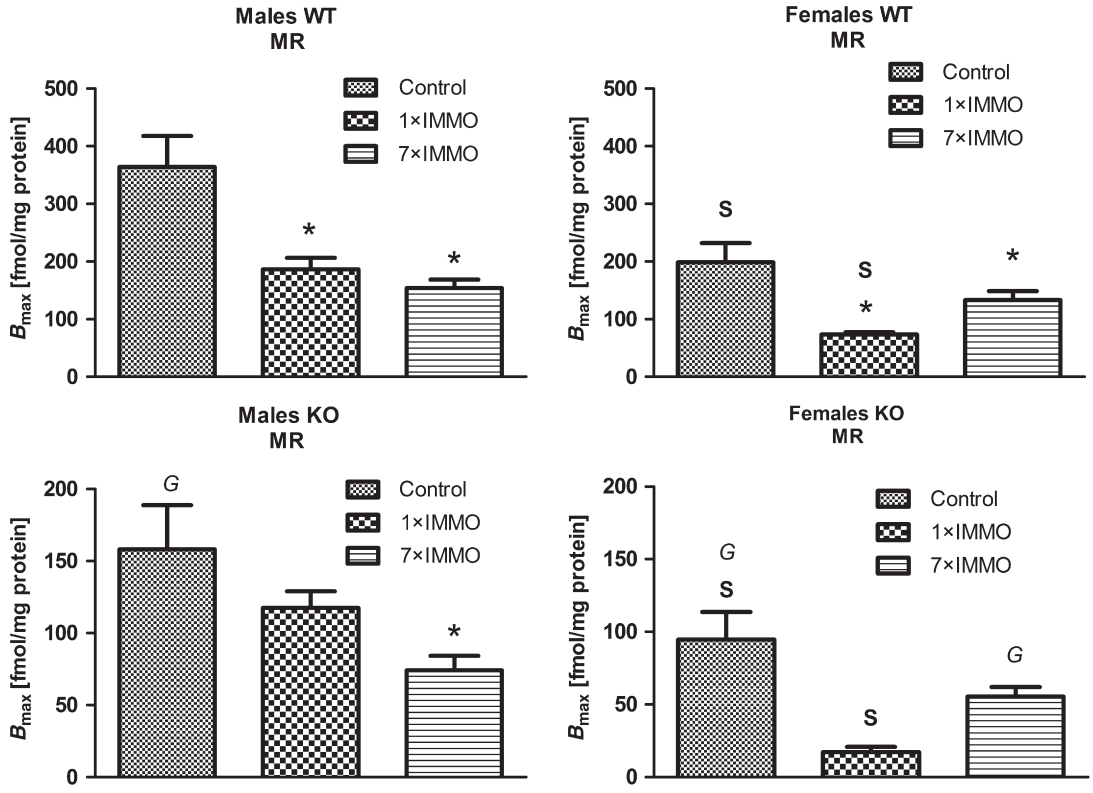


Figure 5. Changes in lung MR densities in WT and KO mice. Left: males, right: females. Symbols and groups are as in Figure 2. *$p < 0.05$ versus control; S, $p < 0.05$ versus the other sex (the difference is indicated only in females); G, $p < 0.05$ versus the other genotype (i.e. WT mice); $n = 5–7$ per group.

($p < 0.001$); MR density in CRH-KO mice (two-way ANOVA, $p = 0.04$, $F = 3.553$, df = 39) was 48% of WT ($p < 0.001$).

Responses to stress. Compared with WT, CRH gene disruption completely abolished the stress reaction (decreases in WT) in the α_{1B}-AR and α_{1D}-AR densities in females (Figures 2 and 3). However, this may reflect the reduced density values for these AR subtypes in control CRH-KO females (Figures 1 and 2). In contrast, the changes in α_{1A}-AR were preserved: acute stress decreased density to 40% ($p = 0.036$), and repeated stress decreased density to 44% of control ($p = 0.011$).

In male CRH-KO mice, there was no change in α_1-AR subtype densities in response to stress (Figure 3).

Similarly, in male CRH-KO mice there was no decrease after stress in β_1-AR density, in contrast with WT mice (Figure 4); however, β_1-AR density in control CRH-KO males was much less than in control WT (Figure 4). Interestingly, in CRH-KO females repeated stress significantly increased the number of β_1-AR (to 462% of control, $p < 0.001$; one-way ANOVA, $p < 0.001$, $F = 59.92$, df = 12).

The decrease in β_2-AR density after stress, seen in WT males, was maintained in the CRH-KO males (one-way ANOVA, $p = 0.0214$, $F = 5.12$, df = 16; decrease to 37% of control, $p < 0.05$; Figure 4). By contrast, the decrease in β_2-AR density seen after stress in WT females was reduced in CRH-KO females, and not significant after repeated immobilization (Figure 4).

MR density was also affected by CRH gene disruption (one-way ANOVA, $p = 0.0265$, $F = 4.597$, df = 18; Figure 5). Male lung MR density was significantly decreased in CRH-KO only after repeated stress (to 47% of control, $p < 0.05$).

Table II. PLC activity.

WT control	KO control	WT 1 × IMMO	KO 1 × IMMO	WT 7 × IMMO	KO 7 × IMMO
8.13 ± 0.94	7.52 ± 0.75	4.59 ± 2.22	8.65 ± 0.33	6.58 ± 0.39	6.63 ± 1.40

PLC activity was measured in males exposed to immobilization stress. Datas are expressed as mean ± SEM. IP_3 nmol mg prot^{-1} min^{-1}. $n = 3$ in all cases. No significant difference between any groups. 1 × IMMO, acute (1) immobilization; 7 × IMMO, repeated (7) immobilizations.

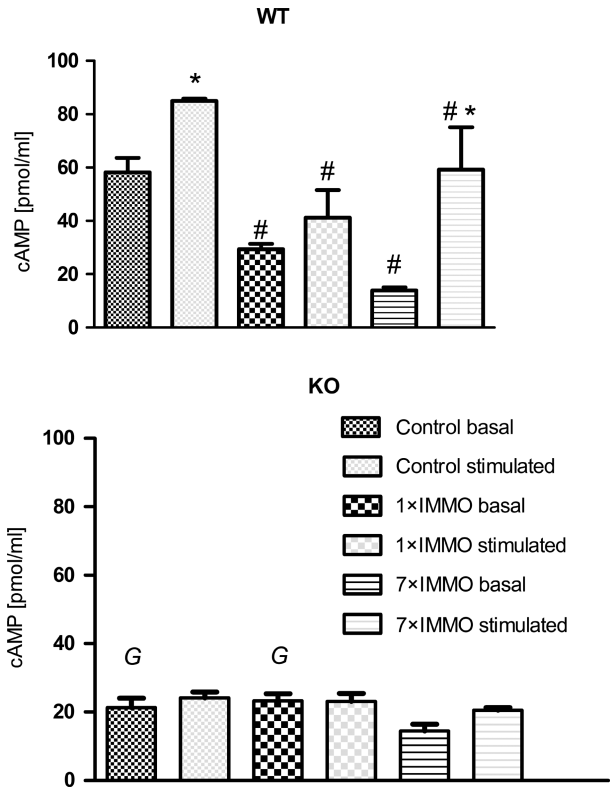


Figure 6. Changes in basal levels in lung of cAMP and AC activity (stimulated by 40 μmol l^{-1} forskolin) in males. See inset for explanation of symbols. #$p < 0.05$ versus respective control (i.e. basal vs. basal and stimulated vs. stimulated), $\star p < 0.05$ versus basal. G, $p < 0.05$ versus the other genotype (i.e. WT mice); $n = 5–6$ per group.

In females, the reduced MR density after stress seen in WT was not significant in CRH-KO females (Figure 5).

The effects of immobilization on PLC activity. There were no significant differences in PLC activity between groups of male mice (Table II).

The effects of immobilization on AC activity. CRH gene disruption completely abolished the respective stimulatory effects of forskolin and inhibitory effects of immobilization on cAMP levels seen in WT mice (one-way ANOVA, $p = 0.1806$, $F = 1.766$, df = 20; Figure 6).

Discussion

We found that male and female mice showed sex differences in the changes in lung parenchyma receptor densities after stress. In males, β_1-AR and MR showed decreases, but in females more complex receptor changes (i.e. decreased density of α_{1A}-AR, α_{1B}-AR, α_{1D}-AR, β_2-AR, and MR) occurred. β_1-AR density was not affected by stress in females. These phenomena have not been observed before and are interesting in the context of the CRH-KO phenotype, in which a sexually dimorphic response to stress has been observed while in WT mice no sex differences were seen (Muglia et al. 1995). Moreover, the findings may be important since some lung pathologies such as asthma show gender differences (Carey et al. 2007; Almqvist et al. 2008) and, more importantly, stress is one of the factors that contribute to the development of asthma (Vig et al. 2006). The sex differences in the reactions of AR and MR to stress may illuminate this gender difference in asthma. The adjusted prevalence of asthma in the North American population is higher in males until the 20th year of age, then it decreases and becomes again higher shortly before the 80th year (Carey et al. 2007). In our model, accepting limitations in comparing murine and human ages, our mice were young adults. Mortality rates for asthma are higher in males (Carey et al. 2007), and female airways are less responsive (i.e. less bronchoconstriction) to cholinergic challenges (Chang and Mitzner 2007). This fits with our finding of almost twofold greater MR density in lung tissue from male than from female mice (and no difference in β-AR).

As mentioned above, plasma EPI and NE concentrations were changed differently in WT and CRH-KO mice and EPI levels were dependent also on gender. Although the plasma catecholamines were measured in trunk blood samples (i.e. in which concentrations are expected to be higher than in arterial or cardiac samples (Kvetnansky et al. 1978)), EPI concentrations were higher in WT mice than in CRH-KO mice, especially in females, which indicates reduced EPI synthesis in the KO mice, and agrees with previous results (Jeong et al. 2000; Kvetnansky et al. 2006). In chromaffin cells of the adrenal medulla, stressor-induced gene expression of PNMT, which converts NE to EPI, requires normal hypothalamo-pituitary–adrenocortical (HPA) axis activity, implying a role for glucocorticoids in PNMT gene expression and action (Jeong et al. 2000; Kvetnansky et al. 2006) and thus probably explaining the altered concentrations of circulating catecholamines in CRH-KO mice (Jeong et al. 2000; Kvetnansky et al. 2006).

The presence of GRE on AR gene promoters (i.e. on β_2-AR (Cornett et al. 1998); β_1-AR (Tseng et al. 2001); α_{1B}-AR (Gao and Kunos 1993) and α_{1A}-adrenergic receptor (Scanga and Schwinn 1998) genes, indicates a mechanism for glucocorticoid hormone actions during stress. Data so far reported about the effects of stress on these receptor systems are rather incomplete. Some have not shown effects of repeated immobilization on either lung α- or β-ARs in rats (Torda et al. 1981, 1985). Others have employed a model of oxidative stress and also have not found effects on rat muscarinic and β-adrenergic receptor function (pD2 values to agonists did not change; van Hoof et al. 1996). By contrast, here we provide

evidence that the AR and MR systems are involved in the reaction of lung tissue to stress. Moreover, we observed important sexual dimorphism in these stress responses. Overall, our results show that the α_1-AR could be an important player in lung physiology in females and that these receptors could have a minor role in regulation of lung function in males. Further investigation is needed to follow these findings.

Some cells in the lung tissue (epithelial cells, bronchial smooth muscle cells, the lung parenchyma cells) could represent the basis for a heterologous regulation system, as they express the pair of receptors with remarkable antagonism (i.e. MR and β-AR). Hence, we could hypothesize about effects of AR activation on MR density. The importance of balance between the MR subtypes was discussed above, and the balance between the muscarinic and adrenergic receptors may also be important. Such heterologous regulation, the effect of one receptor system on another, has been demonstrated repeatedly (Krohn and Hildebrandt 2004; Myslivecek and Kvetnansky 2006). Another possibility is that changes in MR level are a consequence of direct effects of the absence or presence of glucocorticoid. Tentative evidence of the presence of a GRE in the MR M_1 gene has been presented (Klett and Bonner 1999), although the presence of GRE in the promoter region of MR genes has not been confirmed.

We also found sex differences in the basal levels of α_1-AR subtypes (females had higher density of receptors than males) and MR (males had higher density than females) in lung parenchyma. By contrast, β-AR density did not differ between sexes. In relation to the issue of receptor balance, different reactions to stress could be expressed in male and female lung parenchyma, and in particular, a likely higher density of bronchoconstrictive MR in males could be a factor responsible for gender differences in asthma, as discussed above.

Also, the values of B_{max} were different for the three subtypes of α_1-AR, and between males and females. The low B_{max} values for α_{1A}-, iα_{1B}- and α_{1D}-AR in male mice (Figure 2) likely contributed to the general absence of significant differences after stress.

The cAMP signal is neutralized by hydrolysis of cAMP to AMP by phosphodiesterases. Hence, the concentration of cAMP in a cell is a function of the ratio of the rate of synthesis from ATP by AC and its rate of breakdown to AMP. Here, we found that cAMP levels in lung parenchyma from males decrease during the stress response and resemble changes in β_1-AR and MR levels in males. This could imply that the balance between cAMP production by stimulating receptors (i.e. β_1-AR) and cAMP production by inhibiting receptors (i.e. MR) is disrupted. Evidently, stress decreased the expression of AC (as revealed by the inability of forskolin to stimulate cAMP production). By contrast, PLC activity was not changed by stress. This may be related to unchanged (or modestly changed) density of α_1-AR subtypes in males.

We describe here that elimination of the CRH gene led to a decrease in AR and MR receptor levels in lung tissue in mice under basal conditions. Density of all of these receptors in both sexes (except β_1-AR in females) was decreased (see Figures 3–5). This is an important finding as mice lacking the CRH gene have reduced levels of glucocorticoids in basal conditions and in response to stress stimuli (Jeong et al. 2000; Kvetnansky et al. 2006). Similarly, CRH-KO mice fail to increase glucocorticoid levels during the circadian peak (Muglia et al. 1997). In addition, the levels of PNMT are reduced, NE is not efficiently methylated to EPI and plasma NE concentrations are increased. In this paper, we similarly report increased plasma NE concentrations in CRH-KO male mice under basal conditions (Figure 1), which is consistent with decreased PNMT mRNA and protein levels (Jeong et al. 2000). The present results therefore indicate that different reactions in EPI but not NE release to the immobilization stress could contribute to the different AR and MR changes in response to the stress in male and female CRH-KO mice.

We observed essentially unresponsiveness to stress in lung α_1-, β_1-, β_2-ARs and MR in the CRH-KO mice. Specifically, α_1-AR subtypes and β_2-AR were more affected (reduced to one half approximately) than β_1-AR in the female CRH-KO mice. While there was almost no change in β_1-AR density (no change in females, reduction to 62% in males), the β_2-AR density was down-regulated in CRH-KO mice of both sexes. CRH-KO had a greater effect on β_2-AR density. This could be caused by their higher responsiveness to glucocorticoids than other receptors (Myslivecek et al. 2003).

Paradoxically, the lung receptor response to stress was less affected in CRH-KO males than in females. This is similar to the findings that responses to stress in CRH-KO mice are sexually dimorphic (Muglia et al. 1995).

Moreover, it is also necessary to note that intact KO mice have fewer receptor-binding sites than their WT counterparts for all receptors studied here, therefore the absent or reduced stress reactions of the receptors in KO mice may result from the lower basal levels, which were in the range of the inhibited values after stress in WT mice.

In summary, we hypothesize that: (a) lung ARs may play an important role in various pulmonary diseases (e.g. asthma) and in lung development; (b) there are sex differences in levels of AR expression in the lung; and (c) the HPA axis, particularly CRH, may play an important role linking AR and lung pathology.

Our data describe for the first time the effects of stress (immobilization) on lung receptor systems that could underlie impaired lung function in CRH-KO mice.

Thus, we conclude that: (1) CRH gene knock-out changes the densities of MR, α-AR, and β-AR in the adult lung tissue. Our initial hypothesis that CRH gene disruption can affect the densities of these receptor-binding sites in the lung has been supported; (2) our hypothesis that lung receptors in CRH-KO mice would be down-regulated has been supported; and (3) also, we have discovered a sexually dimorphic stress response in these receptor systems that is significantly reduced in CRH-KO mice; although the role of α_1-AR subtypes in lung function is considered minor, we have found that all these subtypes play important role in the stress response in the female lung.

Acknowledgements

We would like to thank Miss Eva Šimáková for technical assistance. This work was supported by Grant 11/2006 from the Grant Agency of Charles University, by the Slovak Research, Development Agency under the Contract No. APVV-0148-06, and Grant VEGA (2/0133/08).

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

Abraham G, Kottke C, Dhein S, Ungemach FR. 2003. Pharmacological and biochemical characterization of the beta-adrenergic signal transduction pathway in different segments of the respiratory tract. Biochem Pharmacol 66:1067–1081.

Abraham G, Kottke C, Ungemach FR. 2007. Equine recurrent airway obstruction does not alter airway muscarinic acetylcholine receptor expression and subtype distribution. J Veter Pharmacol Therap 30:401–409.

Aksoy MO, Mardini IA, Yang Y, Bin W, Zhou S, Kelsen SG. 2002. Glucocorticoid effects on the beta-adrenergic receptor–adenylyl cyclase system of human airway epithelium. J Aller Clin Immunol 109:491–497.

Almqvist C, Worm M, Leynaert B. 2008. Impact of gender on asthma in childhood and adolescence: A GA2LEN review. Allergy 63:47–57.

Alonso-Llamazares A, Zamanillo D, Casanova E, Ovalle S, Calvo P, Chinchetru MA. 1995. Molecular cloning of alpha 1d-adrenergic receptor and tissue distribution of three alpha 1-adrenergic receptor subtypes in mouse. J Neurochem 65:2387–2392.

Carey MA, Card JW, Voltz JW, Arbes SJ Jr, Germolec DR, Korach KS, Zeldin DC. 2007. It's all about sex: Gender, lung development and lung disease. Trend Endocrinol Metabol 18:308–313.

Carswell H, Nahorski SR. 1983. Beta-adrenoceptor heterogeneity in guinea-pig airways: Comparison of functional and receptor labelling studies. Br J Pharmacol 79:965–971.

