PŘÍLOHA III – PUBLIKACE

Available online at www.sciencedirect.com

SCIENCE DIRECT

Developmental Brain Research 151 (2004) 67–73

DEVELOPMENTAL BRAIN RESEARCH

www.elsevier.com/locate/devbrainres

Research report

Increased baclofen-stimulated G protein coupling and deactivation in rat brain cortex during development

Jiri Stöhr[a,b], Lenka Bourova[a,b], Lucie Hejnova[a,b], Ivanna Ihnatovych[c], Jiri Novotny[a,b], Petr Svoboda[a,b,*]

[a] *Departments of Membrane Receptors, Institute of Physiology, Academy of Sciences, Videnska 1083, 142 20, Prague 4, Czech Republic*
[b] *Department of Physiology, Faculty of Natural Sciences, Charles University, Vinicna 7, 120 00, Prague 2, Czech Republic*
[c] *Department of Developmental Epileptology, Institute of Physiology, Academy of Sciences, Videnska 1083, 142 20, Prague 4, Czech Republic*

Accepted 30 March 2004
Available online 1 June 2004

Abstract

The number and affinity of $GABA_B$ receptors (assayed by the specific antagonist [^{3}H]CGP54626A) was unchanged when compared in carefully washed cerebrocortical membranes from young (12-day-old) and adult (90-day-old) rats. In contrast, high-affinity GTPase activity, both basal and baclofen-stimulated was significantly higher (by 45% and 56%, respectively) in adult than in young rats. Similar results were obtained by concomitant determination of agonist (baclofen)-stimulated GTPγS binding. Under standard conditions, baclofen-stimulated GTPase activity was further considerably enhanced by exogenously added regulator of G protein function, RGS1, but not by RGS16. RGS16 was able to affect agonist-stimulated GTPase activity only in the presence of markedly increase substrate (GTP) concentrations. RGS1 alone slightly increased GTPase activity in adult rats, but neither RGS1 nor RGS16 influenced GTPase activity in membrane preparations isolated from young animals. These findings indicate increasing functional activity of trimeric G protein(s) involved in GABAergic transmission in the developing rat brain cortex and suggest a high potential of RGS1 in regulation of high-affinity GTPase activity.

Theme: Development and regeneration
Topic: Neurotransmitter systems and channels

Keywords: Rat brain cortex; High-affinity GTPase; $GABA_B$ receptor; Baclofen; RGS1 and RGS16

1. Introduction

The major inhibitory signalling system in the mammalian brain receives information transmitted by γ-aminobutyric acid (GABA) through two types of membrane-bound receptors—ionotropic ($GABA_A$ and $GABA_C$) and metabotropic ($GABA_B$) receptors. Metabotropic $GABA_B$ receptors initiate a variety of cellular responses via pertussis toxin-sensitive GTP-binding regulatory proteins (G proteins) of the G_i/G_o family [1,16,17,21,28]. This family of G proteins is highly abundant in brain tissue [11,22]. Activation of G_i/G_o as well as other G proteins proceeds by an agonist-induced GDP/GTP exchange reaction, which can be measured as GTPγS binding. This step is immediately followed by stimulation of an endogenous, high-affinity GTPase residing in the Gα subunit, which converts GTP to GDP and inorganic (ortho)phospate. In this way, all heterotrimeric G proteins composed of α and βγ subunits undergo activation/deactivation cycles initiated by agonist-occupied receptors [23]. Since activation of G proteins is initiated by the release of GDP from Gα, measurement of the subsequent binding of GTP (GTPγS) and/or agonist-stimulated GTPase activity serve as a valuable tools for assessment of direct functional coupling between the receptors and their cognate G proteins [27].

It was reported earlier that the number of rat neocortical $GABA_B$ receptors increases during the first 2–3 weeks after birth and then slightly decreases in adulthood [12,32]. The content of $GABA_B$ receptor-coupled $G_i\alpha/G_o\alpha$ proteins in cerebrocortical membranes was shown to rise during post-

* Corresponding author. Department of Biochemistry of Membrane Receptors, Institute of Physiology, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20, Prague 4, Czech Republic. Tel.: +420-241062478; fax: +420-241062488.
E-mail address: svobodap@biomed.cas.cz (P. Svoboda).

0165-3806/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.devbrainres.2004.03.014

natal development [15,18]. Temporary activation of adenylyl cyclase, which was observed around postnatal day (PD) 12, suggests that this period might represent a critical step in postnatal development of G protein-mediated signalling [14,20].

Here we have analysed the high-affinity GTPase activity in cerebrocortical membranes isolated from young (12-day-old) and adult (90-day-old) rats. The 12- and 90-day-old rats were selected from the wide range of age intervals studied previously in our laboratory [12,14,15]. In order to evaluate the potential developmental changes in transduction efficiency of the $GABA_B$-ergic signalling system, baclofen-stimulated GTPase activity was determined. Beside that we have investigated the effect of some regulators of G protein function, namely RGS1 and RGS16, which terminate the signalling by accelerating the intrinsic GTPase activity and promote formation of the inactive GDP-bound state of the Gα subunit. The final result of this reaction sequence is the formation of non-active heterotrimeric form of G protein [6,33]. In parallel, the functional status of GTP-binding proteins in both types of samples was also assessed by GTPγS binding experiments.

2. Materials and methods

[γ^{32}P]GTP (25 Ci/mmol) was from ICN Biomedicals, [^{35}S]GTPγS (1113 Ci/mmol) and [^{3}H]ouabain (17 Ci/mmol) were from Amersham Biosciences International, [^{3}H]CGP54626A (40 Ci/mmol) was obtained from Tocris Cookson. All other chemicals were of highest available purity and purchased from Sigma.

Male Wistar rats were killed by decapitation in deep ether anaesthesia at PD 12 or 90, and their brains quickly removed. The cerebral cortex was rapidly isolated, frozen in liquid nitrogen and stored at − 70 °C. After thawing, the brain tissue was minced with scissors, homogenised in 10 ml STME buffer per gram wet weight (250 mM sucrose, 20 mM Tris HCl, 3 mM $MgCl_2$, 1 mM EDTA; pH 7.6) by using a Potter-Elvehjem homogeniser (Teflon-glass). Unbroken cells and nuclei were removed by low-speed centrifugation ($600 \times g$, 5 min, 4 °C). The supernatant was centrifuged at $200{,}000 \times g$ for 30 min and the resulting membrane pellet resuspended in STME buffer—total volume corresponded to quintuple initial tissue wet weight. Before the final freezing in liquid nitrogen, cerebrocortical membranes were washed twice in STME buffer using high-speed centrifugation ($200{,}000 \times g$, 30 min). For each age group, cerebrocortical membranes were isolated in three independent isolation procedures and five brain cortexes were processed in each preparation.

The properties of $GABA_B$ receptors were assessed by radioligand binding studies using the selective $GABA_B$ antagonist [^{3}H]CGP54626A. These experiments were conducted analogously to [^{3}H]baclofen binding assays described previously [12]. Briefly, 100 μg protein was mixed and incubated with the radioligand in a total volume of 0.5 ml of 50 mM Tris–HCl, 2.5 mM $CaCl_2$; pH 7.4 (TC buffer) for 60 min at 25 °C. Incubation was terminated by filtration through GF/C filters followed by washing (3×3 ml) with ice-cold 50 mM Tris–HCl; pH 7.4. Radioactivity retained on the filters was determined by liquid scintillation. Non-specific binding was defined as that remaining in the presence of 1 mM GABA.

The GTP hydrolysing activity of membrane samples isolated from young and adult rat brain cortex was determined by measuring the radioactivity of inorganic phosphate $^{32}P_i$ released from [γ-^{32}P]GTP as described by Fong and Milligan [7,8]. Samples of cortical membranes (about 10 μg protein) were incubated in the reaction mixture containing 10 mM creatine phosphate, 5 units creatine kinase, 0.1 mM adenosine-5′-*O*-(3-imidotriphosphate (App(NH)p), 1 mM ATP, pH 7.5, 1 mM ouabain, 100 mM NaCl, 5 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM EDTA, pH 7.5, 40 mM Tris–HCl, pH 7.5, 0.5 μM GTP, pH 7.5 and [γ^{32}P]GTP (about 100,000 dpm per assay) for 30 min at 37 °C. GTPase activity was determined in the absence of any additives (basal) or in the presence of 1 mM baclofen (agonist-stimulated level) and/or RGS proteins (0.44 μM). The enzyme reaction was discontinued by chilling the tubes in ice-cold water and charcoal (5% w/v) in 10 mM phosphoric acid. After removal of the charcoal by centrifugation, the radioactivity of $^{32}P_i$ was measured in a liquid scintillation spectrometer. Non-specific GTPase activity was assessed in parallel assays containing 100 μM GTP.

The receptor coupling of GTP-binding proteins was also determined by agonist (baclofen)-stimulated [^{35}S]GTPγS binding as described previously [7,8]. Aliquots of membrane suspension (20 μg of protein) were incubated with (total) or without (basal) 1 mM baclofen in final volume of 100 μl of reaction mix containing 20 mM HEPES (pH 7.4), 3 mM $MgCl_2$, 100 mM NaCl, 2 μM GDP, 0.2 mM ascorbate and 0.5 nM [^{35}S]GTPγS (about 100,000 dpm per assay) for 30 min at 30 °C. The binding reaction was discontinued by dilution with 3 ml of ice-cold 20 mM HEPES (pH 7.4), 3 mM $MgCl_2$ and immediate filtration through Whatman GF/C filters on a Brandel cell harvester. Radioactivity remaining on the filters was determined by liquid scintillation using BioScint cocktail. Affinity of GTPγS binding sites was calculated from competitive displacement curves (5×10^{-6}–1×10^{-10} M GTPγS) by application of the algorithm of DeBlasi [5].

Recombinant RGS1 and RGS16 were expressed and purified as described by [13,34] and were a kind gift from Prof. Graeme Milligan, Glasgow University, UK.

[^{3}H]ouabain binding was used to examine the inhibitor binding sites on Na^+,K^+-ATPase in cerebrocortical membrane preparations from both young and adult rats and this assay was performed as described previously [30].

The Lowry method was used for determination of protein concentrations. An aliquot of each membrane sample was

diluted with water to a final volume of 200 μl, mixed with 0.3 ml of 0.1 N NaOH plus 2% Na_2CO_3 and after 10 min at room temperature, 50 μl of Folin's reagent (diluted 1:1 by volume) was added and mixed immediately. After exactly 30 min, the absorbance was determined at 720 nm. A calibration curve was measured in parallel with 0, 10, 20, 30, 40, 50, 75, 100, 150 and 200 μg of protein standard (Fraction V bovine serum albumin, Sigma). Data were calculated by fitting the calibration curve as a quadratic equation.

If not stated otherwise, all data were obtained from three independent membrane preparations. The numbers represent the average ± S.E.M. of radioligand binding assays or enzyme activity measurements. The data within each group were analysed by one-way ANOVA using the Dunnett's multiple comparison post test and differences between samples from young and adult animals were evaluated by two-way ANOVA followed by the Bonferroni's multiple comparison post test.

3. Results

In order to compare the content of plasma membranes in tested cerebrocortical preparations, specific [^{3}H]ouabain binding was determined as a typical plasma membrane marker. The number of [^{3}H]ouabain binding sites was not significantly different in samples from young (15.6 ± 2.1 pmol mg^{-1} protein) and adult (13.8 ± 2.7 pmol mg^{-1} protein) rats.

$GABA_B$ receptors in cerebrocortical membranes from young and adult animals were determined by radioligand binding using the selective antagonist [^{3}H]CGP54626A. Saturation binding assays were performed in the 0.15–25 nM range of [^{3}H]CGP54626A concentrations. These assays did not reveal any significant difference between the binding characteristics of $GABA_B$ receptors in both tested age groups (Fig. 1). The number and affinity of specific [^{3}H]CGP54626A binding sites in young rats (B_{max} = 0.79 ± 0.22 pmol mg^{-1} protein, K_D = 3.5 ± 1.3 nM) was not different from the number and affinity of these sites in adult animals (B_{max} = 0.85 ± 0.20 pmol mg^{-1} protein, K_D = 5.2 ± 0.6 nM).

The next set of experiments was focused on determination of high-affinity GTPase activity in parallel aliquots of membrane preparations previously used in receptor binding assays. GTPase activity in membranes from adult rats was significantly higher than that determined in membranes isolated from young, 12-day-old rats (Fig. 2). Both basal and baclofen-stimulated GTPase activities were markedly increased (by about 45%) in 90-day-old as compared to 12-day-old rats. The $GABA_B$ receptor agonist baclofen raised GTPase activity in samples from young and adult animals by 57% and 55%, respectively. Whereas RGS16 alone did not influence GTPase activity in any cerebrocortical preparations tested, RGS1 did not affect GTPase activity in young rats but increased it (by 33%) in adult rats. The baclofen-stimulated level of GTPase activity was further enhanced (by about 63%) by the addition of RGS1 in both types of membranes. Interestingly, very little stimulating effect was observed in the case of RGS16.

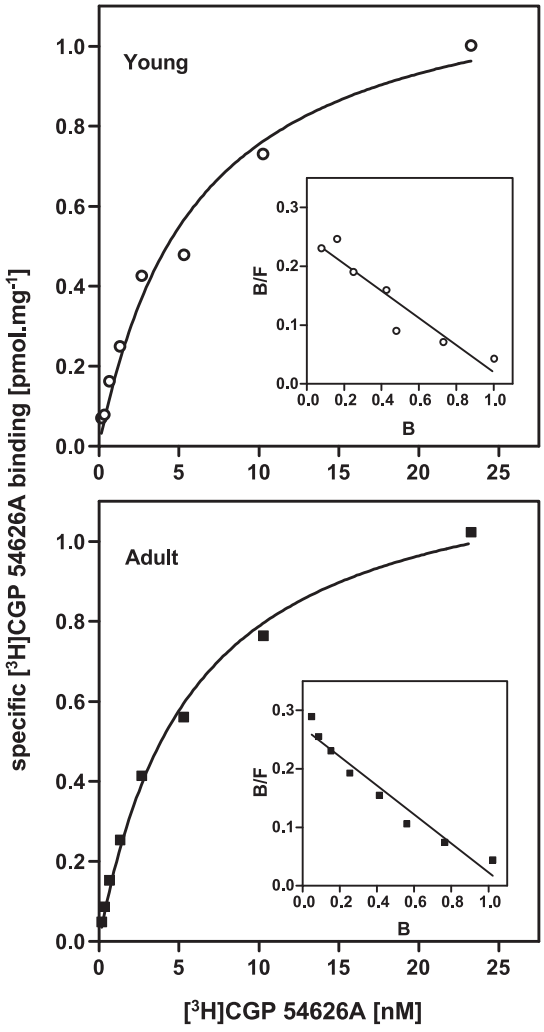


Fig. 1. [^{3}H]CGP 54626A binding in cerebrocortical membranes from young and adult rats. The specific $GABA_B$ antagonist [^{3}H]CGP 54626A was used for determination of number and affinity of $GABA_B$ receptors in 3 × washed membranes prepared from young (12 days old) or adult (90 days old) rats (see Materials and methods). Data represent the mean of three experiments performed in triplicate. Inset: Scatchard representation of the data collected from saturation binding isotherms.

GTPase activity measurements were also performed on these membrane preparations in the presence of a fixed amount of [γ^{32}P]GTP as tracer and increasing concentrations of non-radioactive GTP (10×10^{-9}–10×10^{-6} M) to generate isotopic dilution curves. Enzyme kinetic analysis of such data demonstrated that the $GABA_B$ receptor agonist baclofen raised GTPase V_{max} without altering the measured K_m for GTP (the slope of the line when data were presented as an Eadie-Hofstee transformation) (Fig. 3). This "parallel" type of shift in Eadie-Hofstee plots was observed in 12

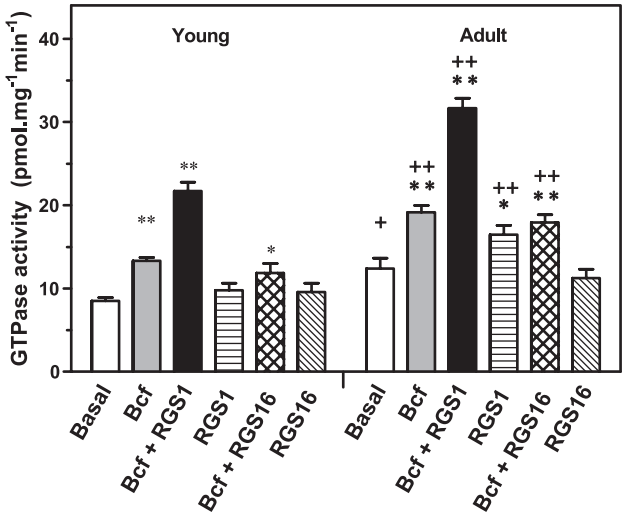


Fig. 2. High-affinity GTPase activity. Basal, baclofen (Bcf)- and/or RGS-stimulated GTPase activity was determined in cerebrocortical membranes isolated from young and adult rats. Data are presented as means ± S.E.M. of at least three experiments performed in triplicates. Asterisks (*$p<0.1$; **$p<0.01$) denote the significant effect of baclofen and/or RGS on basal GTPase activity and statistical difference between corresponding samples from adult and young rats is indicated by small crosses ($^{+}p<0.1$; $^{++}p<0.01$).

days old as well as 90-day-old rats. As noted previously [2,3], these characteristics are incompatible with the agonist-stimulated GTPase activity being regulated by GTPase activating protein such as RGS, possibly because of low amounts of RGS proteins in the membranes. This picture was changed by the addition of exogenous RGS1 and RGS16. Under these conditions, baclofen-stimulated GTPase activity was characterized by increase in V_{max} as well as in the measured K_m for GTP (increased slope of the line). These results indicated that both exogenous RGS1 and RGS16 could function as GAPs (GTPase activating proteins) for receptor-activated G proteins because effects on both V_{max} and K_m for GTP are the anticipated characteristics of such an action [3,4,13]. A significant increase in the K_m and V_{max} values determined in the presence of RGS1 and RGS16 was detected at both age intervals studied (Table 1).

Concomitant measurements of [^{3}H]GTPγS binding indicated that the high-affinity type of this radioligand binding was higher in cerebrocortical membranes isolated from adult rats than in those prepared from young rats (Fig. 4). This difference was found in the case of both basal and baclofen-stimulated levels of [^{3}H]GTPγS binding. The relative increase by baclofen was roughly the same in both types of membranes, by 31% (young) and 28% (adult), respectively. The K_D values, which were calculated from competitive binding displacement curves according to the algorithm of DeBlasi [5], did not significantly differ in samples from young and adult rats—38.6 nM and 33.5 nM, respectively. This indicated an unchanged affinity for the GTPγS binding reaction. However, in both types of membranes, baclofen addition induced a minor left-oriented shift of displacement curves indicating an increase of affinity. This was reflected by the decrease of K_D values from 38.6 to 28.2 nM (33.5–24.3 nM) in samples of young (adult) animals.

