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Functional role of disulphide bonding and extracellular vestibule in the rat P2X4 receptor

Funkční úloha disulfidických můstků a extracelulárního vestibulu potkaního P2X4 receptoru

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Doctoral thesis
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Proclamation:

In accordance with the guidelines for the Ph.D. dissertation publication at the Faculty of Sciences at Charles University in Prague, I, Milos Rokic, declare that the research described within this dissertation has been conducted solely by me. I have created and presented all of the figures, unless otherwise noted, the data has been collected during the course of Ph.D. study. Credit has been given for any data or experimental findings that have been referenced from other studies and publications. This work is not used to obtain any other academic degree.

Prague, January 14, 2013.

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Milos Rokic

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Contents

Abstract

Purinergic P2X receptors are membrane ion channels activated by extracellular ATP. There are seven isoforms of mammalian P2X receptors designated as P2X1-7, which according to their structure represent a specific family of ligand gated ionic channels, with extraordinary structural/functional properties. The P2X receptor consists of three subunits and each subunit has two transmembrane domains. Crystalographic data demonstrate that ionic channel pore is situated between the second transmembrane domains. Crystal structure of P2X4 receptor from the zebrafish (*Danio rerio*) is available in both open and closed state of the channel and the exact structure of ATP binding site is solved. The aim of this thesis was to study the structure-function relationships in a model of recombinant P2X4 receptor of the rat. By employing the point mutagenesis and electrophysiological recording, the functional importance of conserved cysteine residues in the ectodomain and amino acid residues which form the extracellular vestibule was investigated. All ten cysteins were substituted one by one with alanine or threonine and ATP-induced currents were measured from HEK293T cells expressing wild type (WT) and mutated P2X4 receptors. The results indicate that C116A, C126A, C149A and C165A mutations disrupt two disulfide bonds (C116-C126 and C149-C165A) which are needed for the integrity of ATP binding site. The third disulfide bond (C132-C159) is found as unimportant. The fourth (C217-C227) and the fifth (C261-C270) disulfide bonds are supposed to be important for either coupling of ligand binding and channel gating or channel gating itself. Considering the close proximity of C217-C227 to the extracellular vestibule which forms the entrance for ions, the residues V47-V61 and F324-N338 forming the lateral portals of vestibule were also investigated. Alanine mutations at positions F324, G325, V49, Y54 and Q55 have yielded a non-functional receptor indicating that these residues are essential for receptor function. It has been shown that V49 residue is important for expression of the channel on the cell surface. The substitution of Y54 to any other aromatic residue (Y54W and Y54F) resulted in restoring the receptor function, unlike non-aromatic residues (Y54L) which points out the importance of aromatic residue at this position. Furthermore, the Y54A and Y54C receptor function was partially rescued by ivermectin, a positive allosteric modulator of P2X4 receptor, suggesting a rightward shift in the potency of ATP to activate the receptor. In the case of Q55 residue, no substitution restored the receptor function; the only rescue was made by treating Q55E with ivermectin. The F324L, F324Y, and F324W mutations also rescued receptor function partially or completely, ivermectin action on channel gating was preserved in all mutants, and changes in ATP responsiveness correlated with the hydrophobicity and side chain volume of the substituent. The G325P mutant had a normal response to ATP, suggesting that G325 is a flexible hinge. A topological analysis revealed that the G325 and F324 residues disrupt an ectodomain β-sheet upon ATP binding. These results indicate multiple roles of the extracellular vestibule amino acid residues in the P2X4 receptor function.