Chang HY, Mitzner W. 2007. Sex differences in mouse models of asthma. Can J Physiol Pharmacol 85:1226–1235.

Cornett LE, Hiller FC, Jacobi SE, Cao W, McGraw DW. 1998. Identification of a glucocorticoid response element in the rat beta2-adrenergic receptor gene. Mol Pharmacol 54:1016–1023.

Dwivedi Y, Pandey GN. 1999. Repeated administration of dexamethasone increases phosphoinositide-specific phospholipase C activity and mRNA and protein expression of the phospholipase C beta 1 isozyme in rat brain. J Neurochem 73:780–790.

Faure C, Pimoule C, Arbilla S, Langer SZ, Graham D. 1994. Expression of alpha 1-adrenoceptor subtypes in rat tissues: Implications for alpha 1-adrenoceptor classification. Eur J Pharmacol 268:141–149.

Fryer AD, Jacoby DB. 1998. Muscarinic receptors and control of airway smooth muscle. Am J Respir Crit Care Med 158: S154–S160.

Gao B, Kunos G. 1993. Isolation and characterization of the gene encoding the rat alpha 1B adrenergic receptor. Gene 131: 243–247.

Hislop AA, Mak JCW, Kelly D, Reader JA, Barnes PJ, Haworth SG. 2002. Postnatal changes in beta-adrenoceptors in the lung and the effect of hypoxia induced pulmonary hypertension of the newborn. Br J Pharmacol 135:1415–1424.

Hoffert JD, Chou CL, Fenton RA, Knepper MA. 2005. Calmodulin is required for vasopressin-stimulated increase in cyclic AMP production in inner medullary collecting duct. J Biol Chem 280: 13624–13630.

Jeong K-H, Jacobson L, Widmaier EP, Majzoub JA. 1999. Normal suppression of the reproductive axis following stress in corticotropin-releasing hormone-deficient mice. Endocrinology 140:1702–1708.

Jeong KH, Jacobson L, Pacak K, Widmaier EP, Goldstein DS, Majzoub JA. 2000. Impaired basal and restraint-induced epinephrine secretion in corticotropin-releasing hormone-deficient mice. Endocrinology 141:1142–1150.

Kadota H, Kuwahara M, Nishibata R, Mikami H, Tsubone H. 2001. Effect of M2 and M3 muscarinic receptors on airway responsiveness to carbachol in bronchial-hypersensitive (BHS) and bronchial-hyposensitive (BHR) guinea pigs. Exp Anim 50: 49–58.

Kanemi O, Zhang X, Sakamoto Y, Ebina M, Nagatomi R. 2005. Acute stress reduces intraparenchymal lung natural killer cells via beta-adrenergic stimulation. Clin Exp Immunol 139:25–34.

Klett CP, Bonner TI. 1999. Identification and characterization of the rat M1 muscarinic receptor promoter. J Neurochem 72: 900–909.

Krohn M, Hildebrandt JP. 2004. Cross-talk of phosphoinositide- and cyclic nucleotide-dependent signaling pathways in differentiating avian nasal gland cells. J Comp Physiol B 174: 461–470.

Kvetnansky R, Sun CL, Lake CR, Thoa N, Torda T, Kopin IJ. 1978. Effect of handling and forced immobilization on rat plasma levels of epinephrine, norepinephrine, and dopamine-beta-hydroxylase. Endocrinology 103:1868–1874.

Kvetnansky R, Weise VK, Thoa NB, Kopin IJ. 1979. Effects of chronic guanethidine treatment and adrenal medullectomy on plasma levels of catecholamines and corticosterone in forcibly immobilized rats. J Pharmacol Exp Ther 209:287–291.

Kvetnansky R, Kubovcakova L, Tillinger A, Micutkova L, Krizanova O, Sabban EL. 2006. Gene expression of phenylethanolamine *N*-methyltransferase in corticotropin-releasing hormone knockout mice during stress exposure. Cell Mol Neurobiol 26:735–754.

Ma W, Miao Z, Novotny MV. 1998. Role of the adrenal gland and adrenal-mediated chemosignals in suppression of estrus in the house mouse: The Lee–Boot effect revisited. Biol Reprod 59: 1317–1320.

Mak JC, Nishikawa M, Haddad EB, Kwon OJ, Hirst SJ, Twort CH, Barnes PJ. 1996. Localisation and expression of beta-adrenoceptor subtype mRNAs in human lung. Eur J Pharmacol 302: 215–221.

McNeel RL, Mersmann HJ. 1999. Distribution and quantification of beta1-, beta2-, and beta3-adrenergic receptor subtype transcripts in porcine tissues. J Anim Sci 77:611–621.

Muglia L, Jacobson L, Dikkes P, Majzoub JA. 1995. Corticotropin-releasing hormone deficiency reveals major fetal but not adult glucocorticoid need. Nature 373:427–432.

Muglia LJ, Jacobson L, Weninger SC, Luedke CE, Bae DS, Jeong KH, Majzoub JA. 1997. Impaired diurnal adrenal rhythmicity restored by constant infusion of corticotropin-releasing hormone in corticotropin-releasing hormone-deficient mice. J Clin Invest 99:2923–2929.

Myslivecek J, Kvetnansky R. 2006. The effects of stress on muscarinic receptors. Heterologous receptor regulation: Yes or no? Auton Autacoid Pharmacol 26:235–251.

Myslivecek J, Ricny J, Kolar F, Tucek S. 2003. The effects of hydrocortisone on rat heart muscarinic and adrenergic alpha 1, beta 1 and beta 2 receptors, propranolol-resistant binding sites and on some subsequent steps in intracellular signalling. Naunyn Schmiedebergs Arch Pharmacol 368:366–376.

Myslivecek J, Ricny J, Palkovits M, Kvetnansky R. 2004. The effects of short-term immobilization stress on muscarinic receptors, beta-adrenoceptors, and adenylyl cyclase in different heart regions. Ann N Y Acad Sci 1018:315–322.

Myslivecek J, Novakova M, Palkovits M, Krizanova O, Kvetnansky R. 2006. Distribution of mRNA and binding sites of adrenoceptors and muscarinic receptors in the rat heart. Life Sci 79:112–120.

Nobata K, Fujimura M, Ishiura Y, Hirose T, Furusyou S, Myou S, Kurashima K, Kasahara K, Nakao S. 2002. Alpha(1L)-, but not alpha(1H)-, adrenoceptor antagonist prevents allergic bronchoconstriction in guinea pigs *in vivo*. Eur J Pharmacol 452: 97–104.

Novakova M, Myslivecek J. 2005. Identification of all alpha1-adrenoceptor subtypes in rat lung. Gen Physiol Biophys 24: 349–353.

Nozik-Grayck E, Whalen EJ, Stamler JS, McMahon TJ, Chitano P, Piantadosi CA. 2006. *S*-Nitrosoglutathione inhibits alpha(1)-adrenergic receptor-mediated vasoconstriction and ligand binding in pulmonary artery. Am J Physiol—Lung Cell Mol Physiol 290:L136–L143.

Ota A, Matsui H, Asakura M, Nagatsu T. 1993. Distribution of beta 1- and beta 2-adrenoceptor subtypes in various mouse tissues. Neurosci Lett 160:96–100.

Pacak K, Palkovits M. 2001. Stressor specificity of central neuroendocrine responses: Implications for stress-related disorders. Endocr Rev 22:502–548.

Peuler JD, Johnson GA. 1977. Simultaneous single isotope radioenzymatic assay of plasma norepinephrine, epinephrine and dopamine. Life Sci 21:625–636.

Preuss JM, Rigby PJ, Goldie RG. 1998. Age and region-dependent contraction to alpha-adrenoceptor agonists in rat and guinea-pig isolated trachea. Br J Pharmacol 123:1541–1548.

Scanga DR, Schwinn DA. 1998. Transcriptional regulation of alpha-1 adrenergic receptors. Front Biosci 3:d348–d353.

Torda T, Yamaguchi I, Hirata F, Kopin IJ, Axelrod J. 1981. Quinacrine-blocked desensitization of adrenoceptors after immobilization stress or repeated injection of isoproterenol in rats. J Pharmacol Exp Ther 216:334–338.

Torda T, Kvetnansky R, Petrikova M. 1985. Effect of repeated immobilization stress on central and peripheral adrenoceptors in rats. Endocrinol Exp 19:157–163.

Tseng YT, Stabila JP, Nguyen TT, McGonnigal BG, Waschek JA, Padbury JF. 2001. A novel glucocorticoid regulatory unit mediates the hormone responsiveness of the beta1-adrenergic receptor gene. Mol Cell Endocrinol 181:165–178.

van Hoof IH, van Bree L, Bast A. 1996. Changes in receptor function by oxidative stress in guinea pig tracheal smooth muscle. Cent Eur J Public Health 4:3–5.

Venihaki M, Majzoub JA. 1999. Animal models of CRH deficiency. Front Neuroendocrinol 20:122–145.

Vig RS, Forsythe P, Vliagoftis H. 2006. The role of stress in asthma: Insight from studies on the effect of acute and chronic stressors in models of airway inflammation. Ann N Y Acad Sci 1088: 65–77.

Yang M, Reese J, Cotecchia S, Michel MC. 1998. Murine alpha1-adrenoceptor subtypes. I. Radioligand binding studies. J Pharmacol Exp Ther 286:841–847.

Zhong H, Minneman KP. 1999. Alpha1-adrenoceptor subtypes. Eur J Pharmacol 375:261–276.

Zhou C, Fryer AD, Jacoby DB. 2001. Structure of the human M(2) muscarinic acetylcholine receptor gene and its promoter. Gene 271:87–92.

Regulation of Adrenoceptor and Muscarinic Receptor Gene Expression after Single and Repeated Stress

Jaromir Myslivecek,[a] Andrej Tillinger,[b] Martina Novakova,[a] and Richard Kvetňanský[b]

[a]Institute of Physiology, 1st Faculty of Medicine, Charles University, Prague, Czech Republic

[b]Institute of Experimental Endocrinology, Slovak Academy of Sciences, Bratislava, Slovak Republic

Although stress is tightly connected with elevated levels of catecholamines, stress effects on target structures of catecholamine action—adrenoceptors (ARs)—has not been deeply studied yet. Similarly, very little is known about changes of muscarinic receptors (MRs) during stress. We determined changes in these receptors in the individual parts of the heart (right atria and ventricles) of animals (rats and mice) exposed to a single and repeated immobilization stress. Changes of tissue catecholamines, β_2-AR gene expression, protein levels, and binding sites were determined in rat right ventricles, and changes in β_1-, β_2-, and β_3-AR gene expression were followed in murine right atria. Tissue catecholamines were elevated, while β_2-AR mRNA levels and β_2-AR proteins and binding were decreased, in rat right ventricles. In murine right atria, β_1- and β_2-AR gene expression was elevated, while β_3-AR mRNA levels and M_2-MR were reduced. Taken together, our data show that interaction of AR and MR is important for the organism coping with stress and that different heart regions reveal distinct reactions to stress.

Key words: **stress-heart; adrenoceptors; muscarinic receptors**

Introduction

Stress, although the word is promiscuously overused in modern society, is not yet accurately defined. On the other hand, our knowledge about mechanisms of stress reaction dramatically increases every day. It is interesting that although the roles of adrenaline and noradrenaline are widely accepted and have been known for decades, changes in these neurotransmitters' targets—adrenoceptors (ARs)—under stress have been of lesser interest.

This fact is important in view of peripheral organs regulated via two almost antagonistic nervous systems, the sympathetic and the parasympathetic. The initial hypothesis suggests that muscarinic receptors (MRs) can be indirectly affected via ARs activated by increased level of catecholamines. In addition, changes in β_3-AR gene expression opposite to that of β_1- and β_2-AR were expected, as these receptors are supposed to have opposite function to β_1- and β_2-AR.

In this paper, we focused on individual parts of the heart—right atria and right ventricles—as parts with lesser importance to systemic cardiac physiology than left heart, but with importance to lung circulation (right ventricles) and the location of the main cardiac pacemaker (right atria).

Address for correspondence: Jaromir Myslivecek, Institute of Physiology, 1st Faculty of Medicine, Charles University, Albertov 5, 128 00 Prague, Czech Republic. Voice: +420-224 968 485; fax: +420-224 918 816. jmys@lf1.cuni.cz

Stress, Neurotransmitters, and Hormones: Ann. N.Y. Acad. Sci. 1148: 367–376 (2008).
doi: 10.1196/annals.1410.028

Effects of Stress on Adrenoceptors

Data about changes of ARs in the peripheral tissue are not plentiful. In the first reports,[1–3] authors have described changes of ARs in the heart. The next papers[4,5] showed decreased numbers of β-ARs in the heart that could not be prevented by chemical sympathectomy.[6] The role of β_2-AR in the cardiac stress reaction was also shown.[7] Similarly, short-term immobilization selectively changed the numbers of β_1-AR and β_2-AR in different heart regions.[8] In fish, the number of β-AR was increased by hypoxia.[9] On the other hand, oxidative stress (treatment with H_2O_2) decreased the number of heart β-AR.[10] Short-term immobilization leads to a decrease in α_1-AR.[11] Another aspect of receptor changes—heart receptor sensitivity—was reviewed by others.[12]

Effects of Stress on Muscarinic Receptors

Among many stressors, oxidative stress has been shown to impact MRs in peripheral tissue. Stimulation of cardiac M_2 MRs revealed an enhanced negative inotropic response in isolated rat left atria after exposure to hypochlorite-induced oxidative stress.[13] This phenomenon was not observed after stimulation of the cardiac adenosine A_1 receptor, which like the M_2 receptor is coupled to Gi-proteins. In the rat tail artery, the contractile response to M_3 receptor stimulation was not amplified in the rat portal vein. The authors hypothesized an M_2 receptor specificity of the hypochlorite-induced enhancement.[14] Electrical-field stimulation increased the vascular tone in a frequency-dependent way and was attenuated by acetylcholine. It can be concluded that the varied responsiveness among neuronal and cardiac M_2 receptors to hypochlorite may be explained by the different G-protein subunits involved in the activation of the underlying signaling cascade. On the other hand, the eventuality that M_2 receptors in the heart cannot necessarily be located presynaptically (and are mostly expressed by cardiomyocytes, that is, postsynaptically) was not discussed in this paper.[14]

Another report[15] showed that β-ARs, but not MRs are changed in lymphocytes and thymocytes after acute immobilization. Chronic immobilization stress (5 days, for 2 h) decreased MR binding to lymphocytes collected from the spleen and blood, but not from thymus; it caused a significant change in neither the β-ARs binding to thymocytes nor lymphocytes obtained from the blood and spleen.[15]

Short-term immobilization lead to the decrease of MRs,[8] but former data reported increase in MRs after short-term immobilization.[11]

Possible Mechanisms of Receptor Regulation

Heterologous regulation among G-protein-coupled receptors (GPCRs) is a process that could be involved in the regulation of other receptors than ARs. This is an event whereby protein kinases (PKA, PKC) target all receptors despite whether they are occupied or vacant. Heterologous regulation can act either as an amplifier of the targeted receptor response, or as its quencher. Cross-talk is well documented between Gi- and Gq-coupled receptors, as well as between Gs- and Gq-coupled receptors.[16,17]

Moreover, not only receptor phosphorylation but also phosphorylation of Gαi-protein had been suggested to play a role in regulating inhibitory action of the Gi-protein. Phosphorylation of GPCRs via receptors with intrinsic tyrosine kinase activity is another way of "fine-tuning" the GPCRs-mediated signal.[16–18]

Cross-regulation can be comprehended as posttranslational regulation (protein phosphorylation, or desensitization), as posttranscriptional regulation (destabilization of mRNA, or downregulation), or as transcriptional regulation (when the regulation of targeted genes is involved). [19]

Therefore, based on these facts we wanted to know whether:

1. β_1- and β_2-ARs (gene expression, protein levels, receptor binding) are affected by stress in the peripheral tissue (such as the heart),
2. β_3-ARs are affected in a way opposite to that of β_1- and β_2-ARs,
3. MR gene expression is affected as well.

In order to explore these aims we used rats and mice immobilized once or repeatedly for seven times.

Methods

Experimental Protocol and Preparation of Tissue

Animals were treated in accordance with NIH guidelines for animal care, and the experimental protocol was approved by the Committees for Experimental Animals Protection of our Institutes. The experiments were performed on 129SVJ/C57Bl6 mice and on Sprague–Dawley rats, maintained under controlled environmental conditions (12/12 light/dark cycle, 22 ± 1°C, light on at 06.00 h). Food and water were available *ad libitum*. The mice (males weighting 20–25 g) were exposed to immobilization stress for 120 min (one immobilization) or repeatedly for 120 min on 7 consecutive days (seven immobilizations). Animals were sacrificed by decapitation 3 h after the end of the last immobilization (that is, after first or seventh immobilization). Hearts were collected, and right atria and right ventricles were dissected, flash frozen, and kept at −80°C until assays were performed. Male Sprague–Dawley rats, weighting from 300 to 350 g, were exposed to the same immobilization stress protocol as the mice. Hearts were collected, the pericardium carefully removed, and right atria and ventricles dissected.

Noradrenaline and Adrenaline Tissue Levels

Catecholamine concentrations were determined by the modified method for plasma catecholamine quantification.[20] Frozen tissue was weighted and immediately homogenized in 0.1 M $HClO_4$. Homogenate was centrifuged at 10,000 rpm for 15 min. An aliquot of supernatant was taken and diluted with water to a final concentration 1 mg of tissue in 50 μL of solution (pH 8.4). The rest of the method was the same as for plasma catecholamine quantification.[20] Statistical significance of differences between means was evaluated with ANOVA. For multiple comparisons, an adjusted *t*-test with *P*-values corrected by the SNK (Student–Newman–Keuls) method was used.