4. Discussion

The $GABA_B$ receptor-mediated response is representative of the conventional receptor-initiated mode of G protein activation. Therefore, determination of the basic characteristics of $GABA_B$ receptors had to constitute an initial part of our current experimentation which was primarily aimed at the estimate of G protein functional activity in membrane fractions isolated from young and adult rat brain cortex. Ontogenetic distribution of metabotropic $GABA_B$ receptors in the brain tissue was investigated in a number of earlier studies

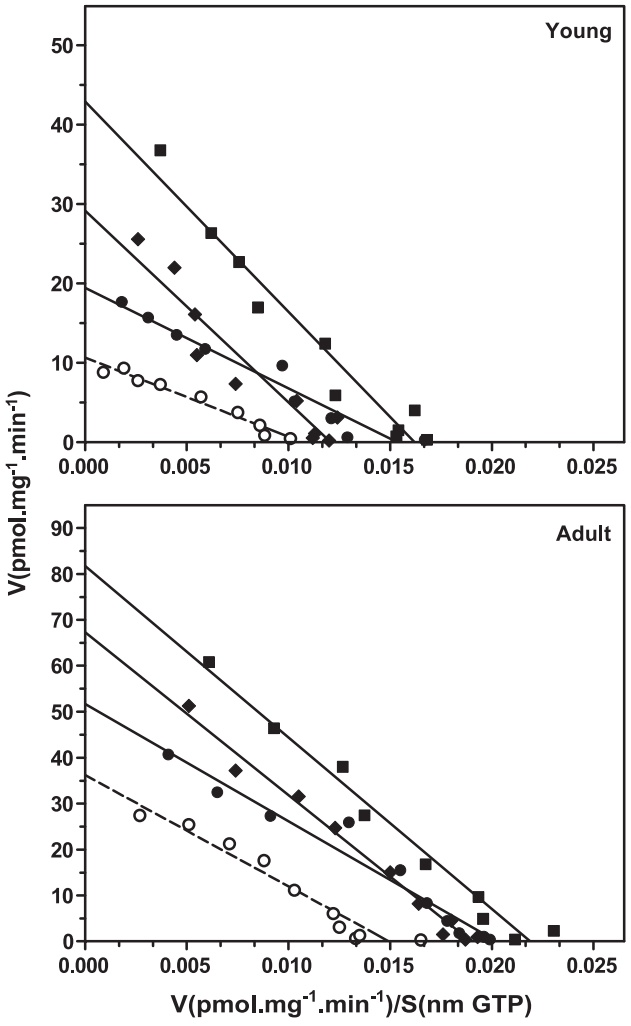


Fig. 3. Kinetic analysis of GTPase activity. Basal- (open circles), baclofen- (closed circles), baclofen + RGS16- (full diamonds) and baclofen + RGS1- (full squares) stimulated GTPase activity was measured in membranes isolated from 12- (Young) and 90-day-old (Adult) rats as function of increasing GTP concentrations. The data are presented as Eadie-Hofstee transformation. The calculated K_m and V_{max} values are shown in Table 1. The data represent the average of three experiments performed in triplicates.

Table 1
Kinetic characteristics of high-affinity GTPase in rat brain cortex

	Basal	Baclofen	Baclofen + RGS1	Baclofen + RGS16
12-day-old				
K_m	0.99 ± 0.09	1.27 ± 0.12	2.65 ± 0.21 $^{\uparrow\uparrow}$	2.41 ± 0.29 $^{\uparrow\uparrow}$
V_{max}	10.7 ± 0.5	19.5 ± 1.1**	42.9 ± 2.6 $^{\uparrow\uparrow\uparrow}$	29.2 ± 2.6 $^{\uparrow\uparrow}$
90-day-old				
K_m	2.43 ± 0.22	2.55 ± 0.19	3.73 ± 0.23 $^{\uparrow\uparrow}$	3.55 ± 0.15 $^{\uparrow\uparrow}$
V_{max}	36.2 ± 2.4	51.7 ± 2.9**	81.7 ± 3.8 $^{\uparrow\uparrow}$	67.3 ± 2.2 $^{\uparrow\uparrow}$

The K_m (μM) and V_{max} (pmol min^{-1} mg^{-1} protein) values were calculated from data presented in Fig. 3. The numbers represent the average of three experiments (means ± S.E.M.). The significant effects of baclofen on basal and those of RGS proteins on baclofen-stimulated kinetic characteristics are indicated by asterisks (**$p < 0.01$) and small arrows ($^{\uparrow\uparrow}p < 0.01$; $^{\uparrow\uparrow\uparrow}p < 0.001$), respectively.

using different experimental approaches [9,10,12,19,29,32]. Our present findings showing no significant change in the receptor density between PD 12 and PD 90 in rat brain cortex are basically in line with most of these investigations.

In order to assess the functional coupling between $GABA_B$ receptors and the inhibitory G proteins, baclofen was employed as an agonist of the receptors involved. Measurement of basal and baclofen-stimulated, high-affinity GTPase activity indicated that both enzyme activities were greatly elevated in samples from adult rats as compared to the young animals. Working with 3 × washed membranes, GTPase activity in both age groups was strongly stimulated by this agonist. Interestingly, the net increment of high-affinity GTPase activity induced by baclofen was virtually the same in both types of membranes. This observation supports the idea that the $GABA_B$ receptor-mediated signalling is already functional in 12-day-old rats but is developed/modified in the course of further maturation.

It has previously been demonstrated that the increase in the brain high-affinity GTPase activity elicited by baclofen does indeed reflect the functional activation of heterotrimeric G proteins coupled with the $GABA_B$ receptors [26]. The brain agonist-stimulated GTPase activity has been analysed in several other studies [24,25,31,35], but these investigations dealt with GTPase activity stimulated by other ligands. Furthermore, most of these studies were performed in regions other than the brain cortex [24,25,31,35]. Thus, there is no direct information available on baclofen-induced changes of high-affinity GTPase activity in the course of postnatal development of rat brain cortex. Here we show that baclofen significantly increased GTPase activity in cerebrocortical preparations from both young and adult rats. Our previous measurements indicated that, between PD 12 and PD 90, the content of $G_i\alpha(1,2)$ and $G_o\alpha$* proteins in rat brain cortex rose by about 50% and 300%, respectively [15]. Higher levels of these G proteins might well account for the increased basal as well as agonist-stimulated GTPase activity in adult rat neocortex. Nevertheless, it is also conceivable that the parallel increases observed in both G protein activation and deactivation might have been brought about by participation of an alternative substrate, such as guanine nucleotide exchange inhibitors that act on G proteins or perhaps G protein kinase/arrestin activity. Kinetic parameters derived from the Eadie-Hofstee plots (unchanged values of K_m) implied that endogenous RGS proteins do not apparently play any significant role in mediating this effect. It has been reported previously that a "parallel" shift in Eadie-Hofstee plots is incompatible with the agonist-stimulated GTPase activity being regulated by a GTPase activating protein such as RGS [2,3].

Next part of our present studies aimed to assess the presumed effect of exogenously added RGS proteins (regulators of G protein signalling), namely RGS1 and RGS16. These two RGS proteins have previously been shown to influence differently agonist-stimulated GTPase activity in COS-7 cells expressing the α_{2A}-adrenoceptor-G protein fusion proteins [13]. Here we took advantage of using cerebrocortical membranes in order to compare the effects of these two closely related RGS proteins on GTPase activity in native tissue containing endogenous receptors and G proteins only. While RGS1 strongly enhanced baclofen-stimulated GTPase activity in samples from both young and adult rats, no such effect was observed for RGS16 in standard experimental conditions. Interestingly, data obtained from the enzyme kinetic analysis indicated that both RGS1 and RGS16 could influence agonist-stimulated GTPase activity. This seeming discrepancy may be most likely explicable on the basis of much weaker regulatory

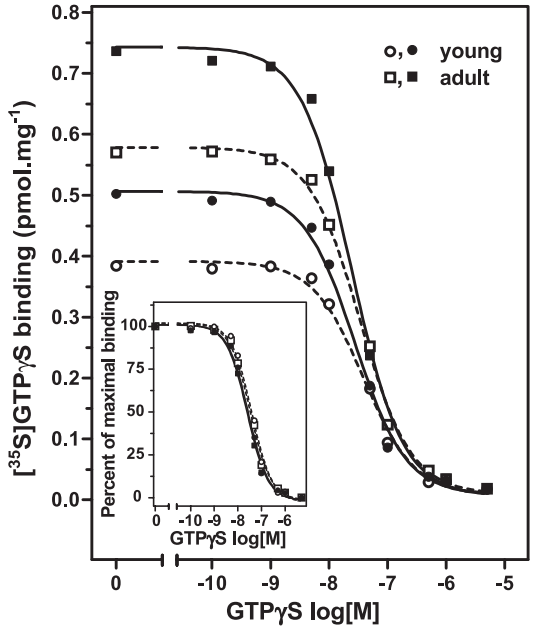


Fig. 4. Competitive binding curves of [^{35}S]GTPγS binding in cerebrocortical membranes from young and adult rats. Points represent the mean of three experiments performed in triplicates for determination of basal (open symbols) and baclofen-stimulated (closed symbols) [^{35}S]GTPγS binding in samples from young (12 days old) and adult (90 days old) rats. Inset: Data were expressed as percentage of the relevant maximal binding.

potential of RGS16 as compared to RGS1. As a strong activator, RGS1 per se increased high-affinity GTPase in preparations from adult animals. Apparently much less potent RGS16 was able to enhance agonist-stimulated GTPase activity only in the presence of high substrate concentrations (>5 μM GTP). Hence, these data provide additional evidence that closely related RGS proteins do not need to function with equal effectiveness [13].

Like high-affinity GTPase activity, GTPγS binding determined in adult rats was higher than that in young animals and baclofen augmented GTPγS binding to similar extent in preparations from both age groups. Hence, these results clearly conform with the increased GTPase activity of cerebrocortical GTP-binding proteins observed in adulthood. We took advantage of using the algorithm of DeBlasi [5] for calculating K_D values for GTPγS binding in both tested membrane preparations and the calculated constants showed not to be significantly different in young and adult rats. Perhaps not too surprisingly, K_D values for baclofen-stimulated GTPγS binding were a bit lower when compared to control conditions, which may reflect the positive effect of agonist-promoted coupling of $GABA_B$ receptors with their cognate G proteins.

In conclusion, our measurements of agonist-stimulated high-affinity GTPase activity and GTPγS binding in the developing rat brain cortex have revealed a markedly higher functional activity of GTP-binding proteins in adult than in young rats. This study further demonstrates that RGS1, by contrast to RGS16, might function as strong regulator of high-affinity GTPase activity in this tissue.

Acknowledgements

This work was supported by The Wellcome Trust, Grant Agency of the Czech Republic (309/01/0255) and by Ministry of Education of the Czech Republic (projects LN00A026 and 11300003). Institutional support was from AVOZ 5011922.

References

[1] T. Asano, M. Ui, N. Ogasawara, Prevention of the agonist binding to γ-aminobutyric acid B receptors by guanine nucleotides and islet-activating protein, pertussis toxin, in bovine cerebral cortex. Possible coupling of the toxin-sensitive GTP-binding proteins to receptors, J. Biol. Chem. 260 (1985) 12653–12658.

[2] D.S. Bahia, N. Sartania, R.J. Ward, A. Cavalli, T.L. Jones, K.M. Druey, G. Milligan, Concerted stimulation and deactivation of pertussis toxin-sensitive G proteins by chimeric G protein-coupled receptor-regulator of G protein signaling 4 fusion proteins: analysis of the contribution of palmitoylated cysteine residues to the GAP activity of RGS4, J. Neurochem. 85 (2003) 1289–1298.

[3] A. Cavalli, K.M. Druey, G. Milligan, The regulator of G protein signaling RGS4 selectively enhances α 2A-adreoreceptor stimulation of the GTPase activity of Go1α and Gi2α, J. Biol. Chem. 275 (2000) 23693–23699.

[4] W. Cladman, P. Chidiac, Characterization and comparison of RGS2 and RGS4 as GTPase-activating proteins for m2 muscarinic receptor-stimulated G(i), Mol. Pharmacol. 62 (2002) 654–659.

[5] A. DeBlasi, K. O'Reilly, H.J. Motulsky, Calculating receptor number from binding experiments using same compound as radioligand and competitor, Trends Pharmacol. Sci. 10 (1989) 227–229.

[6] H.G. Dohlman, J. Thorner, RGS proteins and signaling by heterotrimeric G proteins, J. Biol. Chem. 272 (1997) 3871–3874.

[7] C.W. Fong, G. Milligan, Analysis of agonist function at fusion proteins between the IP prostanoid receptor and cognate, unnatural and chimaeric G-proteins, Biochem. J. 342 (Pt 2) (1999) 457–463.

[8] C.W. Fong, D.S. Bahia, S. Rees, G. Milligan, Selective activation of a chimeric Gi1/Gs G protein α subunit by the human IP prostanoid receptor: analysis using agonist stimulation of high affinity GTPase activity and [^{35}S]guanosine-5′O-(3-thio)triphosphate binding, Mol. Pharmacol. 54 (1998) 249–257.

[9] J.M. Fritschy, V. Meskenaite, O. Weinmann, M. Honer, D. Benke, H. Mohler, GABAB-receptor splice variants GB1a and GB1b in rat brain: developmental regulation, cellular distribution and extrasynaptic localization, Eur. J. Neurosci. 11 (1999) 761–768.

[10] D. Garant, E. Sperber, S. Moshe, The density of $GABA_B$ binding sites in the substantia nigra is greater in rat pups than in adults, Eur. J. Pharmacol. 214 (1992) 75–78.

[11] P. Gierschik, G. Milligan, M. Pines, P. Goldsmith, J. Codina, W. Klee, A. Spiegel, Use of specific antibodies to quantitate the guanine nucleotide-binding protein Go in brain, Proc. Natl. Acad. Sci. U. S. A. 83 (1986) 2258–2262.

[12] L. Hejnova, I. Ihnatovych, J. Novotny, H. Kubova, P. Mares, P. Svoboda, Modulation of adenylyl cyclase activity by baclofen in the developing rat brain: difference between cortex, thalamus and hippocampus, Neurosci. Lett. 330 (2002) 9–12.

[13] M. Hoffmann, R.J. Ward, A. Cavalli, I.C. Carr, G. Milligan, Differential capacities of the RGS1, RGS16 and RGS-GAIP regulators of G protein signaling to enhance α2A-adrenoreceptor agonist-stimulated GTPase activity of G(o1)α, J. Neurochem. 78 (2001) 797–806.

[14] I. Ihnatovych, J. Novotny, R. Haugvicova, L. Bourova, P. Mares, P. Svoboda, Ontogenetic development of the G protein-mediated adenylyl cyclase signalling in rat brain, Brain Res. Dev. Brain Res. 133 (2002) 69–75.

[15] I. Ihnatovych, J. Novotny, R. Haugvicova, L. Bourova, P. Mares, P. Svoboda, Opposing changes of trimeric G protein levels during ontogenetic development of rat brain, Brain Res. Dev. Brain Res. 133 (2002) 57–67.

[16] D.I. Kerr, J. Ong, $GABA_B$ receptors, Pharmacol. Ther. 67 (1995) 187–246.

[17] C. Knott, J.J. Maguire, R. Moratalla, N.G. Bowery, Regional effects of pertussis toxin in vivo and in vitro on GABAB receptor binding in rat brain, Neuroscience 52 (1993) 73–81.

[18] P.P. Li, S. Andreopoulos, C.C. Wong, G.G. Vecil, J.J. Warsh, Effect of thyroid deficiency on Go α-subunit isoforms in developing rat cerebral cortex, Neurochem. Res. 20 (1995) 1249–1255.

[19] B. Malitschek, D. Ruegg, J. Heid, K. Kaupmann, H. Bittiger, W. Frostl, B. Bettler, R. Kuhn, Developmental changes of agonist affinity at GABABR1 receptor variants in rat brain, Mol. Cell. Neurosci. 12 (1998) 56–64.

[20] I. Matsuoka, Y. Suzuki, N. Defer, H. Nakanishi, J. Hanoune, Differential expression of type I, II, and V adenylyl cyclase gene in the postnatal developing rat brain, J. Neurochem. 68 (1997) 498–506.

[21] R. Morishita, K. Kato, T. Asano, GABAB receptors couple to G proteins Go, Go* and Gi1 but not to Gi2, FEBS Lett. 271 (1990) 231–235.

[22] S. Mumby, I.H. Pang, A.G. Gilman, P.C. Sternweis, Chromatographic resolution and immunologic identification of the alpha 40 and alpha 41 subunits of guanine nucleotide-binding regulatory proteins from bovine brain, J. Biol. Chem. 263 (1988) 2020–2026.

[23] E.J. Neer, Heterotrimeric G proteins: organizers of transmembrane signals, Cell 80 (1995) 249–257.

[24] Y. Odagaki, K. Fuxe, 5-HT1A, GABAB, and pirenzepine-insensitive muscarinic receptors are functionally coupled to distinct pools of the same kind of G proteins in rat hippocampus, Brain Res. 689 (1995) 129–135.
[25] Y. Odagaki, S. Dasgupta, K. Fuxe, Additivity and non-additivity between dopamine-, norepinephrine-, carbachol- and GABA-stimulated GTPase activity, Eur. J. Pharmacol. 291 (1995) 245–253.
[26] Y. Odagaki, N. Nishi, H. Ozawa, T. Saito, N. Takahata, P. Riederer, T. Koyama, Measurement of receptor-mediated functional activation of G proteins in postmortem human brain membranes, Brain Res. 789 (1998) 84–91.
[27] Y. Odagaki, N. Nishi, S. Nakagawa, T. Koyama, Activation of G proteins by neuropeptide Y and γ-aminobutyric acid(B) receptor agonists in rat cerebral cortical membranes through distinct modes of action, J. Pharmacol. Exp. Ther. 291 (1999) 1250–1256.
[28] Y. Ohmori, M. Hirouchi, J. Taguchi, K. Kuriyama, Functional coupling of the γ-aminobutyric acidB receptor with calcium ion channel and GTP-binding protein and its alteration following solubilization of the γ-aminobutyric acidB receptor, J. Neurochem. 54 (1990) 80–85.
[29] A. Princivalle, M.C. Regondi, C. Frassoni, N.G. Bowery, R. Spreafico, Distribution of GABA(B) receptor protein in somatosensory cortex and thalamus of adult rats and during postnatal development, Brain Res. Bull. 52 (2000) 397–405.
[30] P. Svoboda, E. Amler, J. Teisinger, Different sensitivity of ATP + Mg + Na (I) and Pi + Mg (II) dependent types of ouabain binding to phospholipase A2, J. Membr. Biol. 104 (1988) 211–221.
[31] M.I. Sweeney, A.C. Dolphin, 1,4-Dihydropyridines modulate GTP hydrolysis by Go in neuronal membranes, FEBS Lett. 310 (1992) 66–70.
[32] S.M. Turgeon, R.L. Albin, Postnatal ontogeny of GABAB binding in rat brain, Neuroscience 62 (1994) 601–613.
[33] N. Watson, M.E. Linder, K.M. Druey, J.H. Kehrl, K.J. Blumer, RGS family members: GTPase-activating proteins for heterotrimeric G-protein α-subunits, Nature 383 (1996) 172–175.
[34] P.J. Welsby, E. Kellett, G. Wilkinson, G. Milligan, Enhanced detection of receptor constitutive activity in the presence of regulators of G protein signaling: applications to the detection and analysis of inverse agonists and low-efficacy partial agonists, Mol. Pharmacol. 61 (2002) 1211–1221.
[35] W.J. Wojcik, D. Cavalla, N.H. Neff, Co-localized adenosine A1 and γ-aminobutyric acid B ($GABA_B$) receptors of cerebellum may share a common adenylate cyclase catalytic unit, J. Pharmacol. Exp. Ther. 232 (1985) 62–66.