Abstrakt

Purinergní P2X receptory jsou membránové iontové kanály aktivované extracelulárním ATP. U savců bylo dosud nalezeno sedm různých podtypů, označovaných jako P2X1-7, které svojí stavbou představují rodinu ligandem řízených iontových kanálů s výjimečnými strukturně/funkčními vlastnostmi. Každý P2X receptor je tvořen třemi podjednotkami, kde každá podjednotka má dvě transmembránové domény. Krystalografická data ukazují, že pór iontového kanálu se nachází mezi druhými transmembránovými doménami. Krystalová struktura P2X4 receptoru zebřičky (*Danio rerio*) je dostupná pro receptor v uzavřeném a otevřeném stavu s navázanou molekulou ATP, a proto je přesná struktura vazebného místa pro ATP vyřešena. Cílem této dizertační práce bylo studium strukturně-funkčních vlastností v modelu rekombinantního P2X4 receptoru. Technikou bodové mutageneze a elektrofyziologickou technikou patch clamp byl zkoumán funkční význam pěti konzervovaných cysteinových párů v ektodoméně a funkční úloha aminokyselinových zbytků, které tvoří extracelulární vestibul P2X4 iontového kanálu potkana. Všech deset cysteinů, jeden po druhém, bylo nahrazeno alaniny nebo threoniny a ATP-stimulované proudy byly snímány z HEK293T buněk nesoucích divoký typ P2X4 receptoru nebo jeho mutace. Výsledky získané u mutací C116A, C126A, C149A a C165A naznačují poruchu ATP-vazebné kapsy při narušení jimi tvořených disulfidických můstků (C116-C126 a C149-C165A). Třetí cysteinový můstek (C132-C159) se jeví jako nedůležitý. Na převodu signálu z ektodomény k transmembránovým doménám či otevírání iontového kanálu se podílí čtvrý a pátý můstek (C261-C270 a C217-C227). Vzhledem k blízkosti cysteinové vazby C217-C227 a brány pro průchod iontů jsme zkoumali také roli aminokyselinových zbytků v polypeptidových řetězcích V47-V61 a F324-N338, které formují extracelulární vestibul P2X4 receptoru. Alaninové mutace v pozici F324, G325, V49, Y54 a Q55 měly za následek nefunkční receptor, což naznačuje klíčovou úlohu těchto aminokyselin ve funkci receptoru. Ukázalo se, že V49 je důležitá pro expresi kanálu na povrchu buňky. Výměnou Y54 za jiné aromatické aminokyseliny (Y54W a Y54F) se podařilo vrátit funkci receptoru, nikoli za nearomatickou (Y54L), což dokládá důležitost aromatického zbytku na této pozici. Funkci mutací Y54A a Y54C bylo možné částečně navrátit také použitím ivermektinu, alosterického modulátoru P2X4 receptoru, což ukazuje na snížení citlivosti k ATP u těchto mutací. U aminokyselinového zbytku Q55 se nepodařilo nijak navrátit funkci receptoru výměnou za jinou aminokyselinou, jediná mutace Q55E byla citlivá k ivermektinu. Mutace F324L, F324Y a F324W částečně či kompletně obnovily funkci receptoru, byly citlivé vůči ivermektinu a změny v citlivosti těchto mutantů korelovaly s velikosti a hydrofobicitou jejich řetězců. Další zasaženou aminokyselinu G325 se podařilo nahradit za prolin, což ukazuje na potřebu flexibilního ohybu v daném místě receptoru. Následná topologická analýza ukázala, že G325 a F324 přerušují po navázání ATP strukturu beta-listu. Výsledky prokazují mnohonásobnou úlohu aminokyselin v oblasti extracelulárního vestibulu ve funkci P2X4 receptoru.

The contribution of Miloš Rokić in author publications

Peer-reviewed publications that form part of the thesis:

(1)
Rokic, M. B., Tvrdonova, V., Vavra, V., Jindrichova, M., Obsil, T., Stojilkovic, S. S. and Zemkova, H. (2010) Roles of conserved ectodomain cysteines of the rat P2X4 purinoreceptor in agonist binding and channel gating. Physiological Research. 59, 927-935, (IF: 1,555)

Performed electrophysiological measurement, cell culturing and transfection, and contributed to preparation of the manuscript.

(2)
Rokic, M. B., Stojilkovic, S. S., Vavra, V., Kuzyk, P., Tvrdonova, V and Zemkova, H. (2013) Multiple roles of the extracellular vestibule amino acid residues in the function of the rat P2X4 receptor. Plos One. Accepted for publication, (IF: 4,092)

Performed electrophysiological measurement, cell culturing and transfection, analysed data, prepared draft and contributed to finalization of the manuscript.

Peer-reviewed publications that do not form part of the thesis:

(3)
Nikolic, L., Rokic M., Todorovic, N., Kartelija, G., Nedeljkovic, M. and Zakrzewska, J. (2010) Effect of alternating the magnetic field on phosphate metabolism in the nervous system of Helix pomatia. Biological Research. 43, 243-250, (IF: 1.029)

Prepared the samples, contributed to the measurements of NMR spectra of garden snail (*Helix pomatia*) cerebral ganglia and contributed to preparation of manuscript.