RNA Isolation and Relative Quantification of mRNA Levels by RT-PCR

RNA was isolated by RNAzol$^{(TM)}$ (Tel-Test, Friendswood, TX). Reverse transcription (RT) was performed using Ready-To-Go You-Prime First-Strand Beads (AP, Biotech, Prague, Czech Republic) and pd$(N)_6$ primer. PCR for specific receptors was carried out afterward using the primers given in Table 1 and the process described in detail therein. For semiquantitative evaluation of PCR, primers for the housekeeper glyceraldehyde-3-phosphate dehydrogenase were used. PCR products were analyzed on 2% agarose gels and visualized by ethidium bromide. Intensity of individual bands was evaluated by analysis system STS 62201 (Ultra-Lum, Inc., Claremont, CA) and PCBAS 2.08e software (Raytest, Inc., Straubenhardt, Germany). Statistical significance of differences among means was evaluated with ANOVA. For multiple comparisons, an adjusted *t*-test with *P*-values corrected by the SNK (Student–Newman–Keuls) method was used.

Radioligand Binding Experiments

The tissue was weighted and homogenized for two or three pulses of 20–30 s in Ultra-Turrax homogenizer in ice cold Tris-EDTA buffer (Tris-HCl 50 mmol/L, EDTA

TABLE 1. Primer Sequences and Protocols Used in PCR Reactions

Gene	Primer sequence	Fragment size	Denaturation (°C)/time (s)	Annealing (°C)/time (s)	Polymerization time (s)	Number of cycles
β_1-adrenoceptors	Sense: 5′-GCC GAT CTG GTC ATG GGA-3′ Antisense: 5′-GTT GTA GCA GCG GCG CG-3′	293	94/60	65/60	60	36
β_2-adrenoceptors	Sense: 5′-ACC TCC TTC TTG CCT ATC CA-3′ Antisense: 5′-TAG GTT TTC GAA GAA GAC CG-3′	111	94/60	65/60	60	36
β_3-adrenoceptors	Sense: 5′-GCA ACC TGC TGG TAA TCA CA-3′ Antisense: GGA TTG GAG TGG CAT TCC TG -3′	381	94/60	60/30	60	36
M_2 muscarinic receptors	Sense: 5′-TAC CCT CTA CAC TGT GAT TGG C-3′ Antisense: 5′-ATG ATG ACA GGC AGA TAG-3′	324	95/45	62/35	40	34
GAPDH	Sense: 5′-AGA TCC ACA ACG GAT ACA TT-3′ Antisense: 5′-TCC CTC AAG ATT GTC AGC AA-3′	309	94/60	60/30	60	37

All polymerizations were performed at 72°C. The initial denaturation was performed at 95°C for 5 min with the exception of M_2 muscarinic receptors, when denaturation lasting 5 min was followed by 35 s annealing at 60°C and 45 s polymerization at 72°C. The final polymerization lasting 7 min was performed at 72°C in all cases.

2 mmol/L, pH adjusted to 7.4), and membranes were prepared using repeated centrifugation at 32,000 *g*. Experiments were performed as has been reported before.[8] Briefly, the amount of muscarinic binding sites (B_{max}) were computed by nonlinear regression of data obtained in saturation experiments with the binding of 65–2000 pmol/L [^{3}H]QNB (Quinuclidinyl benzilate, L-[Benzilic-4,4′-^{3}H]) (PerkinElmer Life and Analytical Sciences, Inc., Waltham, MA) to tissue homogenates performed in duplicates. Nonspecific binding was determined in the presence of 5 μmol/L atropine. The number of β-adrenergic binding sites was investigated using 65–2000 pmol/L [^{3}H]CGP (4-(3-t-butylamino-2-hydroxypropoxy)-[5,7-^{3}H]benzimidazol-2-one) (PerkinElmer Life and Analytical Sciences, Inc.) 12177. Nonspecific binding was determined in the presence of 5 μmol/L propranolol. The incubation was performed at 38°C for 2 h with [^{3}H]QNB and 1 h with [^{3}H]CGP 12177. In all cases, the incubation was terminated by a filtration through Whatman GF/B glass fiber filters presoaked with distilled water in a Brandel cell harvester when the filters were washed three times with ice-cold water (Whatman plc, Maidstone, Kent, UK). Radioactivity retained on the filters was measured by scintillation counting in Bray's solution after desiccation overnight. The affinity constants (K_D) were computed and used for the "single-point" measurement in order to determine the

number of receptors saving the amount of tissue needed, as described bellow.

In "single-point" measurements, numbers of the MR and β-AR binding sites were determined using a single saturating concentration of the radioligand (2000 pmol/L [^{3}H]CGP 12177 for β-ARs and 2000 pmol/L [^{3}H]QNB for MRs), and B_{max} values were computed according to the equation $B_{max} = B \times [(L + K_L)/L]$, where B = radioligand bound (fmol/mg of protein), L = radioligand concentration (fmol/L), and $K_L = K_d$ (fmol/L) of the radioligand. All of the tissue samples were processed in triplicate. β_1- and β_2-ARs were determined in 300 pmol/L of [^{3}H]CGP 12177 and of the subtype-selective antagonists (10^{-7} mol/L CGP 20712A for β_1-ARs or 10^{-8} mol/L ICI 118.552 for β_2-ARs). Radioligand binding data was evaluated as described previously.[6] Statistical significance of differences between means was evaluated with ANOVA. For multiple comparisons, an adjusted *t*-test with *P*-values corrected by the SNK (Student–Newman–Keuls) method was used.

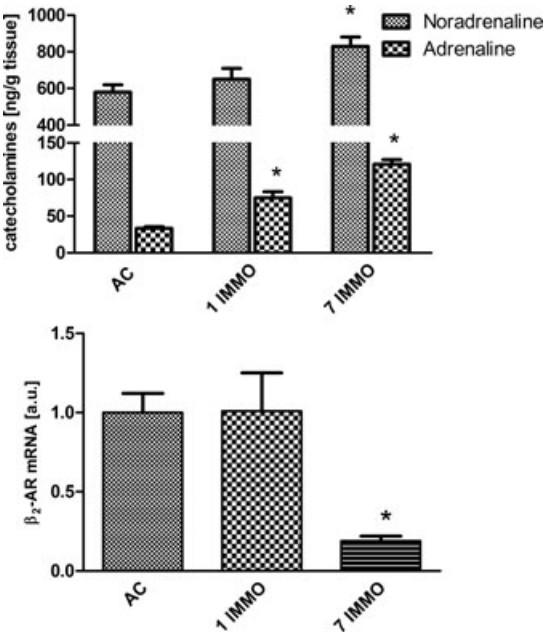


Figure 1. Changes in catecholamine levels (upper panel) and β_2-AR gene expression (lower panel) in the rat right ventricle of animals exposed to immobilization stress. AC, control; 1 IMMO, animals immobilized once; 7 IMMO, animals immobilized 7 times. $^*P < 0.05$ different from control.

Results

Rat Right Ventricles

Noradrenaline and Adrenaline Tissue Levels

We found increased levels of tissue noradrenaline in the rat right ventricles after repeated immobilization (seven immobilizations). Levels of adrenaline were increased after either one or seven immobilizations (Fig. 1).

Measurement of β_2-AR mRNA Levels by RT-PCR

Single immobilization did not change the relative amount of β_2-AR mRNA in rat right ventricles (Fig. 1). On the other hand, we found decreased β_2-AR mRNA levels after seven immobilizations (Fig. 1). In contrast, β_1-AR mRNA levels were not changed in the right ventricles of either once or repeatedly immobilized rats (data not shown).

Radioligand Binding Experiments and Protein Determination

The number of β_2-AR binding sites and the amount of β_2-AR protein were decreased after one immobilization in the rat right ventricles (Fig. 2); however, seven immobilizations did not lead to significant decrease of either β_2-AR protein or number of β_2-AR binding sites (Fig. 2). In contrast, neither β_1-AR protein, the number of β_1-AR, nor MR binding were changed in the rat right ventricles (unpublished data).

Murine Right Atria

Measurement of β-AR mRNA Levels by RT-PCR

In contrast to rats, β_1-AR mRNA levels were increased in the right atria of mice after repeated immobilization stress (Fig. 3). In

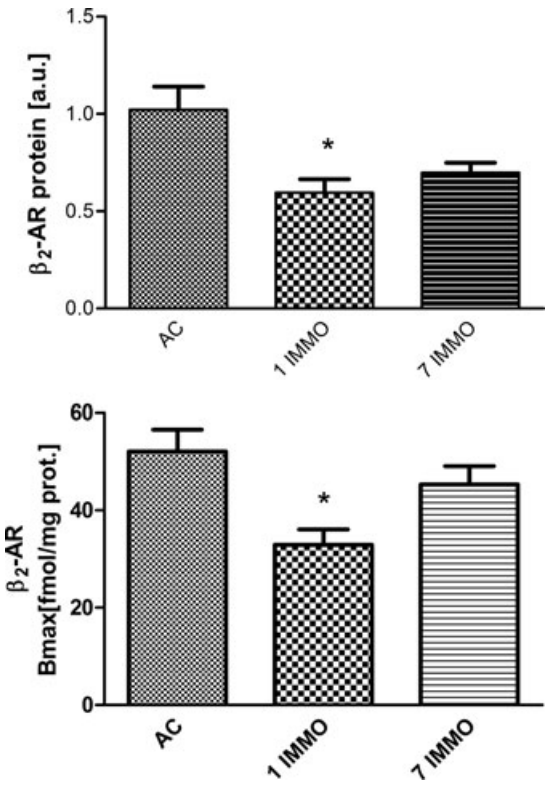


Figure 2. β_2-AR protein levels (upper panel) and β_2-AR receptor binding sites (lower panel) in the rat right ventricle in animals exposed to immobilization stress. AC, control; 1 IMMO, animals immobilized once; 7 IMMO, animals immobilized 7 times. $^*P < 0.05$ different from control.

addition, gene expression of β_2-AR was increased in mice exposed to a single, as well as to repeated, immobilization (Fig. 3). Decreased levels of β_3-AR mRNA (after either one or seven immobilizations) and M_2 MR mRNA (after one immobilization) were seen in murine right atria (Fig. 3).

Discussion

Rat Right Ventricles

Noradrenaline and Adrenaline Tissue Levels

Immobilization stress affected the right ventricle levels of noradrenaline and adrenaline differently. While adrenaline was increased as early as after the first immobilization, the level of noradrenaline was changed only after seven immobilizations. Concentration of noradrenaline in this tissue is more than 20 times higher than that of adrenaline because of noradrenalinergic sympathetic innervation. After repeated stress exposure, there is a more intensive biosynthesis, which might explain the elevated noradrenaline levels.

Cardiac sympathetic innervation containing adrenergic neurons is very low, even if gene expression of phenylethanolamine N-methyltransferase (PNMT), the key enzyme of adrenaline synthesis, has been recently detected in the stellate ganglia.[27] It was believed that uptake from circulation and reduced biosynthesis in some cardiac cells might be responsible for the low adrenaline levels in the heart. Recently, we have described PNMT gene expression in cardiac tissue and its increase, especially after repeated immobilization stress.[28,29] Thus, the found stress-induced increase of adrenaline concentration in the right ventricle of stressed rats is most probably a result of increased adrenaline biosynthesis as the consequence of increased PNMT gene expression.

Changes of mRNA Levels

To our knowledge, no data about changes in AR gene expression after immobilization stress have been published. On the other hand, some data about changes of the β-AR gene expression in the failing heart,[21–23] the situation in which the levels of blood catecholamines are increased, already exist.

Radioligand Binding Experiments and Protein Levels

Our results concerning the changes in binding characteristics are in good agreement with previously published data about changes in the AR binding.[1,4,5] In detail, it was shown[4,5] that the number of β-ARs in the heart is decreased. The role of β_2-AR in the cardiac stress reaction has also been shown.[7] Similarly, short-term immobilization selectively changes the number of β_1-AR and β_2-AR in various heart regions.[8]

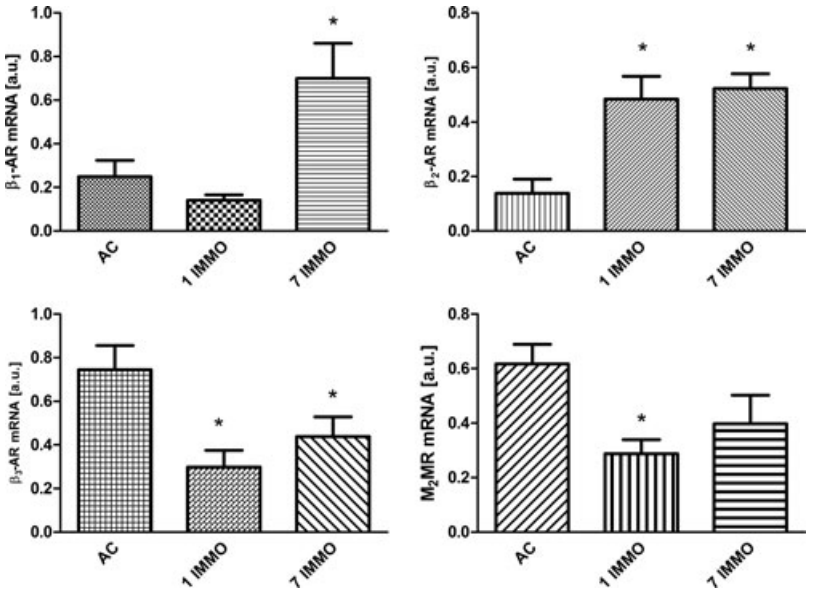


Figure 3. Effects of immobilization stress on murine right atria gene expression of β_1-AR (upper left panel), β_2-AR (upper right panel), β_3-AR (lower left panel), and M_2 MR (lower right panel). AC, control; 1 IMMO, animals immobilized once; 7 IMMO, animals immobilized 7 times. $^*P < 0.05$ different from control.

Short-term immobilization led to a decrease of MR[8] in the heart, but there are reports[15] that β-AR, but not MR, is changed in lymphocytes and thymocytes after an acute immobilization.

Protein levels of β_2-AR were changed similarly to their receptor binding (Fig. 2). On the other hand, physical training did not change the amount of β_2-AR receptor protein in rat left ventricles.[24]

Murine Right Atria

Changes of mRNA Levels

As it can be deduced from Figure 3, changes in AR and MR gene expression in murine right atria differed from that in rat right ventricles. Changes of β_1-, β_2-, β_3-AR gene expression are also different from our investigation of AR gene expression in the rat right atria (unpublished observation). Some data demonstrated changes in β_3-AR gene expression in chronic exercise–induced cardiac hypertrophy,[25] or in human failing myocardium.[26]

Our data demonstrate that the gene expression of cardiostimulative receptors (β_1-AR and β_2-AR) in murine right atria is changed by the same way (increase) and that the gene expression of cardioinhibitive receptors (β_3-AR and MR) is changed by the opposite way (decrease). This could imply their role in maintaining the heart homeostasis. On the other hand, these changes differ from those in rat right atria (unpublished observation), where decrease in β_2-AR gene expression was found. A possible explanation of this discrepancy could lie in species differences between rats and mice.

General Discussion

Taken together, our data show that both AR and MR are important in coping with stress situations. Increase in catecholamine levels brings about not only changes in ARs, but also affects MRs. These changes could be caused by the activation of PKA as well as PKC (see schematic diagram in Fig. 4).

Important findings about the process of receptor regulation can be deduced from the comparison of changes in gene expression, protein levels, and the amount of receptor binding sites. The levels of catecholamines correlate

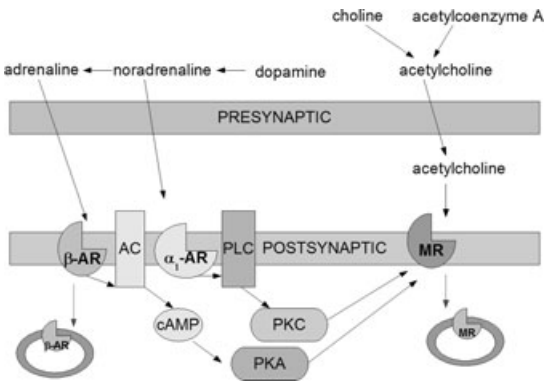


Figure 4. The schematic diagram depicting possible pathways for MR regulation. Increased levels of catecholamines can affect the number of ARs on the membrane. When this occurs, the internalization of β-AR (left part of the diagram) can start. Internalization is a process leading to a decrease of β-AR in the membrane. If receptor activation persists, the increase in cAMP level (via β_1-AR and β_2-AR) or phospholipase C (PLC) activity (via α_1-AR) can affect the activity of protein kinase A (PKA) and protein kinase C (PKC), respectively. The MR can be affected both via PKA or PKC, which can decrease the amount of MR binding sites on the membrane by the process of internalization (right part of the diagram). AC, adenylyl cyclase.

inversely with the levels of protein/receptor binding of β_2-AR. On the other hand, gene expression of this receptor did not change after one immobilization, but did after the seventh immobilization. Moreover, when we compare the changes in protein level and receptor binding, it is possible to deduce that the process of receptor inactivation is mainly involved in receptor regulation. This can be demonstrated by similar changes in the disability of the receptor binding pocket to bind the radiolingand in membrane receptors (as revealed from binding data) as well as in the inability to bind antibodies to the C-terminal of the receptor protein (as revealed from western blots of receptor protein). After the process of receptor inactivation, gene expression is affected. This can be judged from Figure 1 and Figure 2. The gene expression of β_2-AR is not changed after a single immobilization (Fig. 1) but is dramatically decreased after repeated immobilization, while receptor protein and receptor binding are affected after a single but not repeated immobilization (see Fig. 2). A significance of the decrease in gene expression, however, needs further investigation.

Changes in ARs are not the only receptor changes influenced by stress in murine atria. MRs, the receptors with opposite AR function, are affected as well. This could imply that the changes in the receptor milieu are important for homeostatic maintenance.

The question arises, why the number of β_2-AR and level of their protein decreased after a single, but not after repeated, immobilization. In order to hypothesize this phenomenon, it is necessary to take into consideration that the changes in gene expression and receptor binding are a result of adaptation to stress. Therefore, not only β_2-AR, but also β_3-AR and MR, are affected, and the resulting gene expression, protein expression, and binding to receptors should be considered a result of mutual interaction among receptor systems involved in heart regulation caused by huge changes in ARs (see Fig. 4 showing possible mechanisms of another receptor regulation [MR]).

In summary, immobilization stress is able to affect gene expression, protein levels, and receptor binding not only of ARs, but also of MRs in the right heart. We suppose that these changes could help the organism to cope with the stress condition.