JCMM
Available online at
www.jcmm.ro
www.jcmm.org

J. Cell. Mol. Med. Vol 9, No 4, 2005 pp. 940-952

Modulation of adenylyl cyclase activity in young and adult rat brain cortex. Identification of suramin as a direct inhibitor of adenylyl cyclase

Jiri Stöhr [a, b], Jiri Novotny [a, b] *, Lenka Bourova [a, b], Petr Svoboda [a, b]

[a] *Department of Biochemistry of Membrane Receptors, Institute of Physiology, Academy of Sciences, Prague, Czech Republic*
[b] *Department of Physiology, Faculty of Natural Sciences, Charles University, Prague, Czech Republic*

Received: May 27, 2005; Accepted: August 19, 2005

Abstract

Adenylyl cyclase (AC) in brain cortex from young (12-day-old) rats exhibits markedly higher activity than in adult (90-day-old) animals. In order to find some possibly different regulatory features of AC in these two age groups, here we modulated AC activity by dithiothreitol (DTT), Fe^{2+}, ascorbic acid and suramin. We did not detect any substantial difference between the effects of all these tested agents on AC activity in cerebrocortical membranes from young and adult rats, and the enzyme activity was always about two-fold higher in the former preparations. Nevertheless, several interesting findings have come out of these investigations. Whereas forskolin- and Mn^{2+}-stimulated AC activity was significantly enhanced by the addition of DTT, increased concentrations of Fe^{2+} ions or ascorbic acid substantially suppressed the enzyme activity. Lipid peroxidation induced by suitable combinations of DTT/Fe^{2+} or by ascorbic acid did not influence AC activity. We have also observed that PKC- or protein tyrosine kinase-mediated phosphorylation apparently does not play any significant role in different activity of AC determined in cerebrocortical preparations from young and adult rats. Our experiments analysing the presumed modulatory role of suramin revealed that this pharmacologically important drug may act as a direct inhibitor of AC. The enzyme activity was diminished to the same extent by suramin in membranes from both tested age groups. Our present data show that AC is regulated similarly in brain cortex from both young and adult rats, but its overall activity is much lower in adulthood.

Keywords: adenylyl cyclase activity • rat brain cortex • dithiothreitol • peroxidation • suramin

Introduction

Adenylyl cyclase (AC), which catalyses the formation of the ubiquitous second messenger cAMP, plays an important role in neurotransmission in the CNS [1–3]. To date, nine different membrane-bound mammalian isoforms of AC have been cloned and identified in various tissues [4, 5]. Most of AC isoforms may occur in the CNS, but especially type I, II and V are expressed at high levels in rat brain [6, 7]. The unequal tissue distribution of the individual AC isoforms and diversity of their regulatory features may reflect a specific function of this effector molecule in determining the routing of signals to the cAMP pathway.

* Correspondence to: Dr. J. NOVOTNY,
Institute of Physiology, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Prague 4, Czech Republic.
Tel.: + 420-241062703; Fax: + 420-241062488
E-mail: novjiri@biomed.cas.cz

Reprinted from:
Journal of Cellular and Molecular Medicine

Published by:
the CMM Foundation

The regulation of AC activity by G protein-coupled receptors (GPCRs) is a classically described mechanism in which a stimulatory G protein (G_s) couples neurotransmitters to activation of AC, whereas an inhibitory G protein (G_i) couples to inhibition of AC [8]. This simple scheme, however, does not cover significant diversity in regulation of the individual enzyme isoforms. All AC isoforms are activated by the α subunits ($G_s\alpha$) of G_s protein, but only AC type I, V and VI can be inhibited by direct interaction with the α subunits ($G_i\alpha$) of G_i protein [9]. In addition, the different AC isoforms display very distinct patterns of regulation by the βγ subunits of G proteins (Gβγ) as well as by calmodulin (CaM) and protein kinases [5, 10]. All membrane-bound ACs are inhibited by P-site analogues and, except for type IX, stimulated by the diterpene forskolin [11, 12].

Some previous studies revealed marked alterations in AC activity during postnatal development of rodent brain tissue [7, 13–16]. Whereas AC activity is very low shortly after birth, it consecutively increases to reach a sharp maximum by the end of the second postnatal week. Subsequently, activity of AC rapidly decreases to the adult values, which are comparable to those observed shortly after birth. The same developmental profile of the enzyme activity was observed using different stimulators (forskolin, Mn^{2+}, GTPγS, fluoride) of AC [7, 13]. Until now, however, no explanation has been provided for the marked differences between AC activity in young and adult brain.

In the present work we have used different modulators (dithiothreitol, ascorbic acid, Fe^{2+} and suramin) in order to analyse in a more detail AC activity in cerebrocortical membrane preparations from young (12-day-old) and adult (90-day-old) rats. The possible effect of phosphorylation on the enzyme activity was also assed in cerebrocortical homogenates derived from both tested age groups.

Materials and methods

Materials

[α-^{32}P]ATP and [^{3}H]cAMP were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Aluminum oxide 90 (neutral, activity I) was from Merck (Darmstadt, Germany). All other chemicals were obtained from Sigma (St. Louis, MI, USA) and they were of the highest purity available.

Membrane preparation

Young (12-day-old) and adult (90-day-old) male Wistar rats were etherised and killed by decapitation, and their brains quickly removed. Cerebral cortexes were rapidly isolated, frozen in liquid nitrogen and stored at -70°C. The brain material obtained usually from 6 young or 4 adult animals was always combined together to constitute individual experimental groups. After thawing, the brain tissue was minced with scissors, homogenised in 10 ml of STME buffer per gram wet weight (250mM sucrose, 20mM Tris HCl, 3mM $MgCl_2$, 1mM EDTA, pH 7.6) by using a Potter-Elvehjem homogeniser (Teflon-glass). Unbroken cells and nuclei were removed by low-speed centrifugation (600 x g, 5 min, 4°C). The supernatant was centrifuged at 200,000 x g for 30 min and the resulting membrane pellet resuspended in STME buffer (total volume corresponded to quintuple initial tissue wet weight). Cerebrocortical membranes were then washed twice in STME buffer using high-speed centrifugation (200,000 x g, 30 min) and, before final freezing in liquid nitrogen, the membrane pellet was resuspended in TMN buffer (20mM Tris HCl, 3mM $MgCl_2$, 150mM NaCl, pH 7.6).

Adenylyl cyclase activity

Activity of adenylyl cyclase was determined according to the method of White [17]. The reaction mixture (total volume of 0.1ml) contained 30μg of protein (cerebrocortical membranes), 50mM Tris-HCl buffer (pH 7.4), 5mM $MgCl_2$, 50U/ml pyruvate kinase, 10mM potassium phosphoenolpyruvate, 160μg/ml BSA, 0.2 mM 3-isobutyl-1-methylxanthine, 20μM GTP, 0.1mM cAMP, 10,000 cpm per sample of [^{3}H]cAMP and 0.4mM ATP with [α-^{32}P]ATP (about 1x10^6 cpm per sample). Besides forskolin, Mn^{2+}, GTPγS and AlF_4^- (classical activators of AC activity) some other modulators (dithiothreitol, Fe^{2+}, ascorbic acid, suramin) were also used in concentrations stated concretely in the detailed description of the individual experiments. Before starting the reaction, the assay tubes containing all the components except for the isotope were preincubated for 10 min at 30°C. The assay was then run for 30 min at 30°C and the enzyme reaction terminated by adding 0.2 ml of 0.5 M HCl and heating for 5 min at 100°C. The cyclic AMP formed was sepa-

rated from other nucleotides and inorganic phosphate by alumina column chromatography [17].

Assay of lipid peroxidation

Lipid peroxidation was measured using the thiobarbituric acid (TBA) test for malondialdehyde (MDA) formation. This assay was conducted according to the previously described protocol [18], with minor modifications. Briefly, after addition of appropriate amounts of different tested compounds (DTT, Fe^{2+} or ascorbic acid), the reaction mixture (100µl) for AC activity without ATP was incubated for 40 min at 32°C. After this time period, samples were mixed with the same volume (100µl) of ice-cold 10% trichloroacetic acid and centrifuged at 10,000 rpm for 5 min. The resulting supernatant (150µl) was mixed with 0.67% TBA (150µl) and boiled for 10 min. Absorbance was then measured at 535 nm. MDA formation in tested samples was calculated from the calibration curve, which was obtained by parallel measurements of a standard (1,1,3,3-tetramethoxypropane).

Data analysis

All measurements were performed in triplicate in membrane samples prepared by three independent isolation procedures. The numbers represent the average ± S.E.M. and statistically significant differences within each group were calculated using one-way ANOVA with the Dunnett's multiple comparison post test. When desired, differences between samples from young and adult animals were evaluated by two-way ANOVA followed by the Bonferroni's multiple comparison post test.

Results

Activation of AC by forskolin, GTPγS, AlF_4^- and Mn^{2+}

Activity of AC was determined in samples of cerebrocortical membranes prepared from young (12-day-old) and adult (90-day-old) rats. First we measured the dose-response curves of forskolin and GTPγS to find optimum conditions for using these classical stimulators of AC activity (Fig. 1). Forskolin and GTPγS were then used either at maximal stimulating concentrations ($5x10^{-5}$ M and $1x10^{-4}$ M, respectively) or, in some experiments, at lesser concentrations ($1x10^{-5}$ M and $3x10^{-6}$ M, respectively) in order to discern possible effects of some other modulators of AC activity. AlF_4^- (10 mM NaF and 1 mM $AlCl_3$) and Mn^{2+} (10 mM $MnCl_2$) were used at stated standard concentrations in all experiments. In all conditions, AC activity in cerebrocortical membranes from young rats was markedly (about two-fold) higher than in preparations from adult animals.

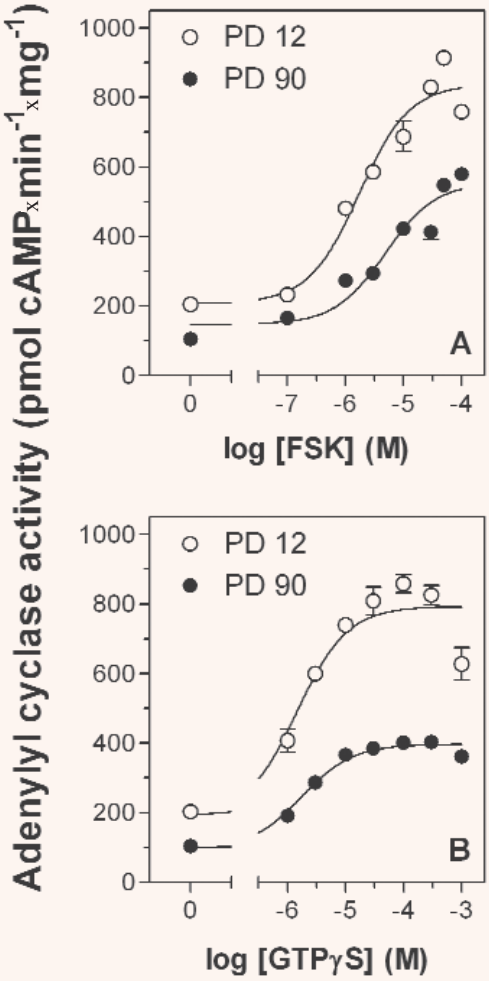


Fig. 1 Stimulation of AC activity by forskolin (FSK) and GTPγS. Activity of AC was determined in cerebrocortical membranes from 12-day-old (PD 12) young (open circles) and adult (closed circles) rats and the dose-response curves for forskolin (A) and GTPγS (B) were constructed. Data are means (± S.E.M.) from a representative experiment assayed in triplicate.

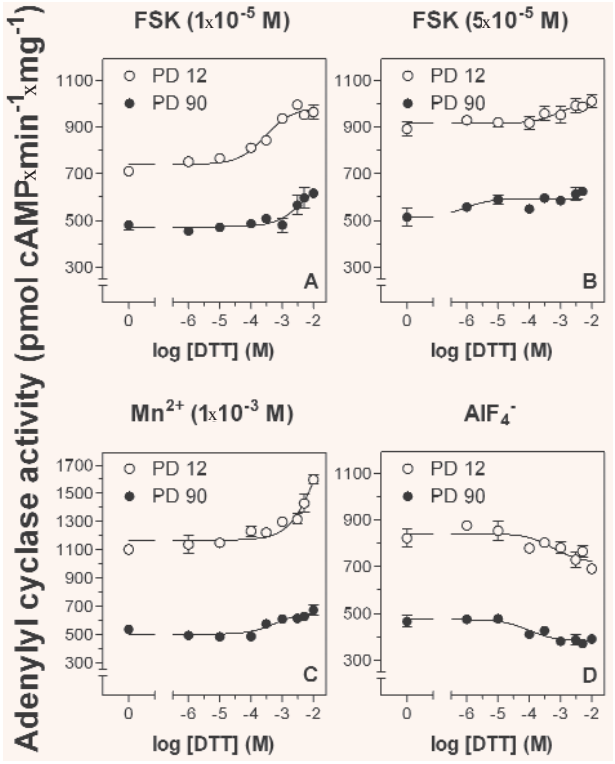


Fig. 2 Effect of DTT on AC activity stimulated by forskolin, Mn^{2+} and AlF_4^-. Increasing concentrations of DTT were applied in order to modulate AC activity stimulated by $1x10^{-5}$ M forskolin (A), $5x10^{-5}$ M forskolin (B), $1x10^{-2}$ M Mn^{2+} (C) and AlF_4^- (D) in cerebrocortical membranes from young (open circles) and adult (closed circles) rats. Data are means (± S.E.M.) from a representative experiment assayed in triplicate.

Effect of dithiothreitol

According to some earlier observations the thiol-reducing agent dithiothreitol (DTT) can influence activity of AC [19, 20]. In our preliminary experiments we observed that DTT did not affect basal AC activity but it was able to enhance forskolin-stimulated activity of the enzyme (data not shown). This augmenting effect of DTT, however, was detectable only at sub-maximal concentrations of forskolin (compare Fig. 2A and 2B). DTT at its most effective concentration (10 mM) increased forskolin-stimulated AC activity by about 37% and 28% in cerebrocortical membranes from young and adult rats, respectively. When used at $1x10^{-5}$ M concentration, forskolin in combination with DTT (10 mM) brought about the same stimulatory effect on AC activity as that achieved by maximal stimulating concentration of forskolin ($5x10^{-5}$ M) alone. At lower forskolin concentrations ($1x10^{-6}$ M), the overall maximal stimulatory effect did not manifest, but the activity of AC was significantly increased (by about 20–30%) in the presence of DTT (data not shown). Interestingly, Mn^{2+}-stimulated AC activity was also enhanced by the addition of DTT, similarly as in the case of forskolin (Fig. 2C). The relative increase in AC activity elicited by DTT in cerebrocortical membranes from young rats was higher by about 20% as compared to the increase in samples from adult animals. By contrast, AlF_4^--stimulated AC activity was rather diminished by DTT (Fig. 2D). Similar decrease (by about 16%) was observed in cerebrocortical membrane preparations both from young and adult rats. Next we analysed the possible effect of DTT on AC activity stimulated by GTPγS, a non-hydrolyzable analogue of GTP. Interestingly, DTT did not significantly influence AC activity stimulated either by maximal or sub-maximal concentrations of GTPγS (Fig. 3).

Effect of Fe^{2+} and ascorbic acid

Some previous studies have demonstrated that free radicals generated by Fe^{2+}/ascorbic acid may alter functioning of the AC signal-transducing system in certain tissues [18, 19, 21, 22]. Our current measurements indicated that the increased concentrations of Fe^{2+} substantially reduce basal as well as forskolin-stimulated AC activity in cerebrocortical membrane preparations both from young and adult rats (Fig. 4). It was shown previously that AC activity in synaptic membrane fraction of rat brain could be potentiated by lipid peroxidation induced by several metal ions (Fe^{2+} inclusive) in the presence of DTT [18, 19]. Surprisingly, here we did not observe any stimulatory effect of any concentration of Fe^{2+} plus DTT (Fig. 5). Results of our concomitant determination of MDA formation demonstrated that Fe^{2+} in the range of micromolar concentrations in the presence of increased levels of DTT (over 1 mM) evidently caused lipid peroxidation (Fig. 6), but activity of AC was not changed under these conditions. Interestingly enough, Fe^{2+} ions at a concentration of $1x10^{-4}$ M induced a quite perceptible increase in MDA formation, which was totally abolished by the addition of 5mM DTT (Fig. 6A). In addition, DTT markedly attenuated the negative

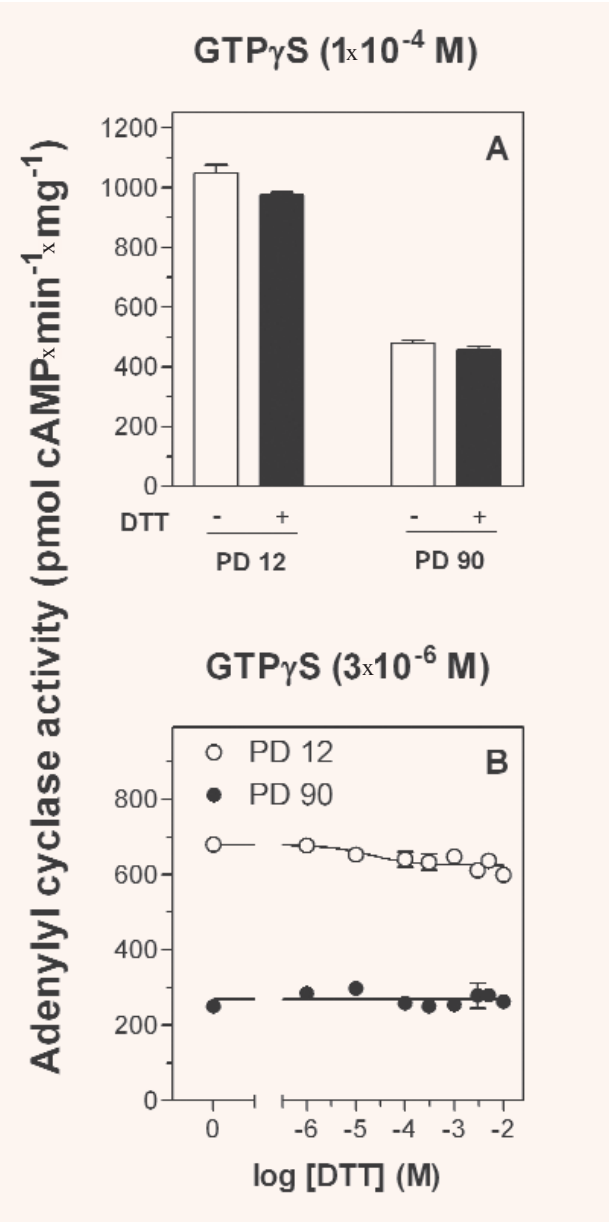


Fig. 3 Effect of DTT on AC activity stimulated by GTPγS. The effect of DTT ($1x10^{-2}$ M) on AC activity stimulated by a maximally effective concentration of GTPγS ($1x10^{-4}$ M) was tested in cerebrocortical membranes from young (PD 12) and adult (PD 90) rats (A). Increasing concentrations of DTT were used in order to modulate AC activity stimulated by a sub-maximal concentration of GTPγS ($3x10^{-6}$ M) in cerebrocortical membranes from young (open circles) and adult (closed circles) rats. Data are means (± S.E.M.) from a representative experiment assayed in triplicate.