Abbreviation list

ATP – adenosin-5'-triphosphate

CTP – cytidine-5'-triphosphate

EC_{50}- concentration of the ligand inducing half-maximum amplitude of the response

GTP – guanosine-5'-triphosphate

HEK293T – human embryonic kidney cells line number 293 T

I_{max}-maximum current amplitude induced by a supramaxinal ligand concentration

IVM – ivermectin

$NMDG^+$- N-methyl-D-glucamine

P2X receptor – ionotropic purinergic receptor

P2Y receptor – G-protein coupled metabotropic purinergic receptors

rP2X receptor– ionotropic purinergic receptor of the rat

SEM- standard error of mean

SS – disulphide bond

TM – transmembrane domain

UTP – uridine-5'-triphosphate

WT – wild type

zfP2X receptor – P2X receptor of the zebra fish (*Danio rerio*)

Amino acid designations

amino acid	three-letter abbreviation	one-letter abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Glutamate	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

1. INTRODUCTION

1.1 Historical background and principles of purinergic signaling

The first physiological action of extracellular adenosin-5'-triphosphate (ATP) was proven in 1929 (Drury and Szent-Gyorgyi 1929) on the model of perfused heart and coronary blood vessels. The cardiovascular and endocrine actions of ATP were further investigated by 1950ties (Green and Stoner 1950; Stoner and Green 1950). Up to these moments the ATP was exclusively considered as the crucial molecule for energy metabolism, as a carrier of chemically bound energy produced during glycolysis and oxidative phosphorylation. Little was known about the purine and pyrimidine derivatives in terms of their signaling function. At present, it is widely accepted that purinergic singling represents the system of chemical communication between cells mediated by the extracellular purine and pirimidine nucleotides (Burnstock 1977; Burnstock 1986; Burnstock 2006; Burnstock 2011). These signaling molecules fulfill numerous physiological roles trough binding to specific membrane receptors called purinoreceptors. The signaling pathways of purines are downregulated by reuptake of purine molecules via membrane transporters (nucleoside transporters) and by a series of extracellular hydrolytic enzymes, ectonucleotidases (Zimmermann 2000; Bonan 2012). Nucleoside transporters are molecules involved in the transport of nucleosides trough plasma membrane. Their role was suggested to be important in restoring the cellular pool of adenosine, needed for the production of ATP, and in preventing the excessive activation of adenosine pathways (Bonan 2012; Choi and Berdis 2012). Up to now, two groups of transporters have been characterized: equilibrative nucleoside transporters and concentrating nucleoside transporters. Equilibrative nucleoside transporters are class of molecules which act by abolishing the concentration gradients of nucleosides across the plasma membrane. There are four isoforms of these transporters, termed ENT1-4 (Baldwin, Beal et al. 2004; Molina-Arcas, Trigueros-Motos et al. 2008; Molina-Arcas, Casado et al. 2009). In contrast, concentrating nucleoside transporters play a role in increasing the intracellular concentration of nucleosides trough co-transport with Na^+ ions (Huang, Yao et al. 1994; Che, Ortiz et al. 1995; Molina-Arcas, Trigueros-Motos et

al. 2008; Molina-Arcas, Casado et al. 2009; Choi and Berdis 2012). There are 3 isoforms of these transporters, termed SLC28A1-3. Enzymes which are degrading purine nucleotides in the extracellular environment are found in a form of membrane proteins with active sites is directed to the exterior of the cell. These enzymes are called ectonucleotidases. Ectonucleotidases have different specificities and kinetic properties towards different purine and pyrimidine agonists and their metabolites, and it is thought that they act in a concerted manner by channeling nucleotides towards nucleosides (Deaglio and Robson 2011; Longhi, Robson et al. 2011). There are four major families of ectonucleotidases: ecto-nucleotide triphosphate diphosphohydrolases (CD39/NTPDases), nucleotide pyrophosphatase/phosphodiesterase (NPP)-type ecto-phosphodiesterases, alkaline phosphatases and ecto-5'-nucleotidases/CD73 (Burnstock 2011; Allard, Turcotte et al. 2012). Since degradable products of purine and pyrimidine molecules can also activate their specific receptors, together they form a complex purinergic signaling with pleiotropism and redundancy as their main property (for example see Figure 1.1). Similarly as with other ligand-gated membrane receptors, receptors mediating the cellular responses to purines are both metabotropic and ionotropic giving the different kinetics and sensitivities of cellular responses *in vivo* (Burnstock 2006). The omnipresence of purines and pyrimidines in extracellular environment makes them physiologically important signaling molecules (Burnstock 1977; Burnstock 1986; Burnstock 2006; Burnstock 2011; Khakh and North 2012). Receptors for nucleotides and nucleosides are widely distributed within the mammalian organisms (Table 1.1).