Acknowledgments

Supported by Grants GAUK 11/06 from Grant Agency of Charles University (Czech Republic), APVV-0148-06 from Agency for Research and Development (Slovakia), and VEGA 2/5252 from The Scientific Grant Agency of the Ministry of Education of the Slovak Republic and of the Slovak Academy of Science and partially supported by research project MSM0021620849 (from Ministry of Education of the Czech Republic).

Conflicts of Interest

The authors declare no conflicts of interest.

References

1. U'Prichard, D.C. & R. Kvetnansky. 1980. Central and peripheral adrenergic receptors in acute and repeated immobilization stress. In *Catecholamines and Stress: Recent Advances*. E. Usdin, R. Kvetnansky & I.J. Kopin, Eds.: 299–308. Elsevier. New York.
2. Yamaguchi, I., T. Torda, F. Hirata & I.J. Kopin. 1981. Adrenoceptor desensitization after immobilization stress or repeated injection of isoproterenol. *Am. J. Physiol.* **240:** H691–H696.
3. Torda, T., I. Yamaguchi & F. Hirata, *et al*. 1981. Quinacrine-blocked desensitization of adrenoceptors after immobilization stress or repeated injection of isoproterenol in rats. *J. Pharmacol. Exp. Ther.* **216:** 334–338.
4. Torda, T., R. Kvetnansky & M. Petrikova. 1984. Effect of repeated immobilization stress on rat central and peripheral adrenoceptors. In *Stress: The Role of Catecholamines and Other Neurotransmitters*. E. Usdin, R. Kvetnansky & J. Axelrod, Eds.: 691–701. Gordon and Breach Sci Publ. New York.
5. Torda, T., R. Kvetnansky & M. Petrikova. 1985. Effect of repeated immobilization stress on central and peripheral adrenoceptors in rats. *Endocrinol. Exp.* **19:** 157–163.
6. Torda, T. & I.J. Kopin. 1985. Lack of preventive effect of 6-hydroxy-dopamine on a decrease of beta-adrenergic receptors induced by repeated stress in the heart of rats. *Endocrinol. Exp.* **19:** 47–51.
7. Kirby R.F. & A.K. Johnson. 1990. Role of beta 2-adrenoceptors in cardiovascular response of rats to acute stressors. *Am. J. Physiol.* **258**(Pt 2): H683–H688.
8. Myslivecek, J., J. Ricny, M. Palkovits & R. Kvetnansky. 2004. The effects of short-term immobilization stress on muscarinic receptors, beta-adrenoceptors, and adenylyl cyclase in different heart regions. *Ann. N. Y. Acad. Sci.* **1018:** 315–322.
9. Gamperl, A.K., M.M. Vijayan, C. Pereira & A.P. Farrell. 1998. Beta-receptors and stress protein 70 expression in hypoxic myocardium of rainbow trout and chinook salmon. *Am. J. Physiol.* **274:** R428–R436.
10. Persad S., H. Rupp, R. Jindal, *et al*. 1998. Modification of cardiac beta-adrenoceptor mechanisms by H2O2. *Am. J. Physiol.* **274**(2 Pt 2). H416–H423.
11. Meerson, F.Z. 1991. Alpha1 desensitization of the heart during adaptation to stress. *Fiziol Zh.* **37:** 3–6.
12. Santos, I.N. & R.C. Spadari-Bratfisch. 2006. Stress and cardiac beta adrenoceptors. *Stress*. **9:** 69–84.
13. Peters, S.L., C. Sand, H.D. Batink, *et al*. 2001. Reactive oxygen species potentiate the negative inotropic effect of cardiac M2-muscarinic receptor stimulation. *NS Arch. Pharmacol.* **364:** 166–171.
14. Sand, C., S.L. Peters, M.J. Mathy, *et al*. 2002. The effects of hypochlorite-induced oxidative stress on presynaptic M2-receptors at sympathetic nerve endings in the rat tail artery. *Auton. Autacoid Pharmacol.* **22:** 127–132.
15. Kubera, M., A. Skowron-Cendrzak, B. Mazur-Kolecka, *et al*. 1992. Stress-induced changes in muscarinic and beta-adrenergic binding sites on rat thymocytes and lymphocytes. *J. Neuroimmunol.* **37:** 229–235.
16. Cordeaux, Y. & S.J. Hill. 2002. Mechanisms of cross-talk between G-protein-coupled receptors. *Neurosignals*. **11:** 45–57.
17. Hur, E.M. & K.T. Kim. 2002. G protein-coupled receptor signalling and cross-talk. Achieving rapidity and specificity. *Cell. Signal.* **14:** 397–405.
18. Selbie, L.A. & S.J. Hill. 1998. G protein-coupled-receptor cross-talk: the fine-tuning of multiple receptor-signaling pathways. *Trends Pharmacol. Sci.* **19:** 87–93.
19. Morris, A.J. & C.C. Malbon. 1999. Physiological regulation of G protein-linked signaling. *Physiol. Rev.* **79:** 1373–1430.
20. Peuler, J.D. & G.A. Johnson. 1977. Simultaneous single isotope radioenzymatic assay of plasma norepinephrine, epinephrine and dopamine. *Life Sci.* **21:** 625–636.
21. Brodde, O.-E., M.C. Michel & H.R. Zerkowski. 1995. Signal transduction mechanisms controlling cardiac contractility and their alterations in chronic heart failure. *Cardiovasc. Res.* **30:** 570–584.
22. Lohse, M.J., S. Engelhardt & T. Eschenhagen. 2003. What is the role of β-adrenergic signaling in heart failure? *Circ. Res.* **93:** 896–906.
23. Port, J.D. 2001. Altered beta-adrenergic receptor gene regulation and signaling in chronic heart failure. *J. Mol. Cell Cardiol.* **33:** 887–905.
24. Barbier, J., F. Rannou-Bekono, J. Marchais, *et al*. 2004. Effect of training on beta1 beta2 beta3 adrenergic and M2 muscarinic receptors in rat heart. *Med. Sci. Sports Exerc*. **36:** 949–954.
25. Barbier, J., F. Rannou-Bekono, J. Marchais, *et al*. 2007. Alterations of beta3-adrenoceptors expression and their myocardial functional effects in physiological model of chronic exercise-induced cardiac hypertrophy. *Mol. Cell Biochem.* **300:** 69–75.
26. Moniotte, S., L. Kobzik, O. Feron, *et al*. 2001. Upregulation of beta(3)-adrenoceptors and altered contractile response to inotropic amines in human failing myocardium. *Circulation* **103:** 1649–1655.

27. Kubovcakova, L. *et al*. 2006. Identification of phenylethanolamine N-methyltransferase gene expression in stellate ganglia and its modulation by stress. *J. Neurochem.* **97:** 1419–1430.
28. Krizanova, O. *et al*. 2001. Existence of cardiac PNMT mRNA in adult rats: elevation by stress in a glucocorticoid dependent manner. *Am.J. Physiol. (Heart Circ. Physiol.)* **281:** H1372–H1379.
29. Kvetnansky, R. *et al*. 2006. Gene expression of phenylethanolamine N-methyltransferase in corticotropin-releasing hormone knockout mice during stress exposure. *Cell Molec. Neurobiol.* **26:** 735–754.

Gene Expression of Adrenoceptors in the Hearts of Cold-Acclimated Rats Exposed to a Novel Stressor

Andrej Tillinger,[a] Jaromír Mysliveček,[b] Martina Nováková,[b] Olga Krizanova,[c] and Richard Kvetňanský [a]

[a] *Institute of Experimental Endocrinology Center of Excellence for Cardiovascular Research SAS, Bratislava, Slovakia*

[b] *1st Faculty of Medicine, Charles University, Prague, Czech Republic*

[c] *Institute of Molecular Physiology and Genetics Center of Excellence for Cardiovascular Research SAS, Bratislava, Slovakia*

Changes in the heart rate and force of contraction are regulated by catecholamines via adrenoceptors (AR). In this work, we measured gene expression of AR in left heart atria and ventricles in rats exposed to cold stress and in cold-acclimated rats exposed to a novel stressor (immobilization). We found a significant increase in β_3-AR in left ventricle of rats exposed to acute (1 day) and long-term (28 days) cold, but no changes in β_1- and β_2-AR mRNA levels. However, single immobilization significantly decreased β_2-AR mRNA levels both in left atria and ventricles compared to acute cold stress. Application of a novel stressor (immobilization) to previously cold-acclimated animals did not show decrease of β_2-AR mRNA levels as seen in intact animals. Moreover, β_1- and β_2-AR did not show any significant changes. Surprisingly, the most prominent changes in the heart were detected for α_{1B}-AR gene expression. We found decreased levels of α_{1B}-AR mRNA in the heart of rats exposed to cold and immobilization. We also found that exposure of cold-acclimated rats to immobilization is responsible for additional decrease of α_{1B}-AR mRNA levels in heart. It seems that while β-AR undergoes adaptation, α_{1B}-AR is probably prepared to modulate heart functions. Proposed mechanism of β-AR adaptation needs to be elucidated. Thus, we have shown that gene expression of different AR subtypes in the heart is regulated differently by various stressors. A protective role of β_2-, β_3-AR, and α_{1B}-AR in the process of heart adaptation to chronic stress exposure is proposed.

Key words: **adrenoceptors; cold; immobilization; heart; gene expression**

Introduction

Catecholamines—norepinephrine (NE) and epinephrine (EPI)—are among the principal compounds released by the organism during a stress reaction, which is related to activation of the sympatho-adrenomedullary system (SAS) as the main source of catecholamines in the organism. NE and EPI play a crucial role in the regulation of many physiological functions in the organism, as in the cardiovascular system, for example. Changes in the heart rate (chronotrophy) and force of muscle contraction (inotrophy) are regulated by catecholamines via adrenoceptors (AR). They belong to two main groups, designated α- and β-AR. At least nine AR subtypes were described (α_{1A}-, α_{1B}-, α_{1D}-, α_{2A}-, α_{2B}-, α_{2C}-, β_1-, β_2-, and β_3-AR). β-AR are the most important AR for the regulation of heart function. The main subtype is β_1-AR, which represents 70–80% of all β-AR in the heart.[1] Despite the fact that β_1-AR are

Address for correspondence: Dr. Andrej Tillinger, Institute of Experimental Endocrinology, SAS, Vlarska 3, 833 06 Bratilsava, Slovakia. Voice: +421 2 5477 2800; fax: +421 2 5477 4247. ueentill@savba.sk

Stress, Neurotransmitters, and Hormones: Ann. N.Y. Acad. Sci. 1148: 393–399 (2008).
doi: 10.1196/annals.1410.024

dominant subtype in the heart,[1] functional responses mediated by β_1- and β_2-AR are not necessarily different. *In vivo* studies have confirmed that both β_1- and β_2-AR are involved in positive inotropic and chronotropic effects.[2,3] In the heart, β_3-AR subtype is also present, but its functional role in the heart has not been clarified. It is supposed that negative inotropic effect induced by β_3-AR might play a protective role during sustained adrenergic stimulation of β_1- and β_2-AR in the heart.[4,5] It is well established that β-AR have a dominant position in the regulation of heart function, but there are also α_1-AR which are contributed in positive inotropic effect in the heart as well.[6,7] All three subtypes of α_1-AR coexist in the heart, but positive inotropic effect is mediated mainly by α_{1A}- and α_{1B}-AR subtypes.[3,6] The maximum positive inotropic effect triggered by α_1-AR is by far less than that evoked by β-AR stimulation.[2] The role of α_1-AR in regulation of the heart function is still a matter of discussion.

Selye's doctrine of stress response nonspecificity[8] has been modified during past years. Nowadays we accept that stress reaction is a specific response of an organism to different stimuli. Individual stressors (immobilization, cold, hypoglycemia, and others) activate the SAS differently. Immobilization increases significantly both NE and EPI plasma levels, while, on the contrary, cold increases NE plasma levels leaving EPI levels practically unchanged.[9] Thus, stressful situations induce increases in plasma levels of catecholamines.[9] Prolonged exposure to stress-induced increases in catecholamine levels could lead to changes in gene expression and protein levels of AR. The aim of this study was to explore changes in mRNA levels of the above-mentioned AR in the heart atria and ventricles in rats exposed to short- and long-term cold exposure as well as in cold-acclimated animals exposed to a novel stressor (immobilization). We wanted to find out whether there is an adaptation in gene expression of the heart AR to chronic stress exposure and how such acclimated rats respond after exposure to a novel stressor.

Materials and Methods

Animals

Male Sprague-Dawley rats (250–300 g, Charles River, Suzfeld, Germany) were used in our experiments. Animals were housed two per cage in a controlled environment (22 ± 2°C, 12 h light–dark cycle, lights on at 6 AM) 1 week before the beginning of the experiment. After the first week, cold-stressed animals were gradually housed two per cage in a cold chamber (4°C, 12 h light–dark cycle, lights on at 6 AM) for 28, 7, and 1 day(s). Animals exposed to 28 days cold (acclimated), as well as animals held at room temperature (control), were exposed to immobilization stress as described before.[10] Animals were immobilized once for 2 h and immediately decapitated. Cold-acclimated rats were immobilized in the cold chamber. All experimental groups (controls, 28 days, 7 days, and 1 day cold exposed, immobilized control and immobilized acclimated rats) were decapitated at the same day. Hearts were rapidly removed, atria and ventricles withdrawn and immediately frozen in liquid nitrogen and stored at −70°C until the assay.

RNA Isolation and Relative Quantification of mRNA Levels by Reverse Transcription with Subsequent Polymerase Chain Reaction (RT-PCR)

Total RNA from frozen heart tissue was isolated by TRI Reagent (MRC Ltd., Cincinnati, OH). The purity and integrity of isolated RNAs was checked on GeneQuant Pro spectrophotometer (Amersham Biosciences, Piscataway, NJ). Reverse transcription was performed using 1.5 μg of total RNAs and Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences) with pd(N6) primer (Amersham Biosciences). PCR specific for AR, primers, and PCR conditions were carried out as described previously.[11] PCR products were analyzed on 2% agarose gels. Intensity of individual bands was evaluated by PCBAS 2.08e software (Raytest, Inc., Dusseldorf, Germany).

Statistical Analysis

Results are presented as a mean ± SEM, and each group represents an average of six animals. Statistical differences among groups were determined by one-way analysis of variance (ANOVA), and for multiple comparisons, an adjusted *t*-test modified by Bonferroni's correction was used. Values of $P < 0.05$ were considered to be significant.

Results

Effect of Long-term Cold on Gene Expression of Adrenoceptors in Rat Heart

There were no changes in gene expression of β_1- and β_2-AR in left atria and ventricles of cold-stressed rats (Fig. 1). In the atria, we also did not find any changes of β_3-AR mRNA levels. On the other hand, we found a significant increase of β_3-AR mRNA levels in left ventricles of animals exposed to acute (1 day) and chronic (28 days) cold exposure (Fig. 1). We found decreased levels of α_{1B}-AR mRNA—the dominant subtype of α_1-AR—in atria and ventricles in cold-stressed animals (Fig. 2).

Effect of Single Immobilization on Adrenoceptors mRNA Levels in Hearts of Rats Acclimated to Long-term Cold Exposure

Immobilization did not change mRNA levels of β_1-AR in left atria and ventricles of rat hearts in either control (held at room temperature) or cold-acclimated animals (Figs. 3 and 4). We have found a decrease in gene expression of β_2-AR in hearts of immobilized control rats. In hearts of cold-acclimated animals exposed to immobilization there were no significant changes in mRNA levels of β_2-AR compared to cold control animals (Figs. 3 and 4). No changes in gene expression of β_3-AR in hearts of control and cold-acclimated rats exposed to immobilization were found (Figs. 3 and 4). The only changes in gene expression

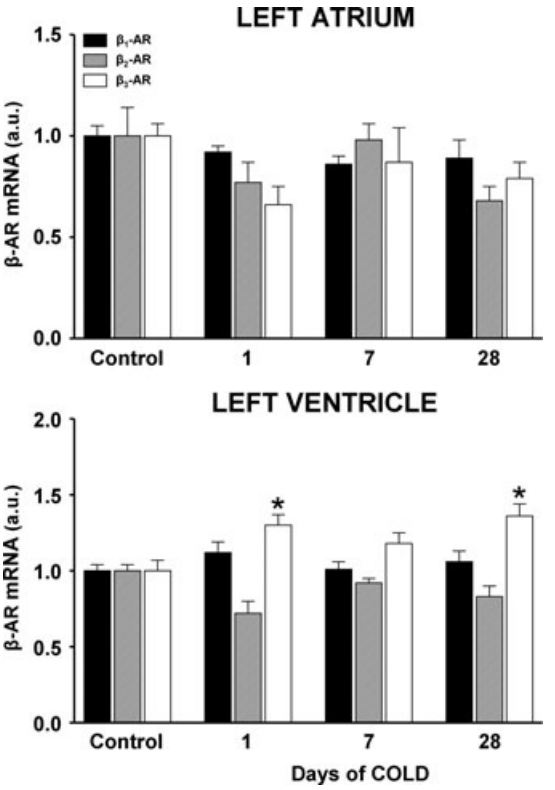


Figure 1. Changes in mRNA levels of various β-AR subtypes in left atrium (*top*) and ventricle (*bottom*) of rats exposed to different time periods of cold exposure (4°C). Results are expressed in arbitrary units as mean ± SEM ($n = 5$–6). Statistical significance between control and cold-stressed group was defined as $^*P < 0.05$.

of AR in hearts of immobilized control and cold acclimated animals were observed in α_{1B}-AR subtype of α_1-AR. There was a decrease in mRNA levels of α_{1B}-AR in hearts of both immobilized control and cold-acclimated animals. We observed also significant additional decreases of α_{1B}-AR mRNA levels in left atria of immobilized cold-acclimated animals (Figs. 3 and 4).