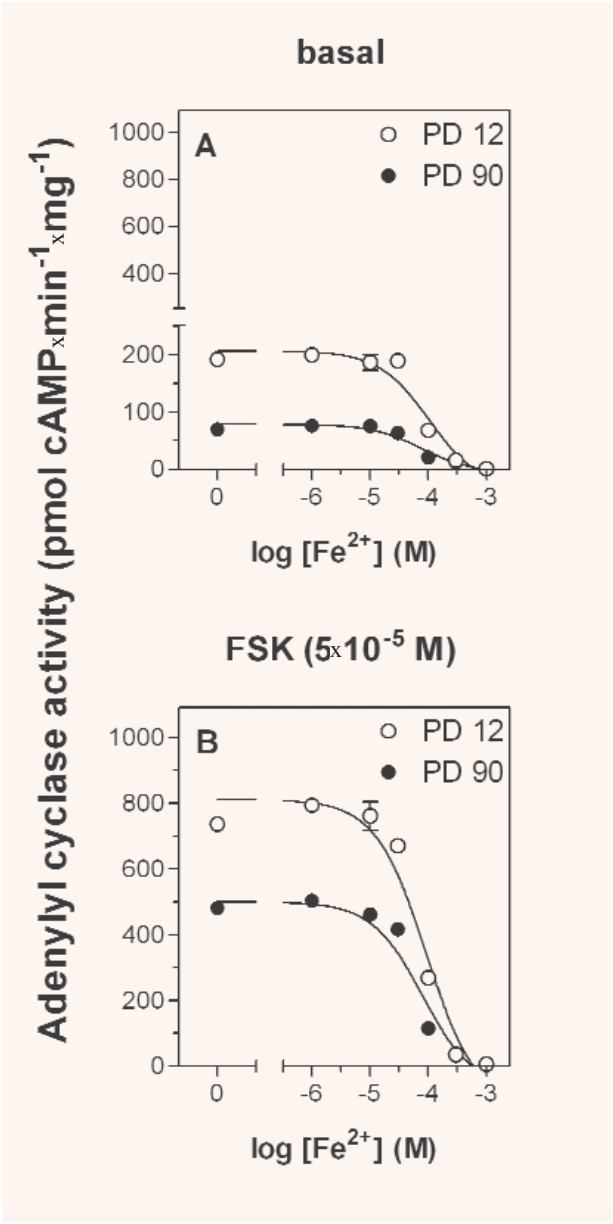


Fig. 4 Effect of Fe^{2+} on basal and forskolin-stimulated AC activity. Increasing concentrations of Fe^{2+} ($FeSO_4$) were applied in order to modulate basal (A) and forskolin-stimulated (B) activity of AC in cerebrocortical membranes from young (open circles) and adult (closed circles) rats. Data are means (± S.E.M.) from a representative experiment assayed in triplicate.

effect of increased Fe^{2+} concentrations on AC activity. While 0.1mM Fe^{2+} reduced basal AC activity by about 67%, the enzyme activity dropped only by 16% in the presence of 5 mM DTT (compare Fig. 4

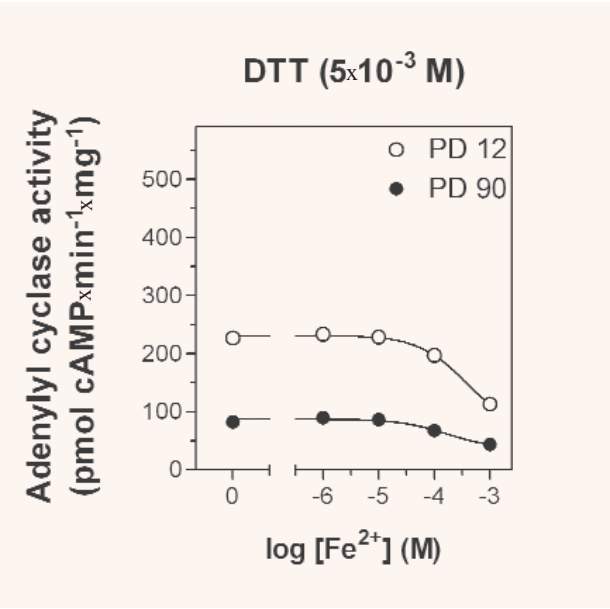


Fig. 5 Effect of Fe^{2+}/DTT on basal AC activity. Activity of AC in cerebrocortical membranes from young (open circles) and adult (closed circles) rats was measured in the presence of $5x10^{-3}$ M DTT and increasing concentrations of Fe^{2+} ($FeSO_4$). Data are means (± S.E.M.) from a representative experiment assayed in triplicate.

and 5). In the following experiments we evaluated the presumed role of ascorbic acid in modulation of AC activity. In spite of our expectations, ascorbic acid at lower concentrations (up to 1mM) did not influence either basal or forskolin-stimulated AC activity and at high concentrations it strongly diminished the enzyme activity in membrane preparations from both young and adult rat brain cortex (Fig. 7). Ascorbic acid at optimal concentration (about $1x10^{-5}$ – $1x10^{-4}$ M) was equally effective in eliciting lipid peroxidation in both types of tested samples (Fig. 6C).

Effect of suramin

Suramin, a polysulphonated naphtylurea, can presumably exert many different biological actions. Besides others, suramin is known to act directly on G protein α subunits and to block their activation by inhibiting the exchange of GDP for GTP [23–25]. In the present study we decided to test this potent G protein antagonist for its possible modulatory effect on AC activity. Quite unexpectedly, suramin exercised a clear inhibitory effect on basal as well as on differently stimulated activity of AC and higher concentrations (above $1x10^{-4}$ M) of this compound totally diminished the enzyme activity (Fig. 8). The pronounced negative effect was manifested to the same extent in cerebrocortical membranes both from young and adult rats. Since the

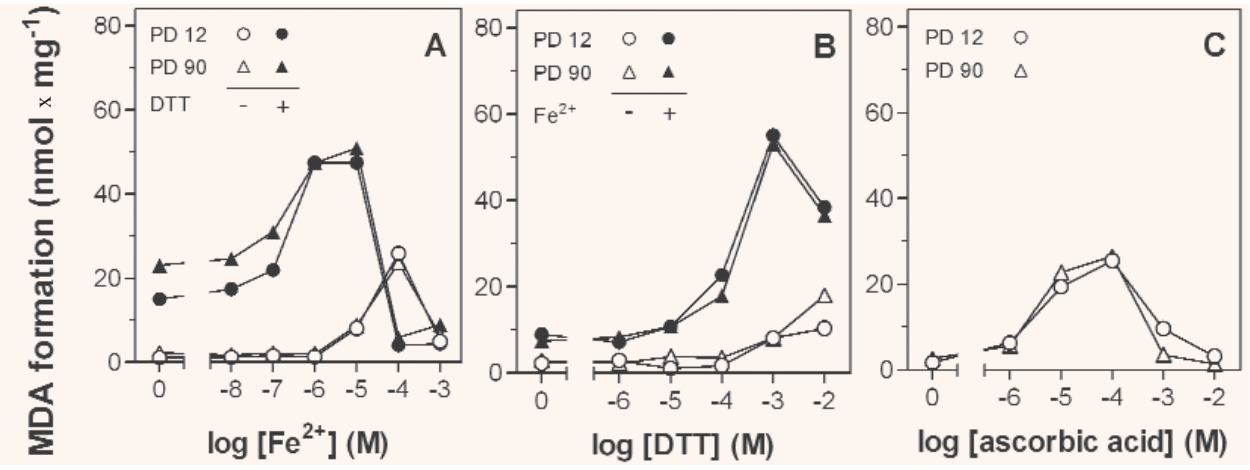


Fig. 6 Effect of Fe^{2+}/DTT and ascorbic acid on lipid peroxidation. Lipid peroxidation in cerebrocortical membranes from young (circles) and adult (triangles) rats was determined as MDA formation using either increasing concentrations of Fe^{2+} ($FeSO_4$) in the absence (open symbols) or presence (closed symbols) of $5x10^{-3}$ M DTT (A) or increasing concentrations of DTT in the absence (open symbols) or presence (closed symbols) of $1x10^{-5}$ M Fe^{2+} (B) or in the presence (open symbols) of increasing concentrations of ascorbic acid (C). Data are means (± S.E.M.) from a representative experiment assayed in triplicate.

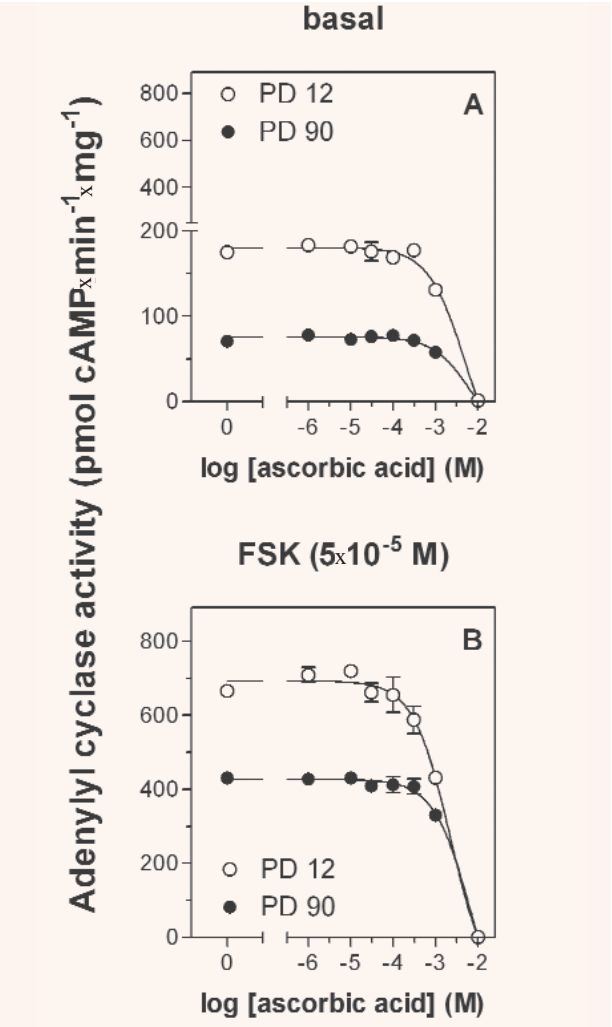


Fig. 7 Effect of ascorbic acid on basal and forskolin-stimulated AC activity. Basal (A) and forskolin-stimulated (B) activity of AC in cerebrocortical membranes from young (open circles) and adult (closed circles) rats was measured in the presence of increasing concentrations ascorbic acid. Data are means (± S.E.M.) from a representative experiment assayed in triplicate.

IC_{50} of suramin (about $3x10^{-5}$ M) was practically the same for basal and directly (Mn^{2+}) stimulated AC activity as well as for the enzyme activity stimulated through the G proteins (AlF_4^-) or both directly and through the G proteins (forskolin), suramin might be considered to act as a direct inhibitor of the catalytic subunits of AC. This assumption was confirmed by subsequent experiments with plasma membranes derived from S49 cyc- lymphoma cells, which lack $G_s\alpha$ protein [26]. Fig. 9 shows that suramin ($1x10^{-4}$ M) substantially reduced AC activity in cyc$^-$ membranes stimulated either by Mn^{2+} or forskolin to the values corresponding to basal enzyme activity, which come close to zero.

Effect of phosphorylation

In order to find possible differences between the regulation of AC activity in cerebrocortical preparations from young and adult rats, finally we evaluated the potential modulatory role of phosphorylation. It is known that some types of AC from higher organisms can be regulated by protein kinases [5, 27, 28]. Posttranslational modification of AC by phosphorylation may either stimulate or inhibit the enzyme activity. Therefore it was of interest to assess the potential effect of AC phosphorylation catalysed by protein kinase C (PKC) as well as by protein tyrosine kinases (PTKs). For this purpose, phorbol myristate acetate (PMA) and pervanadate (PV) were employed as effective activators of PKC and PTKs, respectively [29, 30]. We determined basal as well as foskolin- and AlF_4^--stimulated AC activity in cerebrocortical homogenates prepared from both young and adult rats. All tested samples were either untreated (control) or treated with PMA or PV in order to induce the requisite type of phosphorylation. As seen in Fig. 10, treatment of cerebrocortical homogenates with protein kinase activators apparently did not influence AC activity, because it was not significantly altered in any of the used experimental conditions.

Discussion

In the present work we sought to compare more closely AC activity in cerebrocortical membranes from young (12-day-old) and adult (90-day-old) rats. These two age intervals were chosen because some earlier studies demonstrated that activity of AC dramatically changes during postnatal development of rodent brain and, as a rule, this enzyme activity reaches a sharp maximum around PD 12 and then quickly declines to the adult values [7, 13, 15].

Our current results indicated that AC activity was always considerably (about two-fold) higher in cerebrocortical preparations derived from young than from adult animals. Nevertheless,

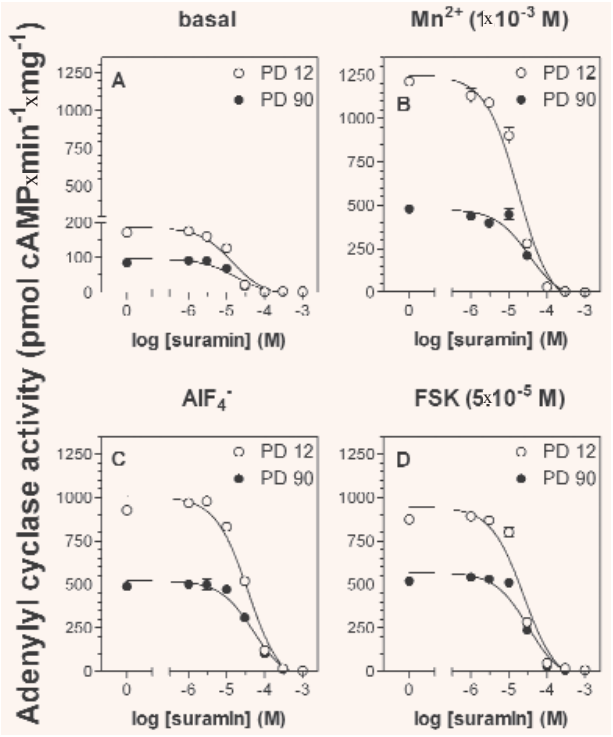


Fig. 8 Effect of suramin on AC activity in cerebrocortical membranes. Increasing concentrations of suramin were applied in order to modulate basal (A) as well as AC activity stimulated by $1x10^{-5}$ M forskolin (A), $1x10^{-2}$ M Mn^{2+} (B) and AlF_4^- (C) and $5x10^{-5}$ M forskolin (D) in membrane preparations from young (open circles) and adult (closed circles) rats. Data are means (± S.E.M.) from a representative experiment assayed in triplicate.

some interesting differences could have been observed between the ability of various activators to stimulate the enzyme activity in both types of tested samples, especially when it was modulated by the addition of dithiothreitol (DTT) to the reaction mixture. Whereas DTT alone did not significantly influence basal activity of AC, the enzyme activity stimulated by forskolin or Mn^{2+} was markedly enhanced by high concentrations (over 1mM) of this agent. The potentiation effect of DTT was detectable only when using sub-maximal stimulating concentrations of forskolin; DTT was not able to further enhance AC activity stimulated by maximally effective forskolin doses. DTT was a bit more effective in exercising these positive effects on AC activity in samples from young than adult rats. Interestingly, DTT did not influence AC activity stimulated by GTPγS and somewhat reduced AlF_4^--stimulated AC activity in samples from both tested age groups. These results indicate that DTT can enhance activity of AC, which is stimulated directly (by forskolin or Mn^{2+}). By contrast, the lack of clear effect of DTT on AC activity stimulated through the G proteins (Gs) suggests that this compound apparently does not strongly influence functioning of these regulatory proteins and their interaction with AC. Our finding of no direct effect of DTT on basal AC activity is in line with some previously published observations [19, 31]. On the other hand, Ozawa and colleagues detected a certain increase

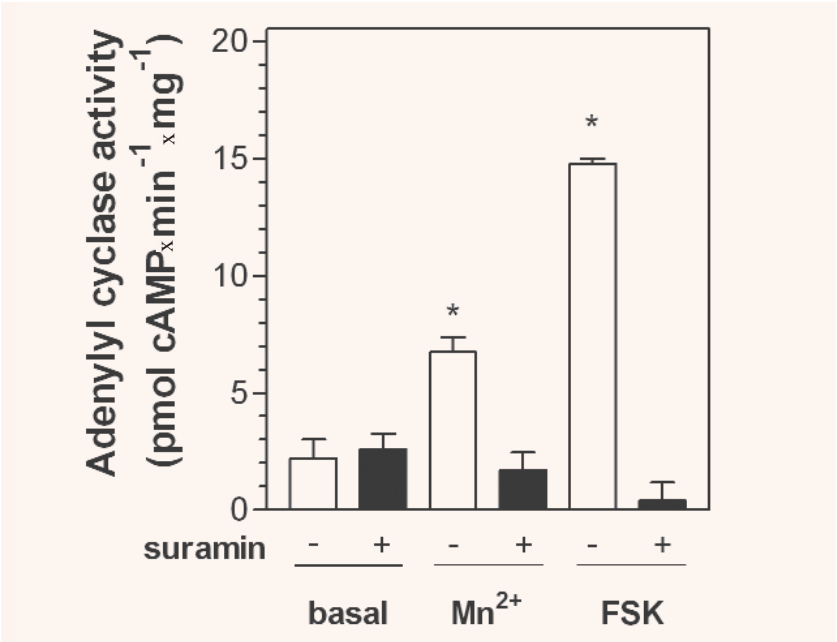


Fig. 9 Effect of suramin on AC activity in cyc- membranes. Basal, Mn^{2+} ($1x10^{-2}$ M) - and forskolin ($1x10^{-5}$ M)-stimulated AC activity in plasma membranes derived from S49 cyc^- lymphoma cells was determined in the absence (empty bars) or presence (solid bars) of $1x10^{-4}$ M sodium suramin. Data are means (± S.E.M.) from a representative experiment assayed in triplicate.