Discussion

Stress is considered a major risk factor in the development of cardiovascular diseases. Stress-induced elevation of plasma levels of catecholamines—NE and EPI—as a

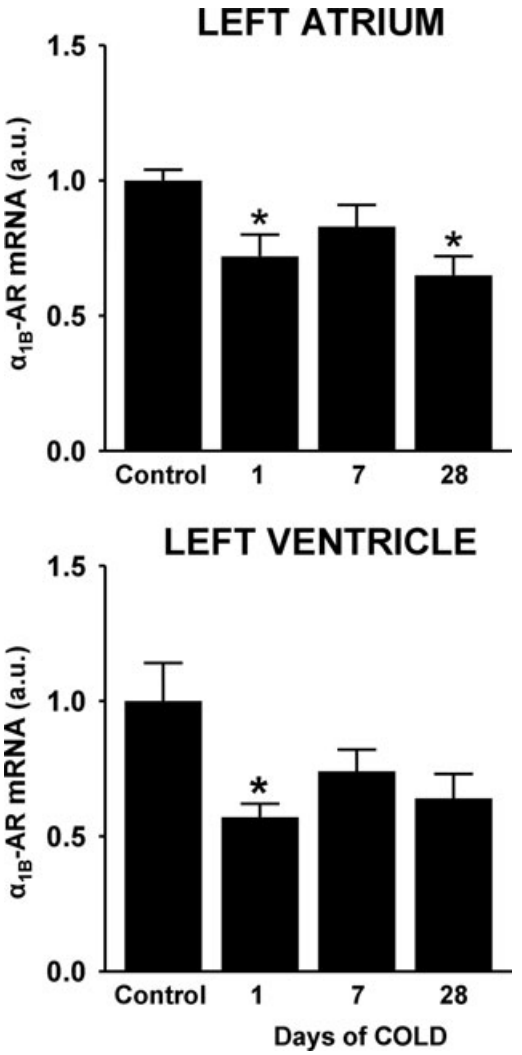


Figure 2. Changes in mRNA levels of α_{1B}-adrenoceptor subtype in left atrium (*top*) and ventricle (*bottom*) of rats exposed to different time periods of cold exposure (4°C). Results are expressed in arbitrary units as mean ± SEM ($n = 5$–6). Statistical significance between control and cold-stressed group was defined as $^{*}P < 0.05$.

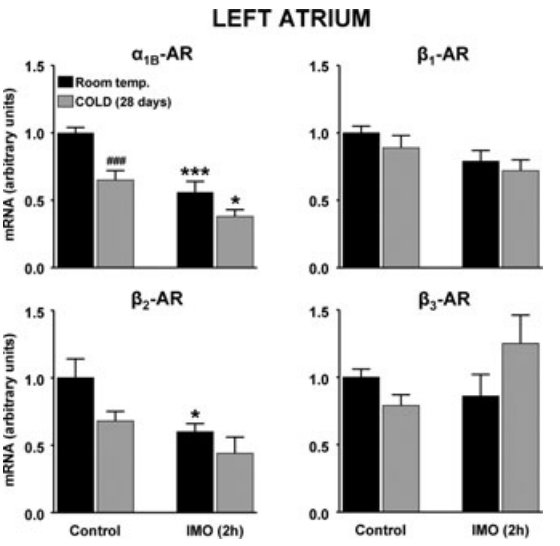


Figure 3. Effect of heterotypic novel stressor (immobilization, IMO) on the gene expression of selected adrenoceptors (α_{1B}, β_1, β_2, and β_3) in left atrium of animals acclimated to long-term cold exposure and in intact, previously unstressed animals. Results are expressed in arbitrary units as mean ± SEM ($n = 5$–6). Statistical significance between control and immobilized group was defined as $^{*}P < 0.05$, $^{***}P < 0.001$ and between group held at room temperature or at cold $^{###}P < 0.001$.

consequence of increased SAS activity is a well-known phenomenon. Changes in mRNA and protein levels of AR in animal models of heart failure and in human failing hearts in relation to increased sympathetic activity have been shown (for review see Brodde and Michel).[1] Due to the increased sympathetic activity, the cardiac NE turnover is enhanced and EPI levels are elevated.[12] Sustained sympathetic stimulation of AR leads to downregulation of β-AR. Decreased levels of β_1-AR, demonstrated by reduced gene expression and protein level and uncoupling of β_2-AR, often seen without changes in mRNA or protein levels, have been described.[1]

In our experiments, we did not find any significant changes of β_1- and β_2-AR mRNA levels in atria and ventricles of animals exposed to acute (1 day) or long-term (28 days) cold. Nevertheless, elevated plasma levels of NE in animals exposed to acute and long-term cold were described.[13,14] Neither exposure of animals to acute cold nor exposure to single immobilization stress was efficient to change levels of β_1-AR mRNA in the left heart. Unchanged levels of β_1-AR mRNA may be related to higher resistance of β_1-AR to agonist-induced downregulation.[15] Contrary to findings on β_1-AR, we found a significant decrease of β_2-AR mRNA levels in animals exposed to single

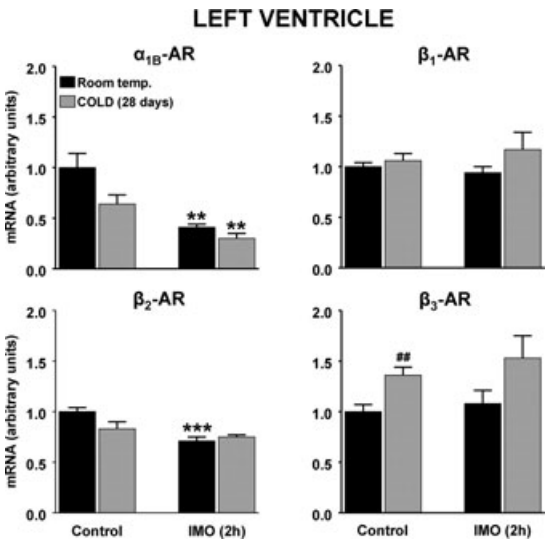


Figure 4. Effect of heterotypic novel stressor (immobilization, IMO) on the gene expression of selected adrenoceptors (α_{1B}, β_1, β_2, and β_3) in left ventricle of animals acclimated to long-term cold exposure and in intact, previously unstressed animals. Results are expressed in arbitrary units as mean ± SEM ($n = 5$–6). Statistical significance between control and immobilized group was defined as $^{**}P < 0.01$, $^{***}P < 0.001$ and between group held at room temperature or at cold $^{\#\#}P < 0.01$.

immobilization stress, while in animals exposed to acute cold stress no changes of β_2-AR mRNA levels in left atria and ventricles were seen (Figs. 1, 3 and 4). These results correspond to decreased density of β_2-AR in immobilized rats observed by Mysliveček and colleagues.[16] As already mentioned, there is a different activation of SAS in relation to the type of stressor. While during immobilization, both plasma levels of NE and EPI are elevated, cold increases only NE plasma levels. So, distinct regulation of β_2-AR gene expression in animals exposed to cold or immobilization is most probably due to the higher affinity of β_2-AR to EPI than to NE.[17]

We also found changes in mRNA levels of the third subtype of β-AR, a significant increase in gene expression of β_3-AR in left ventricle of animals exposed to acute and long-term cold stress (Fig. 1). These results are in good agreement with the observations on animal models of heart failure, in which sustained activation of sympathetic system (elevated levels of NE) led to increased levels of β_3-AR.[18,19] Similarly, in animals exposed to physical training, an increase in the amount of β_3-AR protein in the left ventricle was observed.[20] Stimulation of β_3-AR induces negative inotropic effect, and its overexpression might protect against cardiac damage induced by the high levels of catecholamines present during intense SAS stimulation in stressed healthy heart or in failing heart.[5] Interestingly, in animals exposed to single immobilization, we did not find any changes of β_3-AR mRNA levels in left ventricle compared to acute cold stress (Figs. 1 and 4). Most probably this is a question of stress input duration.

Exposure of cold-acclimated animals (28 days at 4°C) to a novel stressor (immobilization) further increased plasma levels of catecholamines as described earlier,[14] but we did not find any change in the β_1-, β_2-, and β_3-AR mRNA levels. It seems that hearts of acclimated rats are less vulnerable to a novel stressor input. This is evident especially in the β_2-AR mRNA in left atria and ventricles, where immobilization in previously unstressed rats decreases β_2-AR mRNA levels, while in cold-acclimated animals β_2-AR mRNA levels are unchanged. Since, in cold-acclimated animals exposed to a novel stressor (immobilization) a huge increase in plasma levels of EPI was described,[14] we can speculate about a possible adaptation of β_2-AR to repeated stress situations.

Together with dominant β-AR, the heart also expresses subtypes of α_1-AR, predominantly α_{1A}- and α_{1B}-AR in man and rat, respectively. Although catecholamines regulate cardiac contractility primarily by β-AR stimulation, the acute activation of α_1-AR also leads to a positive inotropic effect in the heart.[6,7] We found a decrease in gene expression of α_{1B}-AR in the left atria and ventricles of acute and long-term cold stressed rats (Fig. 2). Also, after single immobilization similar to acute cold exposure, we observed a decrease of α_{1B}-AR

mRNA level in left rat heart (Figs. 3 and 4). Some data about decreased density of total α_1-AR in hearts of animals exposed to immobilization as well as in failing hearts have already been published.[1,2,21,22]

Interestingly, compared to β-AR, we found the most prominent changes in mRNA levels of α_{1B}-AR in atria and ventricles of animals acclimated to cold and then exposed to immobilization. We were able to induce additional decreases of α_{1B}-AR mRNA levels in cold-acclimated animals (Figs. 3 and 4). We can suppose that in cold-acclimated animals there is some kind of reserve in downregulation of α_{1B}-AR in the way of another stressful input. Finally, we can propose that α_{1B}-AR undergo a complex series of adaptive changes generally referred as desensitization. More pronounced changes in gene expression of the α_{1B}-AR in animals exposed to different stress stimuli probably result from their lower density in heart compared to β-AR. Based on our results, we can assume that α_{1B}-AR, in addition to the dominant β-AR, play an important role in the pathway of adaptation to elevated activation of SAS. Crosstalk between α_1- and β-AR was described, and its role in regulation of heart functions is proposed.[3] However, we must be very careful with interpretation of results obtained in rat hearts and its extrapolation to human hearts. In a direct comparative study, it was found that density of α_1-AR is the highest in the rat heart among seven other species, including human.[23] Contrary to human heart, the relative abundance of α_{1B}-AR subtype in the rat heart is higher than that of α_{1A}-AR, which is dominant in the human heart.[24,25]

Our results show different regulation of gene expression of individual types of α and β AR by various stressors. β_2- and α_{1B}-AR are expressed in the opposite manner to β_3-AR, and their role in the process of heart adaptation to repeated or chronic stress exposure is proposed. They may protect heart against cardiotoxic effects of elevated catecholamines levels.

In our previous papers we found that long-term exposure to cold activates the catecholaminergic system in rat adrenals and additional stress input (immobilization) potentiates gene expression of tyrosine hydroxylase, a rate-limiting enzyme in catecholamine biosynthesis, and elevates plasma levels of catecholamines.[13,14] We assume that the catecholaminergic system in cold-acclimated rats is prepared for future stress situations. Question arise, how AR would react on such an activation of the catecholaminergic system in cold-acclimated animals exposed to immobilization.

Prolonged exposure of heart to elevated NE led to changes in gene expression of β-AR that influenced their modulation by additional catecholamine excess. Exposure of cold-acclimated animals to a novel stressor did not change mRNA levels of β-AR in the rat heart that could suggest some kind of adaptation. The mechanism of proposed β-AR adaptation needs to be elucidated. It might be a kind of protection against exaggerated sympathetic stimulation, which could be harmful during long-term and/or additional stress input. Thus, there is a decrease in heart α_{1B}-AR mRNA levels in acclimated animals after exposure to a novel stressor. It appears that α_{1B}-AR is probably involved in modulation of heart functions after exposure to a novel stressor, while the β-AR undergoes adaptation. Nevertheless, further experiments are needed to confirm our results and presumptions and to determine the physiological consequences of our findings.

Acknowledgments

This work was supported by the Slovak Research and Development Agency under the contract No. APVV-0148–06 and by VEGA Grant 2/5125.

Conflicts of Interest

The authors declare no conflicts of interest.

References

1. Brodde, O.E. & M.C. Michel. 1999. Adrenergic and muscarinic receptors in the human heart. *Pharmacol. Rev.* **51:** 651–690.
2. Brodde, O.E. & K. Leineweber. 2004. Autonomic receptor systems in the failing and aging human heart: similarities and differences. *Eur. J. Pharmacol.* **500:** 167–176.
3. Xiao, R.P. *et al*. 2006. Subtype-specific α1- and β-adrenoceptor signaling in the heart. *Trends Pharmacol. Sci.* **27:** 330–337.
4. Gauthier, C., D. Langin & J.L. Ballig. 2000. Beta3-adrenoceptors in the cardiovascular system. *Trends Pharmacol. Sci.* **21:** 426–431.
5. Rozec, B. & C. Gauthier. 2006. β3-Adrenoceptors in the cardiovascular system: Putative roles in human pathologies. *Pharmacol. Therap.* **111:** 652–673.
6. Li, K. *et al*. 1997. Myocardial α1-adrenoceptor: inotropic effect and physiologic and pathologic implications. *Life Sci.* **60:** 1305–1318.
7. Zhang, Y.Y. *et al*. 1999. α1-adrenoceptor subtypes mediating inotropic responses in rat heart. *J. Pharmacol. Exp. Ther.* **291:** 829–836.
8. Selye, H. 1936. A syndrome produced by diverse nocuous agents. *Nature* **138:** 32.
9. Pacak, K. & M. Palkovits. 2001. Stressor specificity of central neuroendocrine responses: implications for stress-related disorders. *Endocr. Rev.* **22:** 502–548.
10. Kvetnansky, R. & L. Mikulaj. 1970. Adrenal and urinary catecholamines in rats during adaptation to repeated immobilization stress. *Endocrinology* **87:** 738–743.
11. Myslivecek, J. *et al*. 2006. Distribution of mRNA and binding sites of adrenoceptors and muscarinic receptors in the rat heart. *Life Sci.* **79:** 112–120.
12. Esler, M. *et al*. 1997. Adrenergic nervous system in heart failure. *Am. J. Cardiol.* **80:** 7–14.
13. Kvetnansky, R. *et al*. 2002. Novel stressors exaggerate tyrosine hydroxylase gene expression in the adrenal medulla of rats exposed to long-term cold stress. In *Stress. Neuronal, Endocrine and Molecular Studies*. R. McCarty, G. Aguilera, E.L. Sabban & R. Kvetnansky, Eds.: 121–128. Taylor & Francis. New York, NY.
14. Dronjak, S. *et al*. 2002. Effects of novel stressors on plasma catecholamine levels in rats exposed to long-term cold. In *Stress. Neuronal, Endocrine and Molecular Studies*. R. McCarty, G. Aguilera, E.L. Sabban & R. Kvetnansky, Eds.: 83–89. Taylor & Francis. New York, NY.
15. Liang, W. *et al*. 2003. Resistance of the human beta1-adrenergic receptor to agonist-mediated down-regulation. Role of the C terminus in determining beta-subtype degradation. *J. Biol. Chem.* **278:** 39773–39781.
16. Myslivecek, J. *et al*. 2004. The effects of short-term immobilization stress on muscarinic receptors, beta-adrenoceptors, and adenylyl cyclase in different heart regions. *Ann. N. Y. Acad. Sci.* **1018:** 315–322.
17. Skeberdis, V.A. 2004. Structure and function of beta3-adrenergic receptors. *Medicina (Kaunas)* **40:** 407–413.
18. Morimoto, A. *et al*. 2004. Endogenous beta3-adrenoreceptor activation contributes to left ventricular and cardiomyocyte dysfunction in heart failure. *Am. J. Physiol. Heart Circ. Physiol.* **286:** H2425–H2433.
19. Zhang, Z.S. *et al*. 2005. Enhanced inhibition of L-type Ca2+ current by {beta}3-adrenergic stimulation in failing rat heart. *J. Pharmacol. Exp. Ther.* **315:** 1203–1211.
20. Barbier, J. *et al*. 2004. Effect of training on beta1 beta2 beta3 adrenergic and M2 muscarinic receptors in rat heart. *Med. Sci. Sports Exerc.* **36:** 949–954.
21. U'Prichard, D.C. & R. Kvetnansky. 1980. Central and peripheral adrenergic receptors in acute and repeated immobilization stress. In *Catecholamines and Stress: Recent Advances*. E. Usdin, R. Kvetnansky & I.J. Kopin, Eds.: 299–308. Elsevier. New York, NY.
22. Kvetnansky, R. & T. Torda. 1983. Changes of heart catecholamine levels, metabolism and adrenergic receptors in acutely and repeatedly stressed rats. In *Cardiac Adaptation to Hemodynamic Overload Training and Stress*. R. Jacob, E. Darmstadt & D. Steinkopf, Eds.: 265–266. Dr. Dietrich Steinkopff Verlag. Darmstadt, Germany.
23. Steinfath, M. *et al*. 1992. Cardiac α1-adrenoceptor densities in different mammalian species. *Br. J. Pharmacol.* **107:** 185–188.
24. Price, D.T. *et al*. 1994. Expression of α1-adrenergic receptor subtype mRNA in rat tissues and human SK-N-MC neuronal cells: Implications for α1-adrenergic subtype classification. *Mol. Pharmacol.* **46:** 221–226.
25. Wolff, D.W. *et al*. 1998. Distribution of α1-adrenergic receptor mRNA species in rat heart. *J. Cardiovasc. Pharmacol.* **32:** 117–122.

4. VŠEOBECNÁ DISKUSE

Z výsledků našich experimentálních prací lze uzavřít, že se nám podařilo identifikovat všechny podtypy α_1-adrenergních receptorů v plicích, identifikovat podtypy muskarinových receptorů v srdci a popsat distribuci mRNA pro příslušné receptory. Tato základní charakteristika receptorů a především zkoumání a porovnání jejich změn u stresovaných zvířat a kontrol nám umožnila některé nové nálezy, které ve známé literatuře dosud popsány nebyly. Za nejdůležitější zjištění považujeme, že se adrenergní a muskarinové receptory mění v plicích vlivem stresu, přičemž jsme prokázali jejich pohlavní závislost a nezbytnost přítomnosti CRH pro aedkvátní průběh stresové odpovědi. Dále jsme prokázali i změny adrenergních a muskarinových receptorů v srdci pod vlivem stresu.

Pokud tyto závěry konfrontujeme s našimi hypotézami, které jsme stanovili v úvodu, pak lze konstatovat, že hypotéza o přítomnosti všech α_1-adrenergních receptorových podtypů v plicích byla potvrzena (hypotéza a). Navíc je třeba zdůraznit, že právě potvrzení přítomnosti všech těchto podtypů nám umožnilo, abychom se dále zabývali jejich významem ve stresové reakci. To se ukázalo jako významné ve stresové reakci v plicích, kde podtypy α_1-adrenergních receptorů hrají zřejmě významnou úlohu. Mimoto jsme také zjistili významné rozdíly v množství vazebných míst mezi samci a samicemi, což by mohlo vzhledem k pohlavní závislosti prevalence astmatu hrát možnou roli v patogenesi jeho vzniku. Hypotéza b) se v zásadě potvrdila: minoritní muskarinový podtyp se nám podařilo v srdečních komorách identifikovat, stejně jako na úrovni genové exprese zaznamenat přítomnost β_3-adrenergních receptorů. Závislost mezi genovou expresí a vazbou na receptory existovala, i když ve všech oblastech množství vazebných míst zcela nekorespondovalo s mírou genové exprese. Zastoupení receptorových typů i podtypů se lišilo podle srdečních oblastí. Tento popis distribuce nám umožnil identifikovat oblasti srdce, u kterých jsme podrobně sledovali stresovou reakci. Tato reakce se lišila dle oblastí srdečních. Ukázalo se také, že i zde hrají

důležitou úlohu α_1-adrenergní receptory. Výsledky sledování vlivu stresu na receptory v plicích potvrdily naše hypotézy: stres nepůsobil pouze na adrenergní receptory, ale ovlivňoval i muskarinové receptory (hypotéza c) a vyřazení genu pro CRH mělo vliv na stresovou odpověď (hypotéza d). Kromě těchto zjištění jsme našli i významné rozdíly ve změnách množství příslušných receptorů mezi samci a samicemi a to jak za u kontrol, tak především u zvířat s vyřazeným genem pro CRH. Toto zjištění by mohlo mít význam v patogenesi různých onemocnění, např. astmatu, onemocnění bezpochyby spojeného se stresovou reakcí a vykazujícího rozdíly v pohlavní prevalenci.

V dalších odstavcích budeme diskutovat konkrétní nálezy našich prací ve vztahu k relevantní literatuře.

4.1 Zastoupení receptorů pro transmitery autonomního nervového systému v plicích

Můžeme tedy shrnout, že jsme v plicích potkanů prokázali výskyt tří subtypů vazebných míst pro α_1-adrenergní receptory.

Použití antagonisté vykazovali specifickou vysokoafinní vazbu a jejich vazebné parametry odpovídaly hodnotám pro klonované α_{1A}- , α_{1B}- , α_{1D} - adrenergní podtypy (pKB1, viz Tab. 1.). Hodnota pKB1 pro RS 17053 (α_{1A}- specifický antagonista) byla prakticky totožná (9.76 *vs.* 9.1-9.9) s hodnotou udávanou Fordem a kol. (Ford, Arredondo et al. 1996) pro klonovaný α_{1A}-adrenoceptor, pKB1 pro L-765,314 (α_{1B}- specifický antagonista) odpovídala (9.28 *vs.* 8.7) hodnotě udávané Patanem a kol. (Patane, DiPardo et al. 1998) pro klonovaný α_{1B}-adrenergní receptor a hodnota pKB1 pro BMY 7378 (specifický antagonista pro α_{1D}-adrenoceptor) byla prakticky totožná (9.37 *vs.* 9.0-9.4) s hodnotou prezentovanou Willemsem a kol. (2001) (Willems, Heiligers et al. 2001) pro klonovaný α_{1D}-adrenergní receptor.

Je však třeba zmínit, že množství vazebných míst s nízkou afinitou (pKB2) poukazuje na suspektní výskyt většího množství vazebných míst s obdobnými vazebnými charakteristikami. Jinak řečeno, tato skutečnost svědčí pro existence jednoho vazebného místa s vysokou afinitou a současně dalšího vazebného místa s nízkou afinitou, které je nejspíše směsí dvou dalších podtypů α_1-adrenergních receptorů. Dále zde existuje další vazebná místa pro ^{3}H-prazosin, která nelze identifikovat za pomoci α_1 antagonistů (viz. Tab. 1.). Tuto skutečnost by mohlo vysvětlovat, že [^{3}H]prazosin se současně minimálně váže i na α_2-adrenergní receptory, tudíž by tato vazebná místa mohla odpovídat α_2-adrenergním receptorům. Tento podtyp α-adrenergních receptorů se v plicích rovněž vyskytuje, jak bylo demonstrováno ve více případech (Goldie, Paterson et al. 1990).

Naše zjištění tří vazebných míst pro ^{3}H-prazosin v plicích koresponduje s výsledky předešlých studií potvrzujících výskyt mRNA pro tyto podtypy α_1-adrenergních receptorů (Faure, Pimoule et al. 1994) a (Alonso-Llamazares, Zamanillo et al. 1995). Stejně tak Hiramatsu a kol.(Hiramatsu, Muraoka et al. 1994) identifikoval na podkladě vazebných studií 3 vazebná místa v plicích potkanů. Vzhledem k úrovni tehdejších znalostí však mohl pouze uzavřít, že se jedná α_{1A}-, α_{1B}- adrenergní podtypy a další třetí neznámý podtyp α_1-adrenergního receptoru.

Na druhou stranu Yang et al. (1998)(Yang, Reese et al. 1998) identifikovali u myší pouze α_{1A} a α_{1B} vazebné podtypy. Obdobně Calzada a kol.(Calzada and De Artinano 2001) dokumentovali expresi pouze dvou typů mRNA pro α_1-adrenergní receptory (α_1 a α_{1B}-adrenergní podtyp).

Naše výsledky, které prokazující tři podtypy α_1 -adrenergních vazebných míst pro [^{3}H]prazosin v plicích, podporují teorii o existenci všech tří podtypů α_1 -adrenergních receptorů v této tkáni: α_{1A}-, α_{1B}- a α_{1D}-podtypů adrenergních receptorů.

4.2 Zastoupení receptorů pro neurotransmitery ANS srdci

Jak je vidět v experimentální části, dá se říci, že množství mRNA kódující adrenergní a muskarinové receptory v dané oblasti srdce koreluje s hustotou příslušných receptorových vazebných míst v této oblasti. Některé výjimky je možné nalézt, pokud sledujeme množství α_{1B} mRNA a množství vazebných míst v síních, ať už v oblastech bohatých na autonomní ganglia, či v oblastech prakticky aganglionárních. Stejně tak jsme zjistili, že největší množství mRNA pro M_2 muskarinové receptory je exprimováno v části levé síně bez neuronálních ganglií (nicméně množství mRNA v komorách je pouze nepatrně menší). V této oblasti odpovídá množství mRNA pro M_2 muskarinové receptory hustotě vazebných míst a jedná se o oblast s nejvyšší hustotou vazebných receptorových míst pro muskarinové ligandy v srdci. Menší množství mRNA pro M_2 muskarinové receptory je exprimováno v levé síni, v části bohaté na gangliové buňky, což je v souladu s hustotou vazebných míst v této oblasti. Na druhou stranu ačkoliv se množství mRNA M_2 muskarinových receptorů příliš nelišilo v srdečních komorách a síních, hustota vazebných míst byla vyšší v levých síních (v části bez nervových ganglií), než v komorách. Tyto výsledky naznačují, že by u muskarinových receptorů mohla existovat receptorová rezerva. Dalo by se spekulovat, že tato rezerva (respektive rezerva mRNA) by mohla v komorách umožnit rychlou akutní proteosyntézu příslušné receptorové bílkoviny a umožnit tak rychlejší funkční změnu. Dále je třeba zmínit, že třebaže jsme nezjistili žádný rozdíl v expresi mRNA pro β_1- a β_2-adrenergní receptory mezi jednotlivými srdečními oddíly, vazba na tyto receptory, zjištěná pomocí radioligandu, se mezi jednotlivými srdečními oblastmi lišila. To platí především pro β_2-adrenergní receptory: mRNA pro tyto receptory byla téměř rovnoměrně exprimována v jednotlivých srdečních oblastech, nicméně množství receptorové bílkoviny (zjišťováno vazebnou studií) bylo nejvyšší v oblasti komorového septa.

Distribuce β_1- a β_2-adrenergní receptorové mRNA se mezi jednotlivými srdečními oddíly nelišila, zatímco vazba odpovídající příslušným receptorům v jednotlivých sledovaných srdečních oblastech srdce rozdílná byla. Množství mRNA pro α_{1B}-receptory se mezi jednotlivými oddíly lišila nespecificky, bez jasného trendu. Také množství receptorových vazebných míst pro α_{1B}-receptory se v jednotlivých srdečních oddílech lišila, především v oblsti síní nekorespondovala s rozdíly v množství mRNA. Množství mRNA pro α_{1A}- a β_3-adrenergní receptory má podobnou distribuci v jednotlivých oblastech srdce, které jsme v rámci našeho experimentu použili.

Stručně řečeno, naše údaje souhlasí s výzkumy na lidském srdci (Brodde O.E. and M.C. 1999) v následujících závěrech:

1) množství β_1-adrenergních receptorů ve všech srdečních oddílech je mnohem vyšší než množství β_2-adrenergních receptorů,
2) množství α_{1B}-adrenergních receptorů v srdečních oddílech je mnohem vyšší než α_{1A}-adrenergních receptorů,
3) množství α_1-adrenergních receptorů je mnohem vyšší v komorách, než v síních
4) hustota muskarinových vazebných míst je mnohem vyšší v atriích než v komorách, ačkoliv množství mRNA se výrazně neliší (v síních je nepatrně vyšší).
5) množství β_3-adrenergní mRNA je velmi nízká ve všech oddílech.

Naše výsledky jsou také v souladu s údaji Wolffa a kol.(Wolff, Dang et al. 1998) o distribuci mRNA pro α_1-adrenergní receptory v různých částech srdce, i když rozdíly v distribuci mRNA pro α_{1A}-adrenergní receptory jsme nedetekovali. Navíc jsme byli schopni identifikovat nejen rozdíly v distribuci mRNA, ale také ve vazbě ligandu na a_{1B}-adrenergní receptory.

Dále jsme získali obdobné výsledky jako (Hardouin, Bourgeois et al. 1998). Tito autoři popsali distribuci mRNA pro β_2-adrenergní receptory v různých částech srdce, nicméně nerozlišovali mezi ganglionárními a aganglionárními částmi síní.

Ačkoliv exprese mRNA pro M_2 muskarinové receptory, β_1-, β_2 a α_{1B}-adrenergní receptory byla opakovaně popsána v myokardu potkana (pro přehled (Brodde, Bruck et al. 2001), stále existují pochybnosti o expresi mRNA pro β_3-adrenergní receptory v srdci. Také α_{1A}-adrenergní receptorová mRNA není v srdeční tkáni pravidelně nacházena a není vyloučeno, že mohou existovat mezidruhové rozdíly (Brodde O.E. and M.C. 1999). Receptory β_3-adrenergní a jejich výskyt v srdci je doposud stále předmětem diskuzí. Zatímco většina autorů ((Gauthier C., Langin D. et al. 2000)) stanovila β_3-adrenergní receptory v srdci funkčně i na úrovni mRNA, některé výzkumné skupiny (Kaumann and Molenaar 1997; Oostendorp and Kaumann 2000) prokázali pouze marginální vliv β_3-adrenergních agonistů na srdeční funkce. Ačkoliv některým skupinám (Krief, Lonnqvist et al. 1993; Berkowitz, Nardone et al. 1995; Evans, Papaioannou et al. 1996) se mRNA pro β_3-adrenergní receptory ve jednotilivých oblastech srdce identifikovat nepodařilo, my jsme identifikovali mRNA pro β_3-adrenergní receptory ve všech námi sledovaných oblastech. Na druhou stranu je třeba si uvědomit, že průměrná genová exprese β_3-adrenergních receptorů (ve všech sledovaných oblastech srdce) byla asi 5× nižší než exprese β_1-adrenergních receptorů a asi 2-3× nižší než exprese β_2-adrenergních receptorů.

Stejně tak jsme v naší studii potvrdili, že v srdeční tkáni je hojnější výskyt mRNA pro α_{1B}-adrenergní receptory, než mRNA pro α_{1A}-adrenergních receptorů. V této souvislosti je však třeba si uvědomit, že exprese mRNA i množství vazebných míst pro α_1-adrenergní receptory je v lidském srdci mnohem menší než u potkanů (Brodde O.E. and M.C. 1999). Podle našich znalostí se jedná o první studii popisující rozdíly v hustotě vazebných receptorových míst a množství mRNA v srdci, kdy bylo použito dělení síní na části bohaté na

autonomní ganglia a na části prakticky aganglionární. Naše data přinesla nové poznatky o rozložení všech podtypů receptorů v oblastech srdce bohaté na buňky neuronálních ganglií. Tato skutečnost může mít velký význam při regulaci receptorů a při vzájemném propojení mezi různými podtypy receptorů jak v myocytech, tak v nervových buňkách.

Zjistili jsme, že srdeční receptory pro neurotransmitery, které jsou uvolňovány na autonomních nervových zakončeních, mají různou a specifickou distribuci v srdečních oblastech. Distribuce mRNA pro tyto receptory vykazovala menší rozdíly, než pozorované rozdíly v distribuci vazebných míst. To naznačuje, že by mohly existovat další mechanismy (např. na úrovni posttranskripčních a posttranslačních modifikací), které jsou schopny determinovat množství receptorových vazebných míst na membránách a regulovat tak funkční efekt na buňku.

4.3 Vliv stresu v plicích a srdci, význam vyřazení genu pro CRH ve stresové reakci

Klíčovým zjištěním naší práce (Novakova, Kvetnansky et al. 2010) je nález významných, pohlavně odlišných změn, ve vazbě specifických ligandů pro receptory autonomního nervového systému v plicním u WT myší a CRH KO myší. Tyto změny jsme pozorovali jak za fyziologických podmínek, tak především pod vlivem stresu.

Stresová reakce způsobila následující rozdílné reakce u samců a samic: u samců se snižovaly β_1-AR a MR, u samic však nastala mnohem komplexnější reakce: došlo ke snížení α_{1A}-AR, α_{1B}-AR, α_{1D}-AR, β_2-AR a MR. β_1-adrenergní receptory nebyly u samic stresem ovlivněny. Tyto jevy nebyly dosud pozorovány a jsou zajímavé v souvislosti s CRH KO fenotypem. Muglia a kol. pozorovali u těchto myší sexuálně dimorfní reakce na stres (zvýšení kortikosteronu ((Muglia, Jacobson et al. 1995)). U WT myší doposud žádné pohlavní rozdíly popsány nebyly. Kromě toho může být nález pohlavního dimorfismu ve stresové odpovědi

důležitý z hlediska výskytu některých plicních onemocnění, především astmatu. Výskyt astma bronchiale jeví prokázané pohlavní rozdíly (Carey, Card et al. 2007; Almqvist, Worm et al. 2008) a stres je prokazatelně jedním z faktorů, které přispívají k rozvoji astmatu (Vig, Forsythe et al. 2006). Pohlavní rozdíly ve změnách adrenergních a muskarinových receptorů jako odpověď na působení stresu by tedy mohly pomoci osvětlit rozdíly mezi pohlavími u astmatu. Prevalence astmatu v populaci severní Ameriky je až do 20. roku věku vyšší u můžů, pak klesá a stává se opět vyšší krátce před 80. rokem (Carey, Card et al. 2007). Úmrtnost na astma je vyšší u mužů (Carey, Card et al. 2007). V našem modelu, porovnáme-li stáří myší a lidský věk, naše myší odpovídaly přibližně mladým dospělým.

Bylo prokázáno, že u myších samic je nižší citlivost dýchacích cest na cholinergní stimulaci, tj. méně intenzivní bronchokonstrikce (Chang and Mitzner 2007). To koresponduje s naším zjištěním téměř dvakrát větší hustoty MR v plicní tkáni u samců než u samic (a žádného rozdílu v denzitě β-AR).

Plazmatické koncentrace adrenalinu a noradrenalinu se u WT a CRH KO myší měnily odlišně. Koncentrace adrenalinu byla závislá i na pohlaví. Plazmatické hladiny katecholaminů byly naměřeny ve vzorcích krve získaných exsanguinací, tj. v koncentracích, které by měly být vyšší než v arteriální krvi nebo v srdeční tkáni (Kvetnansky, Jahnova et al. 1978). Koncentrace adrenalinu byla u WT myší vyšší než u CRH KO myší a to především u samic. Toto zjištění znamená snížení syntézy adrenalinu u KO myší, což souhlasí s předchozími výsledky (Jeong, Jacobson et al. 2000; Kvetnansky, Kubovcakova et al. 2006). V chromafinních buňkách dřeně nadledvin, kde stresor zvyšuje genovou expresi PNMT, která katalyzuje přeměnu noradrenalinu na adrenalin. Pro tento děj je nezbytná normální funkce HPA osy, neboť enová exprese zmíněného enzymu je stimulována působením glukokortikoidů, které jsou nezbytné i pro její správnou funkci (Jeong, Jacobson et al. 2000; Kvetnansky, Kubovcakova et al. 2006), Tímto způsobem je možné vysvětlit změněné

koncentrace cirkulujících katecholaminů u CRH-KO myší (Jeong, Jacobson et al. 2000; Kvetnansky, Kubovcakova et al. 2006).

Přítomnost GRE na promotérových sekvencích genů β_1-AR (Tseng, Stabila et al. 2001), β_2-AR (Cornett, Hiller et al. 1998) a α_{1B}-AR (Gao and Kunos 1993) ukazuje na mechanismus působení glukokortikoidních hormonů během stresu. Dosavadní data o vlivu stresu na tyto receptory jsou neúplná. Někteří autoři efekt opakované imobilizace na β_1-AR u potkanů neprokázali (Torda, Yamaguchi et al. 1981; Torda T. 1985). V jiných pracích se autoři věnovali vlivu oxidativního stresu a také nenašli účinky na muskarinové a β-adrenergní funkce u potkana, pD_2 hodnoty se nezměnily((van Hoof, van Bree et al. 1996)). Naproti tomu v naší práci podáváme důkazy, že BAR a překvapivě významně i AAR, stejně jako MR jsou na úrovni plic do stresové odpovědi zapojeny. Kromě toho jsme pozorovali významný sexuální dimorfismus ve stresové odpovědi. Naše výsledky překvapivě ukazují, že α_1-AR mohou hrát důležitou roli v plicní fyziologii u samic. Současně se ukazuje, že tyto receptory mají zřejmě nevýznamný podíl na regulaci plicních funkcí u samců.