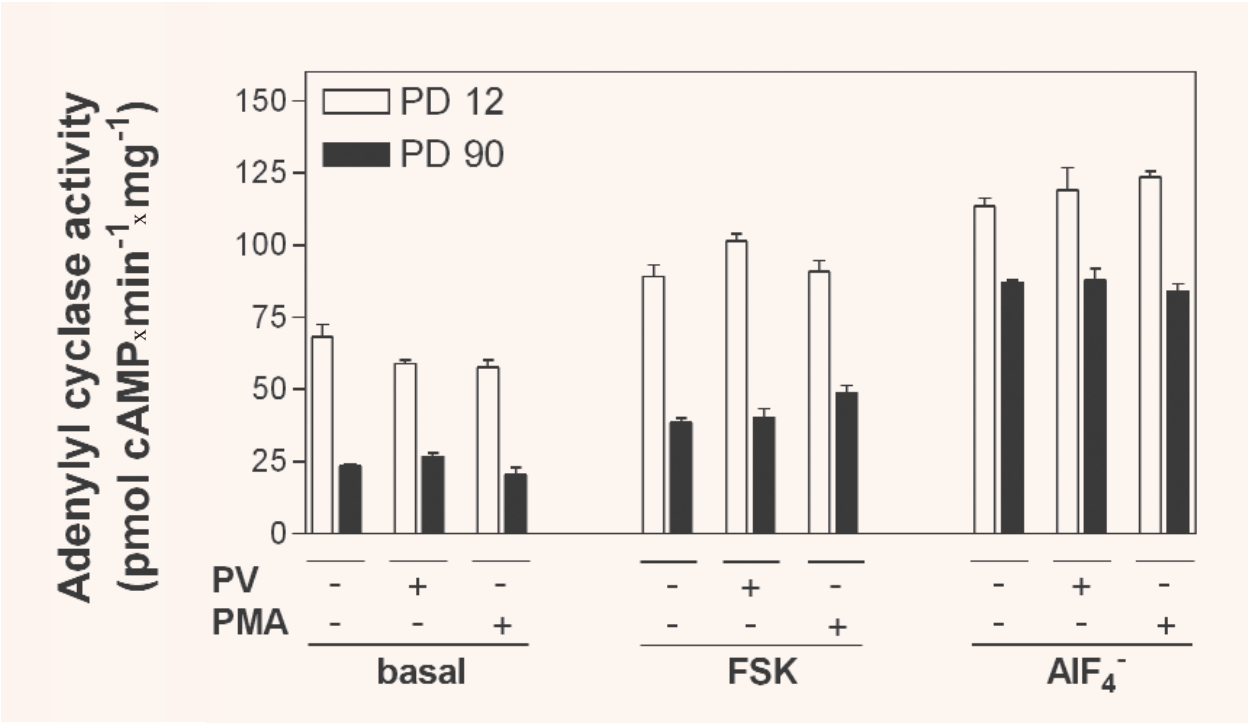


Fig. 10. Effect of phosphorylation on AC activity. Phosphorylation in cerebrocortical homogenates from young (empty bars) and adult (solid bars) rats was catalysed either by protein kinase C (PKC) or protein tyrosine kinases (TRKs). PKC was activated by preincubation of samples with 1μM phorbol myristate acetate (PMA) for 10 min at 30°C [57]. Preincubation (10 min at 30°C) of samples with 10μM pervanadate (PV) was used for activation of TRKs [58]. Basal, forskolin ($1x10^{-5}$ M)- and AlF_4^--activated AC cyclase was then determined as described in Methods. Data are means (± S.E.M.) from a representative experiment assayed in triplicate.

in basal AC activity after pretreatment of thyroid plasma membranes with DTT [32]. The positive modulatory role of DTT on AC activity stimulated by forskolin or Mn^{2+} has not been previously noted. It has been shown, however, that DTT may partially prevent inactivation of AC by some P-site inhibitors [33]. Likewise, Lin and colleagues observed that inhibition of the enzyme activity caused by low concentrations (≤0.1 mM) of organic mercurials could be reversed by the addition of DTT [34]. This is in accord with our present finding that DTT was able to reduce appreciably the negative effect of increased Fe^{2+} concentrations on AC activity. These results suggest that protection of -SH groups of the enzyme from oxidative reactions might be of great importance for proper AC functioning.

Next we examined the potential effects of various concentrations of Fe^{2+} ions on basal and forskolin-stimulated AC activity. Increased levels of Fe^{2+} (over 10μM) diminished AC activity similarly in cerebrocortical membranes from both young and adult animals. As mentioned above, this negative effect was strongly attenuated in the presence of DTT. However, neither combination of Fe^{2+} with DTT had stimulating effect on the enzyme activity, despite lipid peroxidation took place under some tested conditions. Our present results thus do not show any correlation between activity of AC and ambient levels of lipid peroxides. This is a bit surprising, since Baba and co-workers reported earlier that AC in synaptic membrane fraction of rat brain was activated selectively by stimulation of peroxidation [18, 19]. A short transient increase in AC activity was also elicited by lipid peroxidation in sarcolemmal membranes, which was followed by depression of the enzyme activity [22]. In our experimental conditions, however, AC activity was not affected even by lipid peroxidation induced by optimal concentrations of ascorbic acid. By contrast, higher concentrations of ascorbic acid substantially reduced the enzyme activity. All these

observations are in line with data published by [35, 36], who did not find any stimulation of AC in neuroblastoma cells exposed to Fe^{2+}/DTT. Moreover, lipid peroxidation was associated with reduction of AC activity [35]. Likewise, Shin and co-workers have recently reported that AC activity is attenuated by aldehydic products of lipid peroxidation in GH_4C_1 cells [37]. These observations conform to the notion about generally deleterious effects of reactive O_2 species and peroxidation on transmembrane signalling.

In the following set of experiments we wished to test the possibility that the higher AC activity determined in brain cortex preparations from young compared to adult rats could be perhaps somehow related to the enzyme interaction with the G proteins. Therefore we decided to take advantage of using suramin as a known blocker of the G protein function (inhibition of the GDP/GTP exchange) and tried to test this compound for its presumed modulatory effect on AC activity. Rather surprisingly, suramin inhibited not only AC activity stimulated through the G proteins but it was also highly effective in diminishing the basal as well as forskolin- or Mn^{2+}-stimulated enzyme activity. Similar inhibition was found in cerebrocortical preparations from both tested age groups. These observations indicated that suramin may function as a direct inhibitor of the catalytic subunit of AC. Further evidence in favour of this assumption was brought by the assessment of suramin effect on AC activity in plasma membranes derived from S49 cyc^- cells. The addition of suramin to these membranes, which lack $G_s\alpha$ protein [26], completely abolished the stimulating effect of Mn^{2+} and forskolin on AC activity.

Besides showing a high potential of suramin in blocking the G protein function [23–25], a number of previous studies demonstrated its ability to impede binding of several growth factors to their cellular receptors [38–40]. Moreover, suramin has also been identified as an agent strongly interfering with the signal transduction pathways by inhibiting protein kinase C (PKC) and phosphoinisitide synthesis [41–43]. Nevertheless, there is only little information available about the potential relationship between suramin and AC. It has been observed earlier that suramin as a P_2-purinoceptor antagonist could markedly reduce ATP- or UTP-mediated inhibition of forskolin-stimulated cAMP accumulation in cultured mesangial cells [44]. Similarly, suramin in a concentration-dependent manner attenuated ADP-induced inhibition of PGE_1-stimulated cAMP accumulation in human platelets [45]. Suramin alone, however, had no significant effect on the basal levels of intracellular cAMP and it was able to inhibit accumulation of cAMP induced by sub-maximal concentrations of PDE_1 [45]. Interestingly enough, Lopez-Lopez and co-workers reported that treatment of the breast cancer cell line MCF-7 with suramin resulted in rapid and significant enhancement of AC activity (increase in intracellular cAMP levels), but no such effect was observed in the prostate cancer cell line PC3 [46]. Results of many previous experiments have thus disclosed suramin as an agent causing different perturbations in cellular signalling, including variable effects on the cAMP metabolism. Our present data bring a new evidence about direct interaction of suramin with AC and suggest that this drug should be considered not only as a potent inhibitor of G proteins and PKC, but of AC activity as well.

As a last step toward investigating the basis for markedly different activity of AC in brain cortex from young and adult rats, we assessed the possible modulatory effect of phosphorylation. Basal as well as forskolin- and AlF_4^--stimulated AC activity was determined in cerebrocortical homogenates prepared from both age groups. Before measurement of AC activity, the samples were either untreated (control) or treated with phorbol myristate acetate or pervanadate in order to induce phosphorylation catalysed by PKC or protein tyrosine kinases (PTKs), respectively [29, 30]. However, we did not find any significant difference in AC activity in any tested samples after activation of these protein kinases. It has been shown previously that posttranslational modification of some types of AC by phosphorylation may either stimulate or inhibit the enzyme activity [5, 27, 28]. Whereas AC II and AC VII can be stimulated by PKC [47, 48], PKC-mediated phosphorylation of AC IV and AC VI seems to be inhibitory [49, 50]. Some *in vitro* experiments indicated that PKC may enhance activity of AC V [51]. However, treatment of cells with phorbol esters did not alter AC V activity to any significant extent and it remains to be determined whether or not is activity of this type of AC altered by PKC in intact cells [52]. Most recently, it has been noted that AC activity is also regulated by the activation of both receptor- and nonrecep-

tor-linked tyrosine kinases and both stimulatory and inhibitory effects mediated by PTKs have been described [27, 30, 53, 54]. As mentioned above, treatment of cerebrocortical homogenates with any used protein kinase activator did not significantly influence AC activity in our experimental conditions. It might be therefore assumed that the potentially variable effects of phosphorylation are more or less balanced at the level of an overall cerebrocortical activity of AC.

In conclusion, our present observations indicate that AC is regulated similarly in brain cortex from young (12-day-old) and adult (90-day-old) rats, but the enzyme activity is much lower in adult than in young animals. As argued previously, the difference between AC activity in these two age groups is not explicable on the basis of the developmental expression profiles of either AC I, II, IV and VI or different G proteins [13, 55]. It might be speculated, however, that the complement of some other types of AC could be changed in adulthood. This supposition is supported by our recent finding of altered characteristics of [^{3}H]forskolin binding in cerebrocortical membranes from adult compared to young rats [56]. Nevertheless, a possibility can not be ruled out that AC activity in adult rat brain cortex might be perhaps affected by some yet not known negative regulatory factor/mechanism, which is switched on shortly after maturation.

Acknowledgemetns

This work was supported by The Wellcome Trust (GB), Centrum of Neuroscience (LC 554) and Academy of Sciences of the Czech Republic (AV0Z50110509).

References

1. **Mons N, Cooper DM.** Adenylate cyclases: critical foci in neuronal signaling. *Trends Neurosci.* 1995; 18: 536–42.
2. **Herrero I, Sanchez-Prieto J.** cAMP-dependent facilitation of glutamate release by β-adrenergic receptors in cerebrocortical nerve terminals. *J Biol Chem.* 1996; 271: 30554–60.
3. **Dohovics R, Janaky R, Varga V, Hermann A, Saransaari P, Oja SS.** Regulation of glutamatergic neurotransmission in the striatum by presynaptic adenylyl cyclase-dependent processes. *Neurochem Int.* 2003; 42: 1–7.
4. **Hurley JH.** Structure, mechanism, and regulation of mammalian adenylyl cyclase. *J Biol Chem.* 1999; 274: 7599–602.
5. **Patel TB, Du Z, Pierre S, Cartin L, Scholich K.** Molecular biological approaches to unravel adenylyl cyclase signaling and function. *Gene* 2001; 269: 13–25.
6. **Mons N, Harry A, Dubourg P, Premont RT, Iyengar R, Cooper DM.** Immunohistochemical localization of adenylyl cyclase in rat brain indicates a highly selective concentration at synapses. *Proc Natl Acad Sci USA.* 1995; 92: 8473–7.
7. **Matsuoka I, Suzuki Y, Defer N, Nakanishi H, Hanoune J.** Differential expression of type I, II, and V adenylyl cyclase gene in the postnatal developing rat brain. *J Neurochem.* 1997; 68: 498–506.
8. **Levitzki A, Bar-Sinai A.** The regulation of adenylyl cyclase by receptor-operated G proteins. *Pharmacol Ther.* 1991*;* 50: 271–83.
9. **Simonds WF.** G protein regulation of adenylate cyclase. *Trends Pharmacol Sci.* 1999; 20: 66–73.
10. **Hanoune J, Defer N.** Regulation and role of adenylyl cyclase isoforms. *Annu Rev Pharmacol Toxicol.* 2001; 41: 145–74.
11. **Desaubry L, Shoshani I, Johnson RA.** Inhibition of adenylyl cyclase by a family of newly synthesized adenine nucleoside 3'-polyphosphates. *J Biol Chem.* 1996; 271: 14028–34.
12. **Premont RT, Matsuoka I, Mattei MG, Pouille Y, Defer N, Hanoune J.** Identification and characterization of a widely expressed form of adenylyl cyclase. *J Biol Chem.* 1996; 271: 13900–7.
13. **Ihnatovych I, Novotny J, Haugvicova R, Bourova L, Mares P, Svoboda P.** Ontogenetic development of the G protein-mediated adenylyl cyclase signalling in rat brain. *Brain Res Dev Brain Res.* 2002; 133: 69–75.
14. **Harden TK, Wolfe BB, Sporn JR, Perkins JP, Molinoff PB.** Ontogeny of β-adrenergic receptors in rat cerebral cortex. *Brain Res.* 1977; 125: 99–108.
15. **Keshles O, Levitzki A.** The ontogenesis of β-adrenergic receptors and of adenylate cyclase in the developing rat brain. *Biochem Pharmacol.* 1984; 33: 3231–3.
16. **Rius RA, Streaty RA, Peng Loh Y, Klee WA.** Developmental expression of G proteins that differentially modulate adenylyl cyclase activity in mouse brain. *FEBS Lett.* 1991; 288: 51–4.
17. **White AA.** Separation and purification of cyclic nucleotides by alumina column chromatography. *Methods Enzymol.* 1974; 38: 41–6.
18. **Baba A, Lee E, Ohta A, Tatsuno T, Iwata H.** Activation of adenylate cyclase of rat brain by lipid peroxidation. *J Biol Chem.* 1981; 256: 3679–84.
19. **Baba A, Kihara T, Lee E, Iwata H.** Activation of rat brain adenylate cyclase by copper plus dithiothreitol. *Biochem Pharmacol.* 1981; 30: 171–4.
20. **Oyama M, Kubota K.** Inhibition by EDTA and enhancement by divalent cations or polyamines of the dithiothreitol-induced activation of adenylate cyclase in the cellular

slime mold, *Dictyostelium discoideum. Biochim Biophys Acta* 1991; 1092: 85–8.

21. **Schimke I, Haberland A, Will-Shahab L, Kuttner I, Papies B.** Free radical-induced damage of cardiac sarcolemma (SL) and activity loss of β-receptor adenylate cyclase system (β-RAS). A comparison of the time courses. *Biomed Biochim Acta* 1989; 48: S69–72.
22. **Schimke I, Haberland A, Will-Shahab L, Kuttner I, Papies B.** *In vitro* effects of reactive O2 species on the beta-receptor-adenylyl cyclase system. *Mol Cell Biochem.* 1992; 110: 41–6.
23. **Butler SJ, Kelly EC, McKenzie FR, Guild SB, Wakelam MJ, Milligan G.** Differential effects of suramin on the coupling of receptors to individual species of pertussis-toxin-sensitive guanine-nucleotide-binding proteins. *Biochem J.* 1988; 251: 201–5.
24. **Freissmuth M, Boehm S, Beindl W, Nickel P, Ijzerman AP, Hohenegger M, Nanoff C.** Suramin analogues as subtype-selective G protein inhibitors. *Mol Pharmacol.* 1996; 49: 602–11.
25. **Beindl W, Mitterauer T, Hohenegger M, Ijzerman AP, Nanoff C, Freissmuth M.** Inhibition of receptor/G protein coupling by suramin analogues. *Mol Pharmacol.* 1996; 50: 415–23.
26. **Ross EM, Howlett AC, Ferguson KM, Gilman AG.** Reconstitution of hormone-sensitive adenylate cyclase activity with resolved components of the enzyme. *J Biol Chem.* 1978; 253: 6401–12.
27. **Tan CM, Kelvin DJ, Litchfield DW, Ferguson SS, Feldman RD.** Tyrosine kinase-mediated serine phosphorylation of adenylyl cyclase. *Biochemistry* 2001; 40: 1702–9.
28. **El-Mowafy AM, White RE.** Evidence for a tyrosine kinase-dependent activation of the adenylyl Cyclase/PKA cascade downstream from the G-protein-linked endothelin ETA receptor in vascular smooth muscle. *Biochem Biophys Res Commun.* 1998; 251: 494–500.
29. **Patrizio M, Slepko N, Levi G.** Opposite regulation of adenylyl cyclase by protein kinase C in astrocyte and microglia cultures. *J Neurochem.* 1997; 69: 1267–77.
30. **Tan CM, McDonald CG, Chorazyczewski J, Burry AF, Feldman RD, Macdonald CJ.** Vanadate stimulation of adenylyl cyclase: an index of tyrosine kinase vascular effects. *Clin Pharmacol Ther.* 1999; 66: 275–81.
31. **Suen ET, Kwan PC, Clement-Cormier YC.** Selective effects of an essential sulfhydryl group on the activation of dopamine- and guanine nucleotide-sensitive adenylate cyclase. *Mol Pharmacol.* 1982; 22: 595–601.
32. **Ozawa Y, Chopra IJ, Solomon DH, Smith F.** The role of sulfhydryl groups in thyrotropin binding and adenylate cyclase activities of thyroid plasma membranes. *Endocrinology* 1979; 105:1221–5.
33. **Skurat AV, Yurkova MS, Baranova LA, Gulyaev NN, Bulargina TV, Severin ES.** Evidence for the existence of a sulfhydryl group in the adenylate cyclase active site. *Biochem Int.* 1985; 10:451–61.
34. **Lin MC, Cooper DM, Rodbell M.** Selective effects of organic mercurials on the GTP-regulatory proteins of adenylate cyclase systems. *J Biol Chem.* 1980; 255: 7250–4.
35. **Murphy MG.** Membrane fatty acids, lipid peroxidation and adenylate cyclase activity in cultured neural cells. *Biochem Biophys Res Commun.* 1985; 132:757–63.
36. **Murphy MG.** Studies of the regulation of basal adenylate cyclase activity by membrane polyunsaturated fatty acids in cultured neuroblastoma. *J Neurochem.* 1986; 47: 245–53.
37. **Shin Y, White BH, Uh M, Sidhu A.** Modulation of D1-like dopamine receptor function by aldehydic products of lipid peroxidation. *Brain Res.* 2003; 968:102–13.
38. **Coffey RJ Jr, Leof EB, Shipley GD, Moses HL.** Suramin inhibition of growth factor receptor binding and mitogenicity in AKR-2B cells. *J Cell Physiol.* 1987; 132: 143–8.
39. **Mills GB, Zhang N, May C, Hill M, Chung A.** Suramin prevents binding of interleukin 2 to its cell surface receptor: a possible mechanism for immunosuppression. *Cancer Res.* 1990; 50:3036–42.
40. **Minniti CP, Maggi M, Helman LJ.** Suramin inhibits the growth of human rhabdomyosarcoma by interrupting the insulin-like growth factor II autocrine growth loop. *Cancer Res.* 1992; 52:1830–5.
41. **Hensey CE, Boscoboinik D, Azzi A.** Suramin, an anticancer drug, inhibits protein kinase C and induces differentiation in neuroblastoma cell clone NB2A. *FEBS Lett.* 1989; 258: 156–8.
42. **Mahoney CW, Azzi A, Huang KP.** Effects of suramin, an anti-human immunodeficiency virus reverse transcriptase agent, on protein kinase C. Differential activation and inhibition of protein kinase C isozymes. *J Biol Chem.* 1990; 265: 5424–8.
43. **Kopp R, Pfeiffer A.** Suramin alters phosphoinositide synthesis and inhibits growth factor receptor binding in HT-29 cells. *Cancer Res.* 1990; 50: 6490–6.
44. **Schulze-Lohoff E, Bitzer M, Ogilvie A, Sterzel RB.** P2U-purinergic receptor activation mediates inhibition of cAMP accumulation in cultured renal mesangial cells. *Ren Physiol Biochem.* 1995; 18:219–30.
45. **Hall DA, Hourani SM.** Effects of suramin on increases in cytosolic calcium and on inhibition of adenylate cyclase induced by adenosine 5'-diphosphate in human platelets. *Biochem Pharmacol.* 1994; 47: 1013–8.
46. **Lopez-Lopez R, Langeveld CH, Pizao PE, van Rijswijk RE, Wagstaff J, Pinedo HM, Peters GJ.** Effect of suramin on adenylate cyclase and protein kinase C. *Anticancer Drug Des.* 1994; 9: 279–90.
47. **Jacobowitz O, Iyengar R.** Phorbol ester-induced stimulation and phosphorylation of adenylyl cyclase 2. *Proc Natl Acad Sci USA.* 1994; 91:10630–4.
48. **Watson PA, Krupinski J, Kempinski AM, Frankenfield CD.** Molecular cloning and characterization of the type VII isoform of mammalian adenylyl cyclase expressed widely in mouse tissues and in S49 mouse lymphoma cells. *J Biol Chem.* 1994; 269: 28893–8.
49. **Zimmermann G, Taussig R.** Protein kinase C alters the responsiveness of adenylyl cyclases to G protein alpha and betagamma subunits. *J Biol Chem.* 1996; 271: 27161–6.
50. **Lai HL, Lin TH, Kao YY, Lin WJ, Hwang MJ, Chern Y.** The N terminus domain of type VI adenylyl cyclase mediates its inhibition by protein kinase C. *Mol Pharmacol.* 1999; 56: 644–50.