Některé buňky v plicní tkáni (epiteliální buňky, bronchiální hladké svalové buňky, buňky plicního parenchymu) by mohly představovat základ pro systém heterologní regulace, protože exprimují receptory s významným antagonismem (tj. MR a β-AR) Proto bychom mohli předpokládat, že změny v množství adrenergních receptorů může mít v dalším kroku vliv na množství receptorů muskarinových. Důležitost rovnováhy mezi podtypy MR byla uvedena výše, a rovnováha mezi muskarinovými a adrenergními receptory by v tomto kontextu mohla být rovněž důležitá. Tato heterologní regulace, tedy vliv jednoho systému na jiný receptorový systém, byly opakovaně prokázány (Krohn and Hildebrandt 2004; Myslivecek and Kvetnansky 2006). Další možností je, že změny v množství MR jsou důsledkem přímého účinku nepřítomnosti nebo přítomnosti glukokortikoidů. Předběžný důkaz o přítomnosti GRE

na genu pro M_1-muskarinový receptor byl publikován (Klett and Bonner 1999), ale přítomnost GRE v promoterové oblasti genů MR nebyla zatím definitivně potvrzena.

Také u α_1-AR podtypů jsme zjistili pohlavní rozdíly v bazálních hladinách (samice měly vyšší hustotu receptorů než samci). Pohlavní rozdíly jsme pozorovali i u MR (samci mají vyšší hustotu než samice). Naproti tomu, receptorová denzita β-adrenergních receptorů se nelišila mezi pohlavími. Ve vztahu k problematice receptorové rovnováhy, by rozdíl v reakci na stres mohl být důsledkem rozdílů v receptorových denzitách v plicích mezi samci a samicemi přítomných již za fyziologických podmínek. Zajímavé je zejména vyšší množství muskarinových receptorů u samců, tedy receptorů vyvolávající bronchokonstrikci, což by mohlo být faktorem zodpovědným za rozdíly mezi pohlavími u astmatu, jak bylo uvedeno výše.

Tím, že se hodnoty B_{max}, tj. celková receptorová vazba, se lišila mezi samci a samicemi, samci mají celkově nižší množství receptorů, což se týkalo všech podtypů. Tato skutečnost by mohla vést k tomu, že u samců nedochází k výraznějším změnám vmnožství receptorů pod vlivem stresu.

Signál přenášený přes cAMP je neutralizován hydrolýzou cAMP na AMP enzymem fosfodiesteráza. Koncentrace cAMP v buňce je tedy výsledkem poměru aktivity adenylátcyklázy a fosfodiesterázy. V naší práci jsme zjistili, že hladiny cAMP v plicním parenchymu u samců klesají během stresové reakce a připomínají tak změny v množství vazebných míst β_1-adrenergní a muskarinové receptory. To by mohlo znamenat, že rovnováha mezi syntézou cAMP, jež je stimulována β_1-adrenergními receptory a inhibicí této produkce, jež je výsledkem aktivace muskarinových receptorů, je u stresovaných zvířat narušena. Je zřejmé, že stres snižuje expresi adenylátcyklázy (jak lze usuzovat z neschopnosti forskolinu stimulovat produkci cAMP u stresovaných zvířat). Naproti tomu aktivita fosfolipázy C se

neměnila. Tento jev by mohl mít souvislost s tím, že množství α_1-adrenergních receptorů u samců se pod vlivem stresu výrazněji nemění.

Dále jsme se v rámci našich experimentů zajímali o důsledek vyřazení genu pro CRH. Nepřítomnost genu pro CRH vedla ke snížení AR a MR receptorů v plicní tkáni u myší za fyziologických podmínek. Množství všech receptorů u obou pohlaví (s výjimkou β_1-AR u samic) bylo sníženo. Toto je nutné zmínit, neboť myši, kterým chybí gen CRH, mají snížené koncentrace glukokortikoidů jak za fyziologických podmínek, tak při reakci na stres (Jeong, Jacobson et al. 2000; Kvetnansky, Kubovcakova et al. 2006). Podobně, u CRH KO myší nedochází ke zvýšení hladin glukokortikoidů v cirkadiánním peaku (Muglia, Jacobson et al. 1997). Kromě toho se snižují hladiny PNMT, noradrenalin není efektivně methylován na adrenalinu a hladiny noradrenalinu v plazmě se zvyšují. Toto pozorování je v souladu s poklesem mRNA PNMT a nízkou plazmatickou koncentrací PNMT proteinu (Jeong, Jacobson et al. 2000). V rámci naší práce obdobně nacházíme zvýšené plazmatické koncentrace noradrenalinu u CRH KO samců za fyziologických podmínek. Takto odlišné koncentrace adrenalinu a noradrenalinu stejně jako zvýšení koncentrace noradrenalinu během imobilizačního stresu by mohly přispívat k rozdílným změnám v množství adrenergních a muskarinových receptorů během stresu u samců a samic u CRH KO myší.

Pozorovali jsme hlavně neschopnost CRH KO myší reagovat na stres změnami v množství α_1-, β_1 -, β_2-AR a MR. Konkrétně, α_1-AR a β_2-AR byly u samic CRH KO myší ovlivněny více (sníženy na přibližně polovinu) než β_1-AR. Zatímco β_1-adrenergní receptory se u samic CRH KO myší neměnily, snižovaly se u samců až na 62% u samců. β_2-AR byly sníženy u samců i samic CRH KO myší. Vyřazení genu pro CRH mělo tedy větší účinek na β_2-AR. To může mít souvislost s jejich zvýšenou citlivostí na glukokortikoidy ve srovnání s jinými receptory (Myslivecek, Ricny et al. 2003).

Reakce receptorů na stres byla v plicích méně ovlivněna u CRH KO samců než u samic. To koresponduje se zjištěním, že reakce na stres CRH KO myši jsou sexuálně dimorfní (Muglia, Jacobson et al. 1995).

Kromě toho je také nutné si uvědomit, že intaktní KO myši mají celkově méně receptorových vazebných míst, než WT myši, což platí pro všechny receptory, které jsme sledovali. Proto může chybějící nebo snížená receptorová reakce na stres u KO myší být v první řadě odrazem nižšího bazálního množství receptorů. Tento rozdíl v množství receptorů zhruba proporčně odpovídá poklesu v množství receptorů pod vlivem stresu u WT myší.

V souhrnu můžeme předpokládat, že:

a) plicní zastoupení adrenergních a muskarinových receptorů a rozdíly v jejich pohlavním zastoupení mohou hrát důležitou roli v rozvoji některých plicních onemocnění (např. astma),
b) HPA osa, především CRH, hrájí důležitou roli propojení AR a plicní patologie. Naše práce jako první popisuje účinky stresu (imobilizace) na receptorové systémy v plicní tkáni, které by mohly být podkladem poruchy plicních funkcí u CRH-KO myší, tak, jak byly popsány výše.

Můžeme tedy uzavřít, že:

1) CRH KO zvířata vykazují změny v množství MR, α_1-AR a β-AR na úrovni plicní tkáně za fyziologických podmínek. Naše původní hypotéza, že vyřazení genu pro CRH může mít vliv na počet vazebných receptorových míst v plicích, se tedy potvrdila.
2) Naše hypotéza, že receptory autonomního nervového systému (adrenergní i muskarinové) v plicích u CRH-KO myší budou sníženy se potvrdila.
3) Prokázali jsme pohlavně odlišné změny v množství zmíněných receptorů jako odpověď na působení imobilizačního stresu. U CRH KO myší je intenzita změn

v množství receptorů pod vlivem stresu snížena ve srovnání se situací u WT myší. Ačkoliv je úloha α_1-adrenergních podtypů obecně považována za minoritní, naše výsledky ukazují, že podtypy α_1-adrenergních receptorů mění své množství v závislosti na působení stresu a mohou tedy hrát významnější roli v odpovědi na stresové podněty, než se obecně předpokládá. Tyto změny byly pozorovatelné ovšem pouze u samic, u samců se α_1-adrenergní receptory v rámci stresové odpovědi významněji neměnily.

Vliv stresu na srdeční receptory pro působky autonomního nervového systému vykazoval podobné charakteristiky, jaké jsme pozorovali u adrenergních a muskarinových receptorů v plicní tkáni. Naše nálezy jsou v dobré shodě s dříve publikovanými daty o změnách počtu vazebných míst pro β-adrenergní ligandy vlivem stresu (U'Prichard 1980; Torda T. 1984; Torda, Kvetnansky et al. 1985; Torda T. 1985; Kirby and Johnson 1990). Naše nálezy rozvíjejí tyto poznatky o regionální rozdíly v množství receptorů jako odpověď na působení imobilizačního stresu. Mimoto nález, že se vlivem stresu v srdci snižují i muskarinové receptory, je zcela nový (jedna dřívější práce ruských autorů, našla up-regulaci muskarinových receptorů (Meerson, Kopylov et al. 1991)).

Celkově vzato, naše data popisují současné změny AR a MR, které jsou důležité v rámci reakce na stres. Těmto změnám předchází masivní nárůst koncentrace katecholaminů (viz data naší práce (5) a také dřívější pozorování diskutované v této práci).

Důležitým bodem v procesu receptorových regulací je porovnání genové exprese, změn proteinu a změn počtu vazebných míst. Hladiny katecholaminů negativně korelují s množstvím proteinu či vazebných míst pro adrenergní receptory. Nicméně je třeba poznamenat, že se v mnoha pracích je změna proteinu a změny vazebných míst považována

za identický jev, ale to nemusí vždy odpovídat skutečnosti. Často se totiž může stát, že v procesu degradace receptoru, imunodetekce proteinu metodou Western blotting zachytí protein i přesto, že již žádná vazba na receptor (a tedy žádný funkční receptor) není přítomen. Je tomu tak proto, že se protilátka váže na specifický fragment proteinu, aniž by byla schopná zachytit intaktní vazebné místo, jak to dokáže vazebná studie. Proto by bylo vhodnější v rámci receptorových změn spíše sledovat vazbu ligandů na receptory než množství receptorového proteinu. Genová exprese nemusí být stresem ovlivněna paralelně s receptorovými a proto, jako další, spíše metodický závěr naší práce, bychom doporučili sledovat paralelně změny genové exprese, proteinu a vazby, čímž lze dosáhnout podrobného obrazu změn daných stresovou reakcí.

5. ZÁVĚRY

Na základě výsledků a diskuze můžeme tedy shrnout:

1) identifikovali jsme mRNA a vazebná místa pro všechny podtypy α_1-adrenergních receptorů v plicích potkana,
2) identifikovali jsme podtypy muskarinových receptorů v srdci: identifikovali jsme podtyp M_1 a další podtyp, který by nejspíše mohl odpovídat podtypu M_5, ale nikoliv M_3.
3) popsali jsme distribuci mRNA pro podtypy adrenergní receptorů a pro receptory muskarinové. Současně jsme stanovili množství vazebných míst odpovídajících těmto receptorům v sledovaných deseti srdečních oddílech, u některých receptorů jsme v regionech našli rozdíly v množství mRNA ve srovnání s množstvím vazebných míst (α_1-adrenergní receptory, M_2-receptory v srdečních komorách)
4) prokázali jsme změny v množství adrenergních (překvapivě i významné změny podtypů α_1-adrenergních receptorů) a muskarinových receptorů v plicích myší vlivem stresu. Prokázali jsme pohlavní závislost těchto změn a vliv přítomnosti či absence CRH na receptorovou stresovou odpověď v plicích,
5) prokázali jsme změny adrenergních a muskarinových receptorů v srdci vlivem stresu a odlišnost těchto změn v jednotlivých srdečních oddílech.

6. LITERATURA K ÚVODU A DISKUSI

"IUPHAR receptor database." (http://www.iuphar-db.org/GPCR/; Ki).

Abraham, G., C. Kottke, et al. (2003). "Pharmacological and biochemical characterization of the beta-adrenergic signal transduction pathway in different segments of the respiratory tract." Biochem Pharmacol **66**(6): 1067-81.

Ahlquist, R. (1948). "A study of the adrenotropic receptors." Northwest Med. **47**(7): 493-6.

Aksoy, M. O., I. A. Mardini, et al. (2002). "Glucocorticoid effects on the beta-adrenergic receptor-adenylyl cyclase system of human airway epithelium." J Allergy Clin Immunol **109**(3): 491-7.

Almqvist, C., M. Worm, et al. (2008). "Impact of gender on asthma in childhood and adolescence: a GA2LEN review." Allergy **63**(1): 47-57.

Alonso-Llamazares, A., D. Zamanillo, et al. (1995). "Molecular cloning of alpha 1d-adrenergic receptor and tissue distribution of three alpha 1-adrenergic receptor subtypes in mouse." J Neurochem **65**(6): 2387-92.

Berkowitz, D. E., N. A. Nardone, et al. (1995). "Distribution of beta 3-adrenoceptor mRNA in human tissues." Eur J Pharmacol **289**(2): 223-8.

Brodde O.E. and M. M.C. (1999). "Adrenergic and muscarinic receptors in the human heart." Pharmacol. Rev **51**(4): 651-690

Brodde, O. E., H. Bruck, et al. (2001). "Presence, distribution and physiological function of adrenergic and muscarinic receptor subtypes in the human heart." Basic Res Cardiol **96**(6): 528-38.

Bunemann, M. and M. M. Hosey (1999). "G-protein coupled receptor kinases as modulators of G-protein signalling." J Physiol **517 (Pt 1)**: 5-23.

Bylund, D. B., D. C. Eikenberg, et al. (1994). "International Union of Pharmacology nomenclature of adrenoceptors." Pharmacol Rev **46**(2): 121-36.

Bymaster, F. P., D. L. McKinzie, et al. (2003). "Use of M1-M5 muscarinic receptor knockout mice as novel tools to delineate the physiological roles of the muscarinic cholinergic system." Neurochem Res **28**(3-4): 437-42.

Calzada, B. C. and A. A. De Artinano (2001). "Alpha-adrenoceptor subtypes." Pharmacol. Res. **44**: 195-208.

Carey, M. A., J. W. Card, et al. (2007). "It's all about sex: gender, lung development and lung disease." Trends in Endocrinology & Metabolism **18**(8): 308-313.

Colecraft, H. M., J. P. Egamino, et al. (1998). "Signaling mechanisms underlying muscarinic receptor-mediated increase in contraction rate in cultured heart cells." J Biol Chem **273**(48): 32158-66.

Cordeaux, Y. and S. J. Hill (2002). "Mechanisms of cross-talk between G-protein-coupled receptors." Neurosignals **11**(1): 45-57.

Cornett, L. E., F. C. Hiller, et al. (1998). "Identification of a glucocorticoid response element in the rat beta2-adrenergic receptor gene." Mol Pharmacol **54**(6): 1016-23.

Dhein, S., C. J. van Koppen, et al. (2001). "Muscarinic receptors in the mammalian heart." Pharmacol Res **44**(3): 161-82.

Emala, C. W., J. Clancy, et al. (1997). "Glucocorticoid treatment decreases muscarinic receptor expression in canine airway smooth muscle." American Journal of Physiology - Lung Cellular and Molecular Physiology **16**(4): L745-L751.

Endoh, M., T. Hiramoto, et al. (1991). "Myocardial alpha 1-adrenoceptors mediate positive inotropic effect and changes in phosphatidylinositol metabolism. Species differences in receptor distribution and the intracellular coupling process in mammalian ventricular myocardium." Circ Res **68**(5): 1179-90.

Evans, B. A., M. Papaioannou, et al. (1996). "Expression of beta(3)-adrenoceptor mRNA in rat tissues." British Journal of Pharmacology **117**(1): 210-216.

Faure, C., C. Pimoule, et al. (1994). "Expression of alpha 1-adrenoceptor subtypes in rat tissues: implications for alpha 1-adrenoceptor classification." Eur J Pharmacol **268**(2): 141-9.

Ford, A. P., N. F. Arredondo, et al. (1996). "RS-17053 (N-[2-(2-cyclopropylmethoxyphenoxy)ethyl]-5-chloro-alpha, alpha-dimethyl-1H-indole-3-ethanamine hydrochloride), a selective alpha 1A-adrenoceptor antagonist, displays low affinity for functional alpha 1-adrenoceptors in human prostate: implications for adrenoceptor classification." Mol Pharmacol **49**(2): 209-15.

Ford, A. P., D. V. Daniels, et al. (1997). "Pharmacological pleiotropism of the human recombinant alpha1A-adrenoceptor: implications for alpha1-adrenoceptor classification." Br J Pharmacol **121**(6): 1127-35.

Fryer, A. D. and D. B. Jacoby (1998). "Muscarinic receptors and control of airway smooth muscle." Am J Respir Crit Care Med **158**(5 Pt 3): S154-60.

Gamperl, A. K., M. M. Vijayan, et al. (1998). "Beta-receptors and stress protein 70 expression in hypoxic myocardium of rainbow trout and chinook salmon." Am J Physiol **274**(2 Pt 2): R428-36.

Gao, B. and G. Kunos (1993). "Isolation and characterization of the gene encoding the rat alpha 1B adrenergic receptor." Gene **131**(2): 243-7.

Gauthier, C., G. Tavernier, et al. (2000). "Interspecies differences in the cardiac negative inotropic effects of beta(3)-adrenoceptor agonists." J Pharmacol Exp Ther **290**(2): 687-93.

Gauthier C., Langin D., et al. (2000). "β3-adrenoceptors in the cardiovascular system." Trends Pharmacol. Sci. **21**(11): 426-431.

Goldie, R. G., J. W. Paterson, et al. (1990). "Adrenoceptors in airway smooth muscle." Pharmacol Ther **48**(3): 295-322.

Granneman, J. G. (2001). "The putative beta4-adrenergic receptor is a novel state of the beta1-adrenergic receptor." Am J Physiol Endocrinol Metab **280**(2): E199-202.

Guimaraes, S. and D. Moura (2001). "Vascular adrenoceptors: an update." Pharmacol Rev **53**(2): 319-56.

Hall, I. P. (2000). "Second messengers, ion channels and pharmacology of airway smooth muscle." Eur Respir J **15**(6): 1120-7.

Hamilton, S. E., S. N. Hardouin, et al. (2001). "Alteration of cardiovascular and neuronal function in M1 knockout mice." Life Sci **68**(22-23): 2489-93.

Hardouin, S., F. Bourgeois, et al. (1998). "Beta-adrenergic and muscarinic receptor mRNA accumulation in the sinoatrial node area of adult and senescent rat hearts." Mech Ageing Dev **100**(3): 277-97.

Hiramatsu, Y., R. Muraoka, et al. (1994). "Identification of alpha 1-adrenoceptor subtypes in rat lung by binding of [3H]-prazosin and [3H]-WB4101." J Recept Res **14**(2): 75-98.

Hur, E. M. and K. T. Kim (2002). "G protein-coupled receptor signalling and cross-talk: achieving rapidity and specificity." Cell Signal **14**(5): 397-405.

Chang, H. Y. and W. Mitzner (2007). "Sex differences in mouse models of asthma." Can J Physiol Pharmacol **85**(12): 1226-35.

J.Myslivecek, J. Sedl c. S. T. a. S. T. (1996). "Cross-regulation between muscarinic and beta-adrenergic receptors in mammalian and avian heart." Second conference of the

Czech neuroscience society with international participation.Programme and abstracts.: 80.

Jeong, K. H., L. Jacobson, et al. (2000). "Impaired basal and restraint-induced epinephrine secretion in corticotropin-releasing hormone-deficient mice." Endocrinology **141**(3): 1142-50.

Johnson, M. (1998). "The beta-adrenoceptor." Am J Respir Crit Care Med **158**(5 Pt 3): S146-53.

Kadota, H., M. Kuwahara, et al. (2001). "Effect of M2 and M3 muscarinic receptors on airway responsiveness to carbachol in bronchial-hypersensitive (BHS) and bronchial-hyposensitive (BHR) guinea pigs." Exp Anim **50**(1): 49-58.

Kanemi, O., X. Zhang, et al. (2005). "Acute stress reduces intraparenchymal lung natural killer cells via beta-adrenergic stimulation." Clin Exp Immunol **139**(1): 25-34.

Kaumann, A. J. (1997). "Four beta-adrenoceptor subtypes in the mammalian heart." Trends Pharmacol Sci **18**(3): 70-6.

Kaumann, A. J. and P. Molenaar (1997). "Modulation of human cardiac function through 4 beta-adrenoceptor populations." Naunyn Schmiedebergs Arch Pharmacol **355**(6): 667-81.

Kenakin, T. (2004). "Principles: receptor theory in pharmacology." Trends Pharmacol Sci **25**(4): 186-92.

Kirby, R. F. and A. K. Johnson (1990). "Role of beta 2-adrenoceptors in cardiovascular response of rats to acute stressors." Am-J-Physiol **1990**.

Klett, C. P. and T. I. Bonner (1999). "Identification and characterization of the rat M1 muscarinic receptor promoter." J Neurochem **72**(3): 900-9.

Krief, S., F. Lonnqvist, et al. (1993). "Tissue distribution of beta 3-adrenergic receptor mRNA in man." J Clin Invest **91**(1): 344-9.

Krohn, M. and J. P. Hildebrandt (2004). "Cross-talk of phosphoinositide- and cyclic nucleotide-dependent signaling pathways in differentiating avian nasal gland cells." J Comp Physiol [B] **174**(6): 461-70.

Kubera, M., A. Skowron-Cendrzak, et al. (1992). "Stress-induced changes in muscarinic and [beta]-adrenergic binding sites on rat thymocytes and lymphocytes." Journal of Neuroimmunology **37**(3): 229-235.

Kvetnansky, R., E. Jahnova, et al. (1978). "Changes of adrenal catecholamines and their synthesizing enzymes during ontogenesis and aging in rats." Mech Ageing Dev **7**(3): 209-16.

Kvetnansky, R., L. Kubovcakova, et al. (2006). "Gene expression of phenylethanolamine N-methyltransferase in corticotropin-releasing hormone knockout mice during stress exposure." Cell Mol Neurobiol **26**(4-6): 735-54.

Kvetnansky, R., V. K. Weise, et al. (1979). "Effects of chronic guanethidine treatment and adrenal medullectomy on plasma levels of catecholamines and corticosterone in forcibly immobilized rats." J Pharmacol Exp Ther **209**(2): 287-91.

Liang, W. and S. Mills (2001). "Profile of ligand binding to the porcine beta2-adrenergic receptor." J Anim Sci **79**(4): 877-83.

Lohse, M. J., S. Engelhardt, et al. (2003). "What is the role of beta-adrenergic signaling in heart failure?" Circ Res **93**(10): 896-906.

Mak, J. C. W., M. Nishikawa, et al. (1995). "Glucocorticosteroids increase beta(2)-adrenergic receptor transcription in human lung." American Journal of Physiology - Lung Cellular and Molecular Physiology **12**(1): L41-L46.

Marti, D., R. Miquel, et al. (2005). "Correlation between mRNA levels and functional role of alpha1-adrenoceptor subtypes in arteries: evidence of alpha1L as a functional isoform of the alpha1A-adrenoceptor." Am J Physiol Heart Circ Physiol **289**(5): H1923-32.

Maruyama, K., T. Nakamura, et al. (1998). "Tamsulosin: assessment of affinity of 3H-prazosin bindings to two alpha1-adrenoceptor subtypes (alpha1H and alpha1L) in bovine prostate and rat heart and brain." Gen Pharmacol **31**(4): 597-600.

McNeel, R. L. and H. J. Mersmann (1999). "Distribution and quantification of beta1-, beta2-, and beta3-adrenergic receptor subtype transcripts in porcine tissues." J Anim Sci **77**(3): 611-21.

Meerson, F. Z., I. N. Kopylov, et al. (1991). "Alpha1 desensitization of the heart during adaptation to stress.". Fiziol Zh. **37**: 3-6.

Meerson, F. Z., Kopylov, I.N., Baldenkov G.N. (1991). "Alpha1 desensitization of the heart during adaptation to stress." Fiziol Zh(37): 3-6.

Morris, A. J. and C. C. Malbon (1999). "Physiological regulation of G protein-linked signaling." Physiol Rev **79**(4): 1373-430.

Mravec, B. (2008). Neurobiológia chorôb periférnych tkanív. Bratislava, SAP.

Muglia, L., L. Jacobson, et al. (1995). "Corticotropin-releasing hormone deficiency reveals major fetal but not adult glucocorticoid need." Nature **373**(6513): 427-32.

Muglia, L. J., L. Jacobson, et al. (1997). "Impaired diurnal adrenal rhythmicity restored by constant infusion of corticotropin-releasing hormone in corticotropin-releasing hormone-deficient mice." J Clin Invest **99**(12): 2923-9.

Muramatsu, S. F., Tanaka T, Yamamoto H, Morishima S. (2006). "Alpha1-adrenoceptor subtypes and alpha1-adrenoceptor antagonists." Yakugaku Zasshi. **2006 Mar**(126): 187-98.

Myslivecek, J. (1996). "Regulatory mechanisms of receptor-mediated signal transduction." Cesk Fysiol **45**(4): 190-202.

Myslivecek, J. (1998). "Cross-regulation between muscarine and beta-adrenergic receptors." Cesk Fysiol **47**(3): 115-21.

Myslivecek, J. (2003). "Regulation of G-protein-coupled receptors." Cesk Fysiol **52**(1): 4-9.

Myslivecek, J., M. Klein, et al. (2008). "The detection of the non-M2 muscarinic receptor subtype in the rat heart atria and ventricles." Naunyn-Schmiedeberg's Archives of Pharmacology **378**(1): 103-116.

Myslivecek, J. and R. Kvetnansky (2006). "The effects of stress on muscarinic receptors. Heterologous receptor regulation: yes or no?" Auton Autacoid Pharmacol **26**(3): 235-51.

Myslivecek, J., M. Novakova, et al. (2006). "Distribution of mRNA and binding sites of adrenoceptors and muscarinic receptors in the rat heart." Life Sci **79**(2): 112-20.

Myslivecek, J., J. Ricny, et al. (2003). "The effects of hydrocortisone on rat heart muscarinic and adrenergic alpha 1, beta 1 and beta 2 receptors, propranolol-resistant binding sites and on some subsequent steps in intracellular signalling." Naunyn Schmiedebergs Arch Pharmacol **368**(5): 366-76.

Myslivecek, J., J. Ricny, et al. (2004). "The effects of short-term immobilization stress on muscarinic receptors, beta-adrenoceptors, and adenylyl cyclase in different heart regions." Ann N Y Acad Sci **1018**: 315-22.

Myslivecek, J., A. Tillinger, et al. (2008). "Regulation of Adrenoceptor and Muscarinic Receptor Gene Expression after Single and Repeated Stress." Annals of the New York Academy of Sciences **1148**(1): 367-376.

Myslivecek, J. and S. Trojan (2000). "Mechanisms of G-protein coupled receptor regulation." Sb Lek **101**(3): 205-13.

Myslivecek, J. and S. Trojan (2003). "Regulation of adrenoceptors and muscarinic receptors in the heart." Gen Physiol Biophys **22**(1): 3-14.

Nobata, K., M. Fujimura, et al. (2002). "Alpha(1L)-, but not alpha(1H)-, adrenoceptor antagonist prevents allergic bronchoconstriction in guinea pigs in vivo." Eur J Pharmacol **452**(1): 97-104.

Noguchi H, M. R., Kigoshi S, Muramatsu I. (1993). "Alpha 1-adrenoceptor subtype involved in the positive inotropic response to phenylephrine in rat atria." Eur J Pharmacol. (240(2-3)): 291-3.

Nousiainen, O. T., I. Arnala, et al. (1977). "Potentiation of beta-sympathomimetics with alphaadrenoceptor blocking drugs in guinea pig trachea and human bronchus preparations. ." Pharmacology **15**: 469-77.

Novakova, M., R. Kvetnansky, et al. (2010). "Sexual dimorphism in stress-induced changes in adrenergic and muscarinic receptor densities in the lung of wild type and CRH-knockout mice " Stress **13**(1): 22–35.

Oostendorp, J. and A. J. Kaumann (2000). "Pertussis Toxin Suppresses Carbachol-Evoked Cardiodepression but Does Not Modify Cardiostimulation Mediated Through Beta(1)- and Putative Beta(4)-Adrenoceptors in Mouse Left Atria: No Evidence for Beta(2)- and Beta(3)-Adrenoceptor Function." Naunyn-Schmiedebergs Archives of Pharmacology **361**(2): 134-145.

Ota, A., H. Matsui, et al. (1993). "Distribution of beta 1- and beta 2-adrenoceptor subtypes in various mouse tissues." Neurosci Lett **160**(1): 96-100.

Pacak, K. and M. Palkovits (2001). "Stressor specificity of central neuroendocrine responses: implications for stress-related disorders." Endocr Rev **22**(4): 502-48.

Patane, M. A., R. M. DiPardo, et al. (1998). "Selective alpha-1a adrenergic receptor antagonists. Effects of pharmacophore regio- and stereochemistry on potency and selectivity." Bioorg Med Chem Lett **8**(18): 2495-500.

Persad, S., V. Panagia, et al. (1998). "Role of H2O2 in changing beta-adrenoceptor and adenylyl cyclase in ischemia-reperfused hearts." Mol Cell Biochem **186**(1-2): 99-106.

Peters, S., C. Sand, et al. (2001). "Reactive oxygen species potentiate the negative inotropic effect of cardiac M₂-muscarinic receptor stimulation." Naunyn-Schmiedeberg's Archives of Pharmacology **364**(2): 166-171.

Preuss, J. M. H., P. J. Rigby, et al. (1998). "The influence of post-mortem conditions on contractile and relaxant responsiveness of guinea-pig isolated tracheal smooth muscle." Naunyn Schmiedebergs Arch Pharmacol **357**(4): 454-465.

Ramsay, D., I. C. Carr, et al. (2004). "High-affinity interactions between human alpha1A-adrenoceptor C-terminal splice variants produce homo- and heterodimers but do not generate the alpha1L-adrenoceptor." Mol Pharmacol **66**(2): 228-39.

Sand, C., S. L. Peters, et al. (2002). "The effects of hypochlorite-induced oxidative stress on presynaptic M2-receptors at sympathetic nerve endings in the rat tail artery." Auton Autacoid Pharmacol **22**(2): 127-32.

Santos, I. N. and R. C. Spadari-Bratfisch (2006). "Stress and cardiac beta adrenoceptors." Stress **9**(2): 69-84.

Scanga, D. R. and D. A. Schwinn (1998). "Transcriptional regulation of alpha-1 adrenergic receptors." Front Biosci **3**: d348-53.

Selbie, L. A. and S. J. Hill (1998). "G protein-coupled-receptor cross-talk: the fine-tuning of multiple receptor-signalling pathways." Trends Pharmacol Sci **19**(3): 87-93.

Seley, H. (1936). "A syndrome produced by diverse nocious agents." Nature **138**: 32-32.

Skeberdis, V. A. (2004). "Structure and function of beta3-adrenergic receptors." Medicina (Kaunas) **40**(5): 407-13.

Stengel, P. W. and M. L. Cohen (2002). "Muscarinic receptor knockout mice: role of muscarinic acetylcholine receptors M(2), M(3), and M(4) in carbamylcholine-induced gallbladder contractility." J Pharmacol Exp Ther **301**(2): 643-50.

Stengel, P. W., J. Gomeza, et al. (2000). "M(2) and M(4) receptor knockout mice: muscarinic receptor function in cardiac and smooth muscle in vitro." J Pharmacol Exp Ther **292**(3): 877-85.

Takahashi, M., T. Taniguchi, et al. (2000). "Pharmacological characterization of [3H]-JTH-601, a novel alpha1-adrenoceptor antagonist: binding to recombinant human alpha1-adrenoceptors and human prostates." Life Sci **67**(20): 2443-51.

Thiele, R. H., E. C. Nemergut, et al. (2011). "The Physiologic Implications of Isolated Alpha1 Adrenergic Stimulation." Anesthesia & Analgesia(110): 1025-1035.

Torda, T., R. Kvetnansky, et al. (1985). "Effect of repeated immobilization stress on central and peripheral adrenoceptors in rats." Endocrinol Exp **19**(3): 157-63.

Torda, T., I. Yamaguchi, et al. (1981). "Quinacrine-blocked desensitization of adrenoceptors after immobilization stress or repeated injection of isoproterenol in rats." J Pharmacol Exp Ther **216**(2): 334-8.

Torda T., K., I.J. (1985). "Lack of preventive effect of 6-hydroxy-dopamine on a decrease of beta-adrenergic receptors induced by repeated stress in the heart of rats.". Endocrinol. Exp. **19**: 47-51.

Torda T., R. K., Petrikova, M. (1984). Effect of repeated immobilization stress on rat central and peripheral adrenoceptors. Stress: The Role of Catecholamines and Other Neurotransmitters,. R. K. E.Usdin, J.Axelrod. New York, Gordon and Breach Sci Publ**:** 691-701.

Tseng, Y. T., J. P. Stabila, et al. (2001). "A novel glucocorticoid regulatory unit mediates the hormone responsiveness of the beta1-adrenergic receptor gene." Mol Cell Endocrinol **181**(1-2): 165-78.

U'Prichard, D. C., Kvetnansky, R (1980). Central and peripheral adrenergic receptors in acute and repeated immobilization stress. Catecholamines and Stress: Recent Advances. K. Usdin E., R., Kopin, I.J. New York, Elsevier**:** 299-308.

van Hoof, I. H., L. van Bree, et al. (1996). "Changes in receptor function by oxidative stress in guinea pig tracheal smooth muscle." Cent Eur J Public Health **4 Suppl**: 3-5.

Venihaki, M. and J. A. Majzoub (1999). "Animal models of CRH deficiency." Front Neuroendocrinol **20**(2): 122-45.

Vig, R. S., P. Forsythe, et al. (2006). "The Role of Stress in Asthma: Insight from Studies on the Effect of Acute and Chronic Stressors in Models of Airway Inflammation." Ann NY Acad Sci **1088**(1): 65-77.

Wang, Z., H. Shi, et al. (2004). "Functional M3 muscarinic acetylcholine receptors in mammalian hearts." Br J Pharmacol **142**(3): 395-408.

Wess, J., A. Duttaroy, et al. (2003). "M1-M5 muscarinic receptor knockout mice as novel tools to study the physiological roles of the muscarinic cholinergic system." Receptors Channels **9**(4): 279-90.

Willems, E. W., J. P. Heiligers, et al. (2001). "Alpha1-adrenoceptor subtypes mediating vasoconstriction in the carotid circulation of anaesthetized pigs: possible avenues for antimigraine drug development." Cephalalgia **21**(2): 110-9.

Wolff, D. W., H. K. Dang, et al. (1998). "Distribution of alpha1-adrenergic receptor mRNA species in rat heart." J Cardiovasc Pharmacol **32**(1): 117-22.

Yamaguchi, I., T. Torda, et al. (1981). "Adrenoceptor desensitization after immobilization stress or repeated injection of isoproterenol." Am J Physiol **240**(5): H691-6.

Yang, M., J. Reese, et al. (1998). "Murine alpha1-adrenoceptor subtypes. I. Radioligand binding studies." J Pharmacol Exp Ther **286**(2): 841-7.

Zhong, H. and K. P. Minneman (1999). "Alpha1-adrenoceptor subtypes." Eur J Pharmacol **375**(1-3): 261-76.

Zhou, C., A. D. Fryer, et al. (2001). "Structure of the human M(2) muscarinic acetylcholine receptor gene and its promoter." Gene **271**(1): 87-92.

Zimmer, H. G. (1997). "Catecholamine-induced cardiac hypertrophy: significance of proto-oncogene expression." J Mol Med **75**(11-12): 849-59.