51. **Kawabe J, Iwami G, Ebina T, Ohno S, Katada T, Ueda Y, Homcy CJ, Ishikawa Y.** Differential activation of adenylyl cyclase by protein kinase C isoenzymes. *J Biol Chem.* 1994; 269:16554–8.
52. **Jacobowitz O, Chen J, Premont RT, Iyengar R.** Stimulation of specific types of G_s-stimulated adenylyl cyclases by phorbol ester treatment. *J Biol Chem.* 1993; 268: 3829–32.
53. **Hadcock JR, Port JD, Gelman MS, Malbon CC.** Cross-talk between tyrosine kinase and G-protein-linked receptors. Phosphorylation of β2-adrenergic receptors in response to insulin. *J Biol Chem.* 1992; 267: 26017–22.
54. **Valiquette M, Parent S, Loisel TP, Bouvier M.** Mutation of tyrosine-141 inhibits insulin-promoted tyrosine phosphorylation and increased responsiveness of the human β 2-adrenergic receptor. *EMBO J.* 1995; 14: 5542–9.
55. **Ihnatovych I, Novotny J, Haugvicova R, Bourova L, Mares P, Svoboda P.** Opposing changes of trimeric G protein levels during ontogenetic development of rat brain. *Brain Res Dev Brain Res.* 2002; 133:57–67.
56. **Stöhr J, Novotny J, Svoboda P.** Characterization of [3H]forskolin binding sites in young and adult rat brain cortex. Identification of suramin as a competitive inhibitor of [3H]forskolin binding. *Can J Physiol Pharmacol.* 2005; 83: 573–81.
57. **Summers ST, Walker JM, Sando JJ, Cronin MJ.** Phorbol esters increase adenylate cyclase activity and stability in pituitary membranes. *Biochem Biophys Res Commun.* 1988; 151: 16–24.
58. **Mikalsen SO, Kaalhus O.** A characterization of pervanadate, an inducer of cellular tyrosine phosphorylation and inhibitor of gap junctional intercellular communication. *Biochim Biophys Acta* 1996; 1290: 308–18.

Characterization of [^{3}H]-forskolin binding sites in young and adult rat brain cortex: identification of suramin as a competitive inhibitor of [^{3}H]-forskolin binding

Jiri Stöhr, Jiri Novotny, and Petr Svoboda

Abstract: Little is know about forskolin binding in the rat brain during ontogenetic development. For this paper, we have characterized specific binding sites for [^{3}H]-forskolin in cerebrocortical membranes from young (12-day-old) and adult (90-day-old) rats. High-affinity, as well as super-high-affinity, [^{3}H]-forskolin binding sites were detected in samples from both age groups tested, and the binding parameters of these sites differed significantly. Whereas the number of high-affinity [^{3}H]-forskolin binding sites was higher by about 50% in adult than in young rats, their affinity was markedly (about 4 times) lower. In the presence of AlF_4^-, the number high-affinity [^{3}H]-forskolin binding sites in samples from young rats rose to the level determined in samples from adult animals, and the number of super-high-affinity sites considerably increased in both age groups. The different characteristics of [^{3}H]-forskolin binding found in cerebrocortical membranes from young and adult rats may be closely related to markedly diminished adenyl cyclase activity in preparations from adult animals. Results of our experiments with suramin indicated that this drug may act as a competitive inhibitor of [^{3}H]-forskolin binding.

Key words: rat brain cortex; development; forskolin binding; suramin.

Résumé : On sait peu de choses sur la fixation de la forskoline dans le cerveau du rat durant le développement ontogénique. Dans la présente étude, nous avons caractérisé les sites de fixation spécifiques de la [^{3}H]-forskoline dans les membranes cérébrocorticales de rats jeunes (12 jours) et adultes (90 jours). On a détecté des sites de fixation de haute affinité et de très haute affinité dans les échantillons des deux groupes d'âges et les paramètres de fixation de ces sites ont différé de manière significative. Alors que le nombre de sites de fixation de haute affinité a été plus élevé d'environ 50 % chez les rats adultes, leur affinité a été nettement (environ 4 fois) plus faible. En présence de AIF_4^-, le nombre de sites de fixation de haute affinité dans les échantillons des rats jeunes a rejoint celui déterminé dans les échantillons des rats adultes et le nombre de sites de très haute affinité a augmenté de manière considérable chez les deux groupes d'âges. Les différentes caractéristiques de la fixation de [^{3}H]-forskoline, observées dans les membranes cérébrocorticales des rats jeunes et adultes, pourraient être étroitement liées à la diminution significative de l'activité AC dans les préparations des animaux adultes. Les résultats de nos expériences avec de la suramine ont indiqué que ce médicament agit comme un inhibiteur compétitif de la fixation de la [^{3}H]-forskoline.

Mots clés : cortex cérébral du rat, développement, fixation de la forskoline, suramine.

[Traduit par la Rédaction]

Introduction

The diterpene forskolin was originally discovered as a unique efficacious activator of mammalian hormone-sensitive adenylyl cyclase (AC) (Seamon et al. 1981). Forskolin activates the AC catalytic subunit directly or it may synergistically stimulate the enzyme activity in combination with hormones that activate AC via the G_s protein (Seamon and Daly 1981; Daly et al. 1982; Sutkowski et al. 1994). Whereas the direct activation of AC occurs at micromolar concentrations, much lower (nanomolar) concentrations of forskolin are sufficient to elicit cAMP formation by its synergistic interaction with hormones.

The ability of forskolin to interact with AC with high affinity suggests the existence of high-affinity binding sites for forskolin. These high-affinity binding sites can be stud-

Received 17 September 2004. Published on the NRC Research Press Web site at http://cjpp.nrc.ca on 15 July 2005.

J. Stöhr, J. Novotny,[1,2] and P. Svoboda. Department of Biochemistry of Membrane Receptors, Institute of Physiology, Academy of Sciences, Videnska 1083, Prague 4, and Department of Physiology, Faculty of Natural Sciences, Charles University, Vinicna 7, 120 00 Prague 2, Czech Republic.

[1]Corresponding author (e–mail: novjiri@biomed.cas.cz).
[2]Present address: Institute of Physiology, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Prague 4, Czech Republic.

 doi: 10.1139/Y05-048

ied with radioligand binding techniques, and [^{3}H]-forskolin has successfully been used to detect high-affinity binding sites for forskolin in membranes from a number of tissues (Seamon et al. 1985; Nelson and Seamon 1986; Goldman et al. 1988; Post et al. 1996; Musser et al. 1999). Interestingly, several autoradiographic studies performed in brain tissue revealed a highly heterogeneous distribution of [^{3}H]-forskolin binding sites (Gehlert et al. 1985; Gehlert 1986; Poat et al. 1988; Stockmeier and Zhang 1993). This distinct pattern, which does not correspond to the known distribution of AC or receptors in the brain, may reflect association of the high-affinity [^{3}H]-forskolin binding sites with a complex of AC and the activated G_s protein (Insel and Ostrom 2003). This assumption is supported by the finding that guanine nucleotides and AlF_4^-, which are known to promote $G_s\alpha$-AC coupling, may also stimulate [^{3}H]-forskolin binding (Seamon et al. 1985; Gehlert 1986).

Previous studies have indicated that AC activity in rat brain cortex reaches a sharp maximum around postnatal day (PD) 12 and then rapidly declines to the adult values (Keshles and Levitzki 1984; Matsuoka et al. 1997; Ihnatovych et al. 2002*a*). This considerable alteration is apparently not explicable by quantitative changes in expression of crucial components of the AC signalling system. The levels of various isoforms of AC (type I, II, IV, and VI) are either unchanged or increased between PD 12 and PD 90, and the same applies for $G_s\alpha$ protein (Ihnatovych et al. 2002*a*, 2002*b*). We have also observed that different modulators exert comparable effects on AC activity in cerebrocortical membranes prepared from young (12-day-old) and adult (90-day-old) rats (unpublished data). Hence, other factors must account for the diminished activity of AC in adulthood. To assess the properties of AC (and its coupling to $G_s\alpha$ protein) in more detail, here we have sought to analyze the characteristics of [^{3}H]-forskolin binding sites in cerebrocortical membranes derived from young (12-day-old) and adult (90-day-old) rats. Interestingly, despite the binding characteristics (B_{max} and K_D) were quite different in preparations from young and adult rats, Gpp(NH)p and sodium fluoride exerted similar effects on [^{3}H]-forskolin binding in both types of tested samples. We previously identified suramin as a direct inhibitor of AC in our recent experiments (unpublished observations), and so evaluated the potential ability of this agent to interfere with [^{3}H]-forskolin binding. Quite unexpectedly, our current measurements performed on cerebrocortical membrane preparations from both young and adult rats allowed us to reveal that suramin may act as a potent inhibitor of [^{3}H]-forskolin binding.

Materials and Methods

Materials

[^{3}H]-forskolin (34 Ci/mmol) was purchased from Perkin Elmer Life Sciences, Inc. (Boston, Mass.). All other chemicals were obtained from Sigma (St. Louis, Mo.) and they were of the highest purity available.

Membrane preparation

Young (12-day-old) and adult (90-day-old) male Wistar rats were etherised, killed by decapitation, and their brains quickly removed. Cerebral cortexes were rapidly isolated, frozen in liquid nitrogen and stored at –70 °C. The brain material obtained (usually from 6 young or 4 adult animals) was always combined to constitute individual experimental groups. After thawing, the brain tissue was minced with scissors, homogenized in 10 mL STME buffer/g wet mass (250 mmol/L sucrose, 20 mmol/L Tris–HCl, 3 mmol/L $MgCl_2$, 1 mmol/L EDTA, pH 7.6) by using a Potter-Elvehjem homogenizer (Teflon-glass). Unbroken cells and nuclei were removed by low-speed centrifugation (600*g* for 5 min at 4 °C). The supernatant was centrifuged at 200 000*g* for 30 min, and the resulting membrane pellet was resuspended in STME buffer (total volume corresponded to quintuple initial tissue wet mass). Cerebrocortical membranes were then washed twice in STME buffer using high-speed centrifugation (200 000*g* for 30 min) and, before final freezing in liquid nitrogen, the membrane pellet was resuspended in TN buffer (20 mmol/L Tris–HCl, 150 mmol/L NaCl, pH 7.6). The animals used in this study were cared for in accordance with the Guide to the Care and Use of Laboratory Animals (Canadian Council on Animal Care).

[^{3}H]-forskolin binding

High affinity [^{3}H]-forskolin binding sites in cerebrocortical membranes from young and adult rats were determined as described previously (Nelson and Seamon 1986). Briefly, the membrane suspension was diluted with 50 mmol/L Tris buffer (pH 7.4) to a protein concentration of 2 mg/mL. The reaction mixture (total volume of 0.4 mL) contained 200 µg of protein (cerebrocortical membranes), 50 mmol/L Tris–HCl buffer (pH 7.4), 10 mmol/L $MgCl_2$ (if not stated otherwise), 5 mmol/L cytochalasin B and 20 nmol/L [^{3}H]-forskolin (about 6×10^5 dpm/sample). The incubation was carried out at 20 °C for 60 min without any other additives, or in the presence of AlF_4^- (10 mmol/L NaF and 1 mmol/L $AlCl_3$) or Gpp(NH)p (1×10^{-7} – 1×10^{-4} mol/L). The assay was terminated by the addition of 4 mL of ice-cold TM buffer (50 mmol/L Tris, 10 mmol/L $MgCl_2$, pH 7.4) and rapid filtration over Whatman GF/C filters, using a Brandel cell harvester (Gaithersburg, Md.). Filtered samples were rinsed free of unbound radioligand with ice-cold TM buffer (3 washes with 4 mL of buffer) and radioactivity associated with the filters was determined by scintillation counting. Specific [^{3}H]-forskolin binding was defined as that not replaceable by excess unlabelled forskolin (2×10^{-5} mol/L). Nonspecific binding usually represented less than 35% of total [^{3}H]-forskolin binding. To determine the binding parameters, data were analysed using the GraphPad program (PRISM, GraphPad Software Inc.; San Diego, Calif.).

Data analysis

All data were obtained from at least 3 independent membrane preparations. The data within each group were analysed by the one-way ANOVA followed by Dunnett's multiple comparison post-hoc test, and variances between samples from young and adult animals were evaluated by 2-way ANOVA followed by the Bonferroni's multiple comparison post test. Results are presented as mean ± SE, and were significant when $p < 0.05$.

Results

[^{3}H]-forskolin binding and competition binding studies

Specific high-affinity binding sites for [^{3}H]-forskolin were studied in cerebrocortical membrane preparations both from young and adult rats. Our preliminary experiments indicated that incubation of membrane samples with [^{3}H]-forskolin for 60 min at 20 °C is sufficient to achieve the maximal radioligand binding. All subsequent experiments were conducted in the same incubation conditions. Representative saturation isotherms for [^{3}H]-forskolin binding to cerebrocortical membranes, which were measured in the presence of 10 mmol/L $MgCl_2$, are shown in Fig. 1. A curvilinear shape of Scatchard plots constructed from the binding data was indicative of 2 classes of binding sites on cerbrocortical membranes from both young and adult animals. Nevertheless, binding parameters determined in these 2 types of membrane samples were rather different (Table 1). Cerebrocortical preparations from young rats contained a lower number of high-affinity [^{3}H]-forskolin binding sites (B_{max} = 200 pmol/mg protein) than those from adult rats (B_{max} = 296 pmol/mg protein), but affinity of these sites was apparently much higher in young (K_D = 33 nmol/L) than in adult (K_D = 136 nmol/L) animals. Besides these high-affinity binding sites, low amounts of super-high-affinity [^{3}H]-forskolin binding sites were detectable in preparations from both age groups, but their characteristics did not differ markedly from each other (Table 1). Both these types of [^{3}H]-forskolin binding sites were clearly preserved, even when the measurements were performed in the presence of AlF_4^-. In this case, the number of high-affinity [^{3}H]-forskolin binding sites in cerebrocortical membranes from young rats increased by about 75%, and it was similar to that one determined in samples from adult animals (Table 1). Interestingly, whereas the K_D values were not markedly affected by the presence of AlF_4^-, this activator increased considerably (by about 20 times) the number of detectable super-high-affinity [^{3}H]-forskolin binding sites in samples from both young and adult rats (Table 1).

Our subsequent competition binding experiments with unlabelled forskolin did not reveal any major difference between membrane preparations from both tested age groups, except for higher values of [^{3}H]-forskolin binding in samples from young rats achieved at a chosen constant concentration (20 nmol/L) of the radioligand (data not shown). The dissociation constants (K_i) for unlabelled forskolin calculated from these measurements were 87 ± 27 nmol/L and 98 ± 19 nmol/L in samples from young and adult rats, respectively. Thus, the K_i values seem to be relatively very close to the K_D values determined from direct binding studies.

Effect of Mg^{2+} ions and Gpp(NH)p

It has been shown earlier that Mg^{2+} ions play a significant role in binding of [^{3}H]-forskolin (Seamon et al. 1984; Nelson and Seamon 1986). The increased concentration of Mg^{2+} is especially important for the effective enhancement of [^{3}H]-forskolin binding by sodium fluoride or GTP analogues. We determined the effect of increasing concentrations of Mg^{2+} on [^{3}H]-forskolin binding stimulated by low (1×10^{-7} mol/L) and high (1×10^{-4} mol/L) concentrations of Gpp(NH)p (Fig. 2). The binding of [^{3}H]-forskolin was very low in the absence of Mg^{2+} ions and it reached about 40 or 20 fmol/mg protein in cerebrocortical membranes from young or adult rats, respectively. The addition of $MgCl_2$ (10 mmol/L or 100 mmol/L) increased the "basal" values of

Fig. 1. Saturation curve of [3H]-forskolin binding to rat cerebrocortical membranes. Membranes from (A) young and (B) adult rats were incubated with increasing concentrations of [^{3}H]-forskolin in the presence 10 mmol/L $MgCl_2$ at 20 °C for 60 min. Specific (●) [^{3}H]-forskolin binding refers to the difference between total (□) and nonspecific (Δ) binding, which was measured in the presence of 20 µmol/L unlabelled forskolin. Data are mean ± SE from a single representative experiment assayed in triplicate. *Inset:* Scatchard plots of the data collected from saturation binding isotherms.

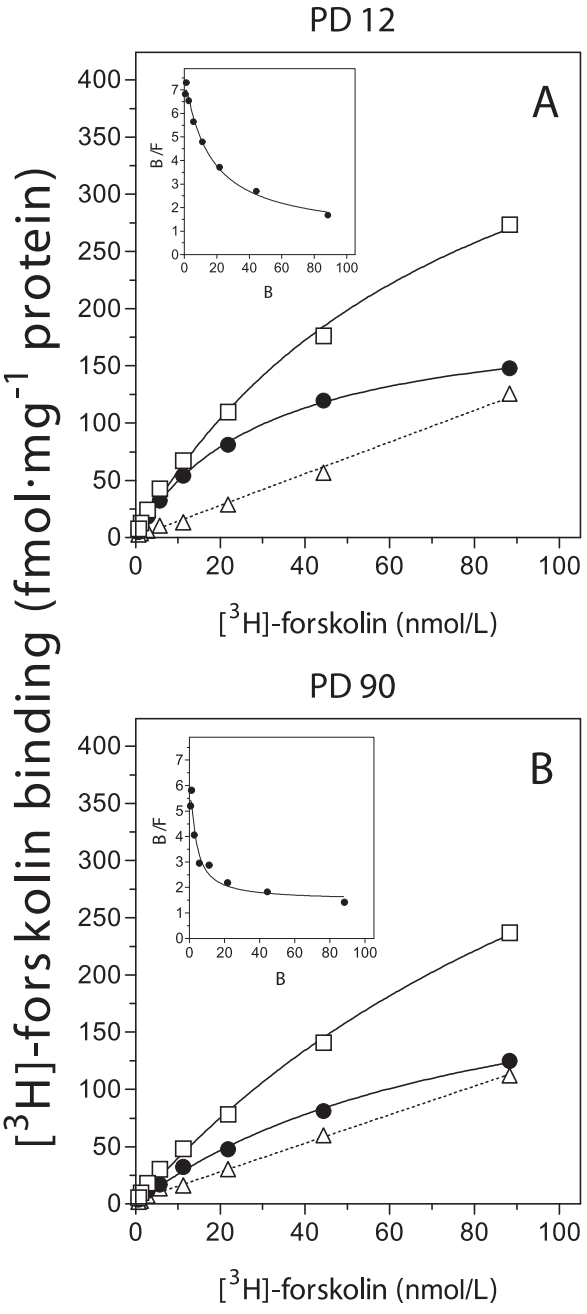


Table 1. Binding of [^{3}H]-forskolin to cerebrocortical membranes from young and adult rats.

		K_D (nmol/L)		B_{max} (fmol/mg)	
		KH	K_{SH}	B_H	B_{SH}
PD 12					
	Mg^{2+}	33±6	3.1±1.8	200±16	3.2±1.6
	Mg^{2+}/AlF_4^-	45±9	4.0±2.3	351±45 †	103±15 †
PD 90					
	Mg^{2+}	136±26*	3.2±1.9	296±39*	8.6±2.9
	Mg^{2+}/AlF_4^-	168±31*	5.6±2.5	335±34	101±18 †

Note: Characteristics of [^{3}H]-forskolin binding to cerebrocortical membranes from young (PD 12) and adult (PD 90) rats in the presence of 10 mmol/L $MgCl_2$(Mg^{2+}) or 10 mmol/L $MgCl_2$ + AlF_4^- (Mg^{2+}/AlF_4^-) were calculated from the corresponding Scatchard plots. Values are mean ± SE of the high-affinity (K_H) and super-high-affinity (K_{SH}) dissociation constants or of the numbers of high-affinity (B_H) and super-high-affinity (B_{SH}) binding sites for [^{3}H]-forskolin obtained from 3 independent experiments measured in triplicate.*$p < 0.05$ vs. PD 12;† $p < 0.05$ vs. Mg^{2+}.

[^{3}H]-forskolin binding to membranes from young and adult animals by about 37% and 131%, respectively (Fig. 3). Higher concentrations of Mg^{2+} ions did not significantly change the level of [^{3}H]-forskolin binding (data not shown). The nonhydrolyzable GTP analogue Gpp(NH)p was able to further enhance [^{3}H]-forskolin binding, but this effect was manifested only in the presence of increased Mg^{2+} concentrations (>100 mmol/L). Similar effects were observed in preparations from both young and adult brain cortex (Fig. 2).

Interestingly, construction of the dose-response curves for Gpp(NH)p (1×10^{-7} – 1×10^{-4} mol/L) allowed us to reveal a certain difference between samples from young and adult animals (Fig. 3). As noticed above, [^{3}H]-forskolin binding determined in the presence of Mg^{2+} ions was significantly higher in samples from young (about 55 fmol/mg protein) than adult (about 40 fmol/mg protein) rats. Whereas the addition of both low (1×10^{-7} mol/L) and high (1×10^{-4} mol/L) concentrations of Gpp(NH)p apparently increased the binding of [^{3}H]-forskolin to cerebrocortical membranes from young rats to the maximal values (about 80 fmol/mg protein), the maximal level of [^{3}H]-forskolin binding in samples from adult animals was achieved only by adding high amounts of Gpp(NH)p (>1×10^{-5} mol/L). Gpp(NH)p did not influence [^{3}H]-forskolin binding to samples from any tested age group in the presence of 10 mmol/L $MgCl_2$ (Fig. 3).

Effect of AlF_4^-, isoproterenol and baclofen

Our assessment of the effect of AlF_4^- (10 NaF mmol/L and 1 $AlCl_3$ mmol/L) indicated that this classical activator of G proteins (in the presence of 100 $MgCl_2$ mmol/L) was able to increase [^{3}H]-forskolin binding to cerebrocortical membranes from both young and adult rats by about 63% and 124%, respectively (Fig. 4). The comparable enhancement of [^{3}H]-forskolin binding (to about 90 fmol/mg protein) induced by AlF_4^- in samples from both tested age groups was even slightly higher than that elicited by the addition of maximally effective concentrations of Gpp(NH)p (about 80 fmol/mg protein).

In the next set of experiments we aimed to modulate Gpp(NH)p-promoted [^{3}H]-forskolin binding by agonists of the receptors coupled either to G_s or G_i/G_o proteins. For this purpose we employed isoproterenol and baclofen as the typical β-adrenergic and $GABA_B$ receptor agonists, respectively. Fig. 4 shows that the binding of [^{3}H]-forskolin enhanced by Gpp(NH)p (1×10^{-7} mol/L) was not significantly altered by the addition of either isoproterenol (1×10^{-5} mol/L) or baclofen (1×10^{-3} mol/L) in any tested samples.

Effect of suramin

Finally, we investigated the potential effect of suramin on the binding characteristics of [^{3}H]-forskolin. This compound is known to affect the function of G proteins (Butler et al. 1988; Beindl et al. 1996), but our recent observations suggested that suramin may also act as a direct inhibitor of AC activity (unpublished data). Interestingly, our preliminary experiments indicated that this agent could strongly inhibit the binding of [^{3}H]-forskolin (Fig. 4). Therefore we performed complete competition binding experiments on cerebrocortical membrane preparations from both young and adult rats (Fig. 5). These studies indicated that suramin could quite effectively inhibit specific binding of [^{3}H]-forskolin, with K_i about 5 μmol/L in samples from both tested age groups. The steepness of competitive binding curves was characterized by the slope factor (Hill coefficient), which was 0.88 ± 0.05 and 0.85 ± 0.08 in samples from young and adult animals, respectively. These data (Hill slopes calculated from competition binding data close to 1.0) suggest that suramin might be considered as a competitive inhibitor of [^{3}H]-forskolin binding.

Discussion

The high-affinity binding sites for forskolin have been identified in various tissues, especially in membranes from brain and platelets (Seamon et al. 1984; Gehlert et al. 1985; Nelson and Seamon 1985; Seamon and Daly 1985; Jackman and Bobik 1986; Nelson and Seamon 1986; Ross et al. 1993). However, little attention has been paid to the possible changes in forskolin binding during ontogenetic development. In the present study, we aimed to determine and compare binding parameters for [^{3}H]-forskolin binding sites in cerebrocortical membranes from young (12-day-old) and adult (90-day-old) rats.

Our current analyses indicate that a two-site model is more appropriate than a single-site model for fitting the binding data obtained from standard [^{3}H]-forskolin satura-

Fig. 2. Effect of Mg^{2+} on specific [^{3}H]-forskolin binding modulated by Gpp(NH)p. Cerebrocortical membranes from (A) young and (B) adult rats were incubated with 20 nmol/L [^{3}H]-forskolin in the presence of 1×10^{-7} mol/L (open circles) or 1×10^{-4} mol/L Gpp(HN)p (closed circles) and increasing concentrations of $MgCl_2$ at 20 °C for 60 min. The "basal" values of [^{3}H]-forskolin binding obtained in the absence of Mg^{2+} and Gpp(NH)p are marked 'B'. Data are mean ± SE from a representative experiment assayed in triplicate.

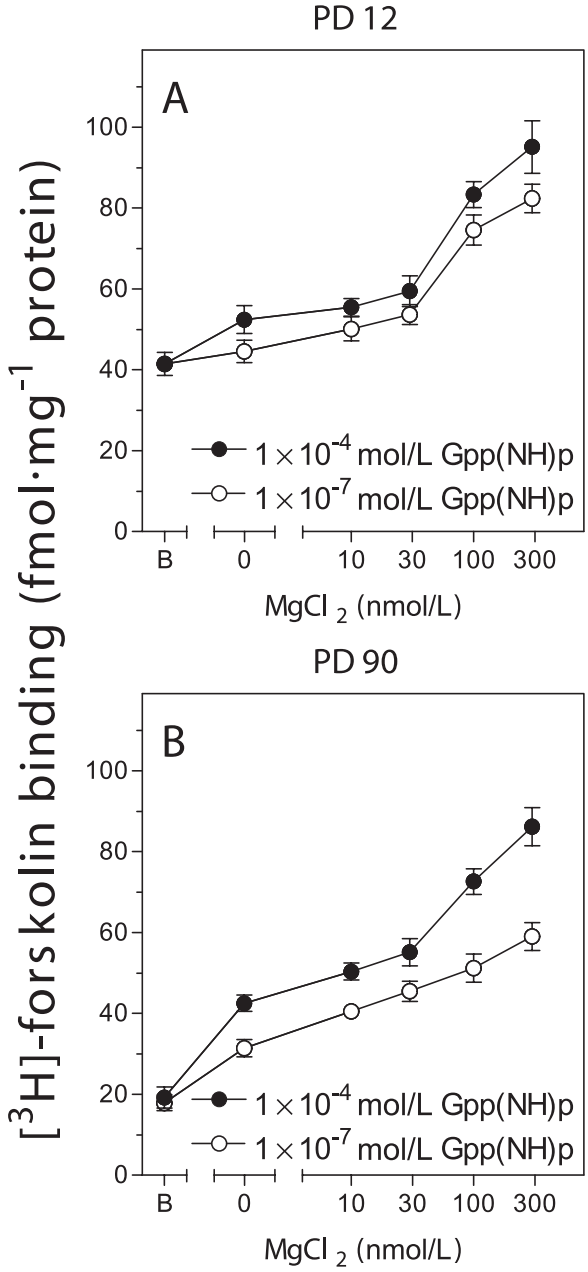
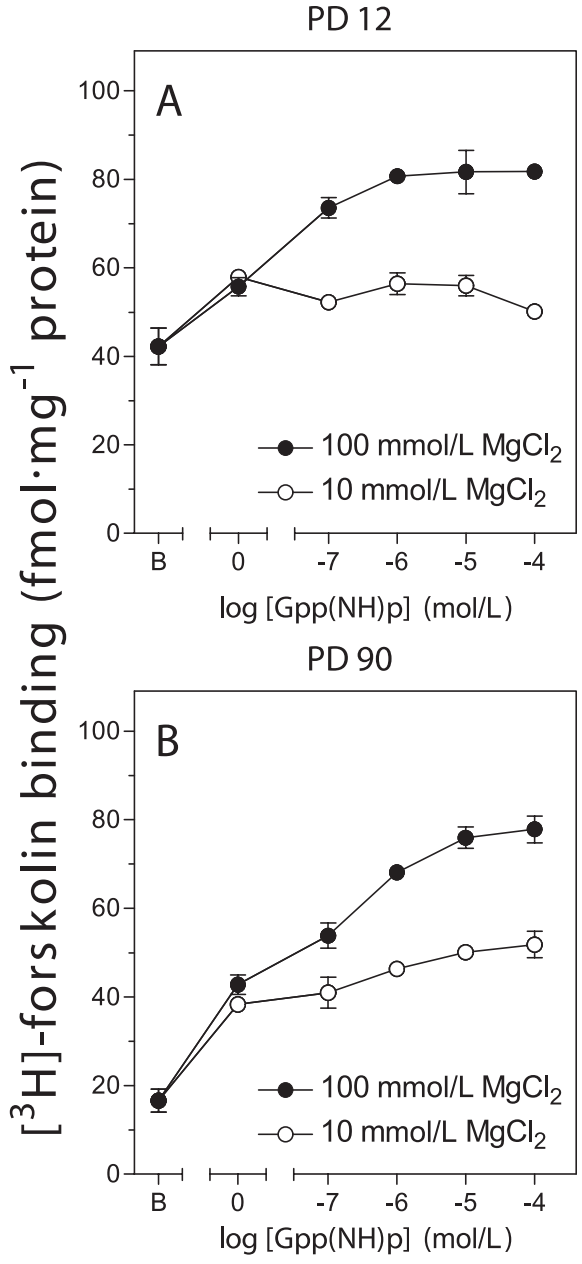


Fig. 3. Effect of Gpp(NH)p on specific [^{3}H]-forskolin binding in cerebrocortical membranes from young (A) and adult (B) rats. Samples were incubated with 20 nmol/L [^{3}H]-forskolin in the presence of 10 mmol/L Mg^{2+} (open circles) or 100 mmol/L Mg^{2+} (closed circles) at 20 °C for 60 min. The "basal" values of [^{3}H]-forskolin binding obtained in the absence of Gpp(NH)p and Mg^{2+} are marked 'B' on the x-axis. Data are mean ± SE from a representative experiment assayed in triplicate.



tion binding assays. In this way, we were able to distinguish high-affinity and super-high-affinity binding sites in cerebrocortical membranes from both young and adult rats. Whereas the total number of prevalent high-affinity [^{3}H]-forskolin binding sites was higher (by about 50%) in samples from adult than from young animals, affinity of these sites was much lower (about 4 times) in adulthood. The number and affinity of high-affinity [^{3}H]-forskolin binding sites determined in our preparations correspond quite well to some previously published data. The following binding parameters of [^{3}H]-forskolin binding to rat brain membranes were previously reported: $B_{max} \approx 400$ fmol/mg and $K_D \approx 26$ nmol/L (Seamon et al. 1984), $B_{max} \approx 200$ fmol/mg and $K_D \approx 15$ nmol/L (Seamon et al. 1985), $B_{max} \approx 102$ fmol/mg and $K_D \approx 42$ nmol/L (Gehlert et al. 1985), $B_{max} \approx 408$ fmol/mg and $K_D \approx 31$ nmol/L (Odagaki et al. 1991).

Fig. 4. Modulation of [^{3}H]-forskolin binding by Gpp(NH)p, isoproterenol, baclofen, AlF_4^-, and suramin. Cerebrocortical membranes from (A) young and (B) adult rats were incubated with 20 nmol/L [^{3}H]-forskolin in the presence of either 1×10^{-7} mol/L Gpp(NH)p alone or in combination with 1×10^{-5} mol/L isoproterenol (ISO), 1×10^{-3} mol/L baclofen (BCF), or 1×10^{-4} mol/L sodium suramin (SUR) at 20 °C for 60 min. The effect of AlF_4^- (10 mmol/L NaF + 1 mmol/L $AlCl_3$) or 1×10^{-4} mol/L suramin alone on [^{3}H]-forskolin binding was assessed under the same conditions. All these data were measured in the presence of 100 mol/L $MgCl_2$ and are representative of 2 experiments performed in triplicate. *$p < 0.01$, ***$p < 0.001$ vs. basal [^{3}H]-forskolin.

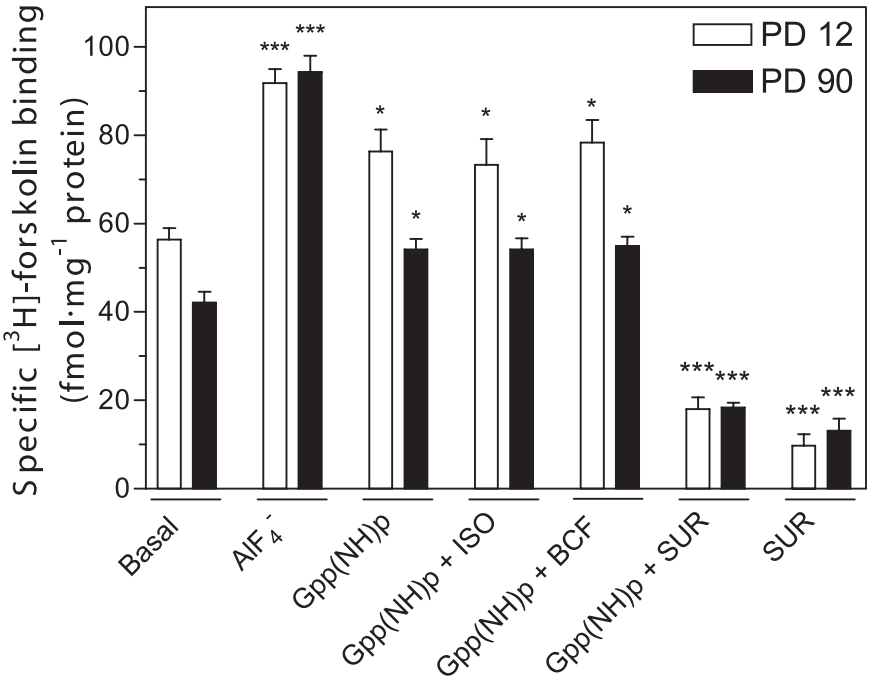
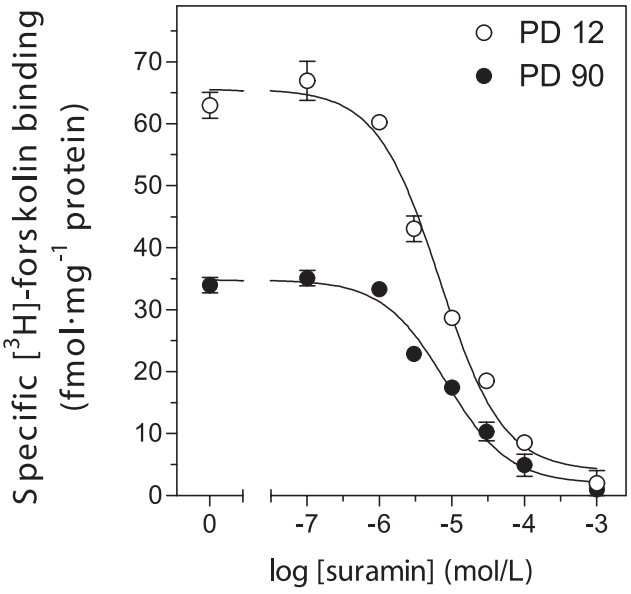


Fig. 5. Effect of suramin on [^{3}H]-forskolin binding. Cerebrocortical membranes from young (open circles) and adult (closed circles) rats were incubated with increasing concentrations of suramin in the presence of 20 nmol/L [^{3}H]-forskolin at 20 °C for 60 min. The competition curve is representative of 2 similar experiments performed in triplicate.



Besides these high-affinity binding sites, we were able to detect low amounts of super-high-affinity [^{3}H]-forskolin binding sites in membrane preparations from both age groups, but their characteristics did not differ markedly from each other (Table 1). Curiously, super-high-affinity binding sites for [^{3}H]-forskolin have not been previously noted in either brain or other tissues. There are 2 possible explanations for our present successful uncovering of this type of [^{3}H]-forskolin binding sites. First, we used relatively low amounts of protein (about 200 µg per assay) as compared with the majority of earlier studies using about 1 mg protein per assay. It is likely that the whole process of [^{3}H]-forskolin binding may have been affected by this condition to at least some extent. Second, our sample preparation differed from previous experiments, and may have influenced the outcome of our [^{3}H]-forskolin binding experiments. Our cerebrocortical membranes were washed 3 times before using in subsequent analyses. This step might have been of crucial importance for removing some endogenous ligands that may have otherwise hampered the binding of [^{3}H]-forskolin to its super-high-affinity binding sites.

Interestingly, both types of [^{3}H]-forskolin binding sites were clearly detectable, even in the presence of the G protein activator AlF_4^-. Whereas AlF_4^- did not significantly influence the amount of high-affinity [^{3}H]-forskolin binding sites in cerebrocortical membranes from adult rats, the number of these sites in young rats was markedly elevated and approached the level found in membranes from adult animals. Additionally, AlF_4^- increased enormously, and to similar extent, the number of super-high-affinity [^{3}H]-forskolin binding sites in samples from both tested age groups. The ability of activated G proteins to enhance [^{3}H]-forskolin binding has been previously described (Seamon et al. 1985;

Gehlert 1986; Goldman et al. 1988), but the exact underlying molecular mechanism is not quite clear. It may be speculated that the interaction between activated G_s protein and AC alters the enzyme conformation, which becomes more easily accessible to forskolin molecules. Such a notion is supported by the observation that activity of AC is much more effectively promoted by low concentrations of forskolin in the presence of activated G_s protein than by forskolin alone (Daly et al. 1982; Seamon and Daly 1985). Interaction of G_s protein with AC apparently favours both substrate catalysis at the active site and binding of forskolin at the pseudoactive site of the enzyme (Dessauer et al. 1998). It has been proposed that high-affinity binding sites for forskolin correspond to a ternary complex of the AC catalytic subunit, G_s protein, and forskolin (Alousi et al. 1991; Post et al. 1995).

In the next set of experiments, we analysed more closely the effect of different concentrations of Mg^{2+} ions and Gpp(NH)p on the specific binding of [^{3}H]-forskolin to cerebrocortical membranes. As expected, the addition of Mg^{2+} ions markedly increased [^{3}H]-forskolin binding in both types of tested membranes, but it was still higher in preparations from young than adult animals. Previously, it was shown that Gpp(NH)p may promote [^{3}H]-forskolin binding, and Mg^{2+} ions were found to be essential for this effect to take place. Whereas some investigators reported that Gpp(NH)p increases [^{3}H]-forskolin binding in the presence of 5 mmol/L $MgCl_2$ (Nelson and Seamon 1985; Seamon et al. 1985), others observed that much higher Mg^{2+} concentrations (100 mmol/L) were necessary (Odagaki et al. 1991). In our experimental conditions, Gpp(NH)p markedly enhanced [^{3}H]-forskolin binding, but this effect was manifested only in the presence of high concentrations of Mg^{2+} (>100 mmol/L). In addition, we noticed a different ability of Gpp(NH)p to promote [^{3}H]-forskolin binding in samples from young and adult rats. Whereas [^{3}H]-forskolin binding in cerebrocortical membranes from young rats was maximally increased by the addition of low amounts of Gpp(NH)p (1×10^{-7} mol/L), much higher concentrations ($>1\times10^{-5}$ mol/L) of this nonhydrolyzable GTP analogue were required for maximal binding enhancement in preparations from adult animals. These data may suggest that the coupling efficiency of the stimulatory G_s protein to AC is lower in samples from adult rather than young animals.

Inspired by the work of Nelson and Seamon (1986), who observed a clear stimulating effect of PGE_1 and PGD_2 on Gpp(NH)p-promoted [^{3}H]-forskolin binding in platelet membranes, we wished to assess the supposable effect of the β-adrenergic receptor agonist isoproterenol and the $GABA_B$ receptor agonist baclofen on [^{3}H]-forskolin binding. In spite of isoproterenol (1×10^{-5} mol/L) and baclofen (1×10^{-3} mol/L) added to the incubation mixture at their maximally effective concentrations usually applied in measurements of AC activity (Ihnatovych et al. 2002*a*), neither of these 2 agents significantly altered the binding of [^{3}H]-forskolin found in the presence of 1×10^{-7} mol/L Gpp(NH)p. Interestingly, isoproterenol has previously been shown to enhance [^{3}H]-forskolin binding in S49 lymphoma cells, but these experiments were conducted on growing cell cultures and not in isolated membranes (Alousi et al. 1991; Post et al. 1996). In contrast, isoproterenol did not significantly influence [^{3}H]-forskolin binding in cardiac myocytes, although this agonist and forskolin exerted remarkable synergistic effect on cAMP production in these cells (Post et al. 1995). Our current data that show no detectable effects of isoproterenol and baclofen indicate that binding of [^{3}H]-forskolin to cerebrocortical membranes is not prone to modulation by these 2 agonists, although their ability to affect AC activity in this kind of samples has been demonstrated (Ihnatovych et al. 2002*a*). Likewise, Odagaki et al. (1991) did not observe any effect of isoproterenol on [^{3}H]-forskolin binding in rat brain tissue.

We have recently observed that suramin may act as a direct inhibitor of AC activity (unpublished data) and therefore considered this compound worth testing for its possible capacity to interfere with [^{3}H]-forskolin binding. The current competition binding experiments performed on cerebrocortical membranes demonstrated that suramin was able to inhibit specific binding of [^{3}H]-forskolin similarly in samples from both age groups. Results of further analyses of competitive binding curves (Hill slopes close to 1.0) suggested that suramin may act as a competitive inhibitor of high-affinity [^{3}H]-forskolin binding. These observations bring new information about this pharmacologically important compound. Suramin has mostly been used as trypanocidal drug for treatment of African sleeping sickness and as salvage therapy in some cancers in the last 2 decades (Jennings et al. 2002; Kaur et al. 2002; Ryan et al. 2002). Nevertheless, the molecular mechanism of its action is not well understood. Suramin exhibits antiproliferative and antiangiogenetic activity by interfering with the binding of several growth factors to their receptors (Mills et al. 1990; Minniti et al. 1992; Firsching et al. 1995). However, suramin may compromise the enzyme functions of protein kinase C and protein-tyrosine phosphatases (Khaled et al. 1995; Zhang et al. 1998), and it has the ability to interfere with transmembrane signalling regulated by trimeric G proteins has also been described (Butler et al. 1988; Huang et al. 1990; Beindl et al. 1996). More recently, suramin has been shown to interact directly with calmodulin-recognition sites and to activate the skeletal-muscle ryanodine receptors (Klinger et al. 1999; Klinger et al. 2001). Our present studies have thus extended the existing array of suramin cellular targets by identifying this drug as a potent direct inhibitor of AC and [^{3}H]-forskolin binding.

Overall, the results presented here demonstrate that high-affinity, as well as super-high-affinity, binding sites for [^{3}H]-forskolin can be detected in rat brain cortex. In addition, we have found that parameters (K_D and B_{max}) of specific [^{3}H]-forskolin binding differ quite significantly in cerebrocortical membranes from young and adult rats. It may be assumed that the markedly different affinity of [^{3}H]-forskolin binding sites in these 2 age groups, which certainly reflects altered ability of forskolin to interact with AC, is above all given by qualitative changes in the enzyme. There are some indications that forskolin need not bind to and activate all AC isoforms with the same rank of potency (Sutkowski et al. 1994). Our present finding of lower affinity of binding sites for [^{3}H]-forskolin and presumably lesser coupling efficiency of G_s protein to AC in adulthood may thus imply that the complement of AC isoforms in young and adult rat brain cortex might be different. This speculation remains to be proved or disproved in future studies.

Acknowledgements

This work was supported by The Wellcome Trust (G.B.), Academy of Sciences of the Czech Republic (AV0Z 5011922) and Ministry of Education of the Czech Republic (projects MSMT 11300003 and LN00A026).

References

Alousi, A.A., Jasper, J.R., Insel, P.A., and Motulsky, H.J. 1991. Stoichiometry of receptor-Gs-adenylate cyclase interactions. FASEB. J. **5**: 2300–2303.

Beindl, W., Mitterauer, T., Hohenegger, M., Ijzerman, A.P., Nanoff, C., and Freissmuth, M. 1996. Inhibition of receptor/G protein coupling by suramin analogues. Mol. Pharmacol. **50**: 415–423.

Butler, S.J., Kelly, E.C., McKenzie, F.R., Guild, S.B., Wakelam, M.J., and Milligan, G. 1988. Differential effects of suramin on the coupling of receptors to individual species of pertussis-toxin-sensitive guanine-nucleotide-binding proteins. Biochem. J. **251**: 201–205.

Daly, J.W., Padgett, W., and Seamon, K.B. 1982. Activation of cyclic AMP-generating systems in brain membranes and slices by the diterpene forskolin: augmentation of receptor-mediated responses. J. Neurochem. **38**: 532–544.

Dessauer, C.W., Tesmer, J.J., Sprang, S.R., and Gilman, A.G. 1998. Identification of a Giα binding site on type V adenylyl cyclase. J. Biol. Chem. **273**: 25831–25839.

Firsching, A., Nickel, P., Mora, P., and Allolio, B. 1995. Antiproliferative and angiostatic activity of suramin analogues. Cancer Res. **55**: 4957–4961.

Gehlert, D.R. 1986. Regional modulation of [^{3}H]forskolin binding in the rat brain by guanylyl-5′-imidodiphosphate and sodium fluoride: comparison with the distribution of guanine nucleotide binding sites. J. Pharmacol. Exp. Ther. **239**: 952–958.

Gehlert, D.R., Dawson, T.M., Yamamura, H.I., and Wamsley, J.K. 1985. Quantitative autoradiography of [^{3}H]forskolin binding sites in the rat brain. Brain Res. **361**: 351–360.

Goldman, M.E., Mallorga, P., Pettibone, D.J., and Sugrue, M.F. 1988. Characterization of [^{3}H]forskolin binding sites in the iris-ciliary body of the albino rabbit. Life Sci. **42**: 1307–1314.

Huang, R.R., Dehaven, R.N., Cheung, A.H., Diehl, R.E., Dixon, R.A., and Strader, C.D. 1990. Identification of allosteric antagonists of receptor-guanine nucleotide-binding protein interactions. Mol. Pharmacol. **37**: 304–310.

Ihnatovych, I., Novotny, J., Haugvicova, R., Bourova, L., Mares, P., and Svoboda, P. 2002*a*. Ontogenetic development of the G protein-mediated adenylyl cyclase signalling in rat brain. Brain Res. Dev. Brain Res. **133**: 69–75.

Ihnatovych, I., Novotny, J., Haugvicova, R., Bourova, L., Mares, P., and Svoboda, P. 2002*b*. Opposing changes of trimeric G protein levels during ontogenetic development of rat brain. Brain Res. Dev. Brain Res. **133**: 57–67.

Insel, P.A., and Ostrom, R.S. 2003. Forskolin as a tool for examining adenylyl cyclase expression, regulation, and G protein signaling. Cell. Mol. Neurobiol. **23**: 305–314.

Jackman, G.P., and Bobik, A. 1986. Forskolin-mediated activation of cardiac, liver and lung adenylate cyclase in the rat. Relation to [^{3}H]forskolin binding sites. Biochem. Pharmacol. **35**: 2247–2251.

Jennings, F.W., Rodgers, J., Bradley, B., Gettinby, G., Kennedy, P.G., and Murray, M. 2002. Human African trypanosomiasis: potential therapeutic benefits of an alternative suramin and melarsoprol regimen. Parasitol. Int. **51**: 381–388.

Kaur, M., Reed, E., Sartor, O., Dahut, W., and Figg, W.D. 2002. Suramin's development: what did we learn? Invest. New Drugs. **20**: 209–219.

Keshles, O., and Levitzki, A. 1984. The ontogenesis of beta-adrenergic receptors and of adenylate cyclase in the developing rat brain. Biochem. Pharmacol. **33**: 3231–3233.

Khaled, Z., Rideout, D., O'Driscoll, K.R., Petrylak, D., Cacace, A., Patel, R., et al. 1995. Effects of suramin-related and other clinically therapeutic polyanions on protein kinase C activity. Clin. Cancer Res. **1**: 113–122.

Klinger, M., Freissmuth, M., Nickel, P., Stabler–Schwarzbart, M., Kassack, M., Suko, J., et al. 1999. Suramin and suramin analogs activate skeletal muscle ryanodine receptor via a calmodulin binding site. Mol. Pharmacol. **55**: 462–472.

Klinger, M., Bofill–Cardona, E., Mayer, B., Nanoff, C., Freissmuth, M., and Hohenegger, M. 2001. Suramin and the suramin analogue NF307 discriminate among calmodulin-binding sites. Biochem. J. **355**: 827–833.

Matsuoka, I., Suzuki, Y., Defer, N., Nakanishi, H., and Hanoune, J. 1997. Differential expression of type I, II, and V adenylyl cyclase gene in the postnatal developing rat brain. J. Neurochem. **68**: 498–506.

Mills, G.B., Zhang, N., May, C., Hill, M., and Chung, A. 1990. Suramin prevents binding of interleukin 2 to its cell surface receptor: a possible mechanism for immunosuppression. Cancer Res. **50**: 3036–3042.

Minniti, C.P., Maggi, M., and Helman, L.J. 1992. Suramin inhibits the growth of human rhabdomyosarcoma by interrupting the insulin-like growth factor II autocrine growth loop. Cancer Res. **52**: 1830–1835.

Musser, B., Mudumbi, R.V., Liu, J., Olson, R.D., and Vestal, R.E. 1999. Adenosine A1 receptor-dependent and -independent effects of the allosteric enhancer PD 81,723. J. Pharmacol. Exp. Ther. **288**: 446–454.

Nelson, C.A., and Seamon, K.B. 1985. Regulation of [^{3}H]forskolin binding to human platelet membranes by GppNHp, NaF, and prostaglandin E1. FEBS. Lett. **183**: 349–352.

Nelson, C.A., and Seamon, K.B. 1986. Binding of [3H]forskolin to human platelet membranes. Regulation by guanyl-5′-yl imidodiphosphate, NaF, and prostaglandins E1 and D2. J. Biol. Chem. **261**: 13469–13473.

Odagaki, Y., Koyama, T., and Yamashita, I. 1991. Effects of long-term treatment with lithium and antidepressants on [^{3}H]forskolin binding to rat cerebral cortical membranes. Prog. Neuropsychopharmacol. Biol. Psychiatry. **15**: 709–716.

Poat, J.A., Cripps, H.E., and Iversen, L.L. 1988. Differences between high-affinity forskolin binding sites in dopamine-rich and other regions of rat brain. Proc. Natl. Acad. Sci. U. S. A. **85**: 3216–3220.

Post, S.R., Hilal–Dandan, R., Urasawa, K., Brunton, L.L., and Insel, P.A. 1995. Quantification of signalling components and amplification in the beta-adrenergic-receptor-adenylate cyclase pathway in isolated adult rat ventricular myocytes. Biochem. J. **311**: 75–80.

Post, S.R., Aguila–Buhain, O., and Insel, P.A. 1996. A key role for protein kinase A in homologous desensitization of the beta 2-adrenergic receptor pathway in S49 lymphoma cells. J. Biol. Chem. **271**: 895–900.

Ross, B., Dawson, D., Dewar, D., Macrae, M., Knowler, J., and McCulloch, J. 1993. Effects of post-mortem delay on high affinity forskolin binding sites and adenylate cyclase activity in rat and human striatum and cerebral cortex. Brain Res. **629**: 225–230.

Ryan, C.W., Vokes, E.E., Vogelzang, N.J., Janisch, L., Kobayashi, K., and Ratain, M.J. 2002. A phase I study of suramin with once- or twice-monthly dosing in patients with advanced cancer. Cancer Chemother. Pharmacol. **50**: 1–5.

Seamon, K.B., and Daly, J.W. 1981. Forskolin: a unique diterpene activator of cyclic AMP-generating systems. J. Cyclic Nucleotide Res. **7**: 201–224.

Seamon, K.B., and Daly, J.W. 1985. High-affinity binding of forskolin to rat brain membranes. Adv. Cyclic Nucleotide Protein Phosphorylation Res. **19**: 125–135.

Seamon, K.B., Padgett, W., and Daly, J.W. 1981. Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. Proc. Natl. Acad. Sci. U. S. A. **78**: 3363–3367.

Seamon, K.B., Vaillancourt, R., Edwards, M., and Daly, J.W. 1984. Binding of [^{3}H]forskolin to rat brain membranes. Proc. Natl. Acad. Sci. U. S. A. **81**: 5081–5085.

Seamon, K.B., Vaillancourt, R., and Daly, J.W. 1985. Modulation of forskolin binding to rat brain membranes. J. Cyclic Nucleotide Protein Phosphorylation Res. **10**: 535–549.

Stockmeier, C.A., and Zhang, Y. 1993. Characterization of [^{3}H]forskolin binding sites in the human caudate nucleus. Brain Res. **610**: 156–161.

Sutkowski, E.M., Tang, W.J., Broome, C.W., Robbins, J.D., and Seamon, K.B. 1994. Regulation of forskolin interactions with type I, II, V, and VI adenylyl cyclases by Gsα. Biochemistry, **33**: 12852–12859.

Zhang, Y.L., Keng, Y.F., Zhao, Y., Wu, L., and Zhang, Z.Y. 1998. Suramin is an active site-directed, reversible, and tight-binding inhibitor of protein-tyrosine phosphatases. J. Biol. Chem. **273**: 12281–12287.