Receptor	Distribution
P2X1	Smooth muscle, platelets, cerebellum, dorsal horn spinal neurons
P2X2	Smooth muscle, brain, pituitary, pancreas, retina, chromaffin cells, autonomic and sensory ganglia
P2X3	Nociceptive sensory neurons, solitary tractus neurons, some sympathetic neurons
P2X4	Microglia, brain, pituitary, testis, colon, endothelial cells
P2X5	Proliferating cells in skin, gut, bladder, thymus, spinal cord, heart, adrenal medulla
P2X6	Brain, motor neurons in spinal cord
P2X7	Macrophages, mast cells, microglia, pancreas, skin, endocrine organs
P2Y1	Brain, epithelial and endothelial cells, platelets, immune cells, osteoclasts
P2Y2	Immune cells, epithelial and endothelial cells, kidney tubules, osteoblasts
P2Y4	Endothelial cells, placenta
P2Y6	Airway and intestinal epithelial cells, spleen, placenta, T-cells, thymus
P2Y11	Spleen, intestine, granulocytes
P2Y12	Platelets, brain (glial cells), microglial cells
P2Y13	Spleen, brain, lymph nodes, bone marrow
P2Y14	Placenta, mast cells, adipose tissue, stomach, intestine, discrete brain regions

Table 1.1 The tissue distribution of particular purinergic recepors. (From Jacobson 2010)

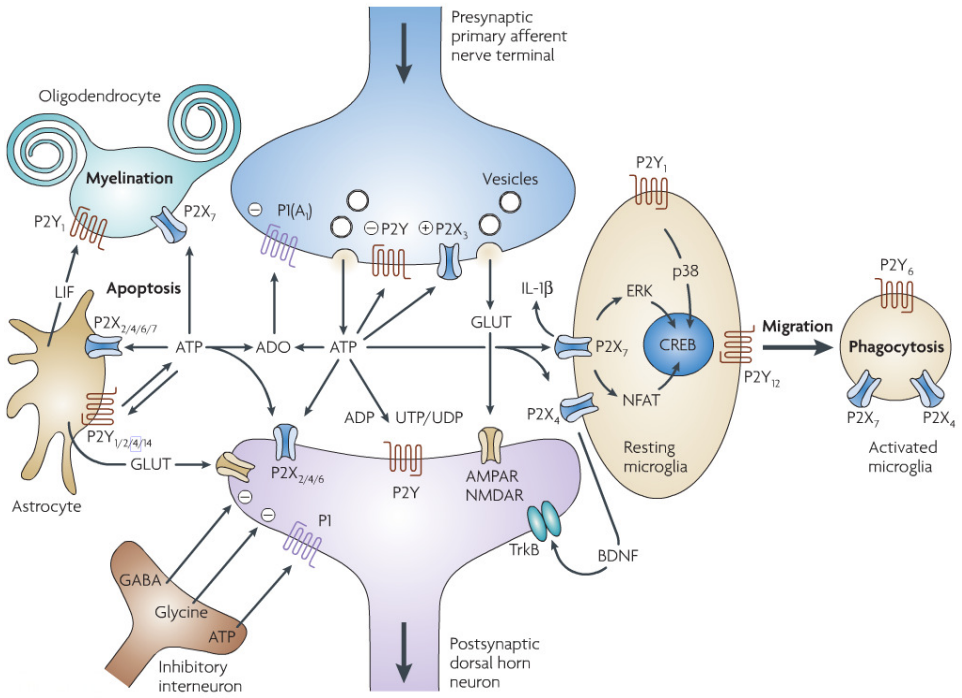

Figure 1.1 The schematic representation of purinergic signaling within the spinal cord. Please note, that pleiotropic properties of purinoreceptors are mediating neuro—remodelation trough increased production of BDNF (brain derived neurotrophic factor) and IL-1β (interleukin - 1β), neurotransmission trough secretion of glutamate, and neuromodulation trough increase of exocytosis of ATP filled vesicles, and immune processes of microglial activation and migration, mediated by P2Y receptors. The molecular factors presented are LIF-Leukemia inhibitory factor, GLUT-Glucose transporter, GABA- γ-amino butyric acid, AMPAR - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor, NMDAR - *N*-methyl-D-aspartate receptor, TrkB - neurotrophic tyrosine kinase receptor type 2, NFAT - Nuclear factor of activated T-cells, CREB - cyclic adenosine – monophosphate response element-binding protein, p38 - mitogen-activated protein kinase, ERK- extracellular-signal-regulated kinase, ADO - adenosine (Burnstock 2008).

1.2 Purinoreceptors

Purinergic receptors are transmembrane protein molecules responsible for triggering and in certain cases eliciting physiological responses to purine or pyrimidine nucleotides and nucleosides in the extracellular environment. These receptors have been categorized into two groups in respect to which agonist they bind. P1 receptors are activated primarily by adenosine while P2 receptors are involved in binding ADP or ATP (Abbracchio and Burnstock 1994). P1 receptors are exclusively metabotropic, these are seven transmembrane domain receptors eliciting responses trough G-protein coupled signaling.

These receptors are also called adenosine receptors and up to present day there are four classes of adenosine receptors (A1, A2A, A2B and A3) (Maenhaut, Van Sande et al. 1990; Moro, Gao et al. 2006). P2 receptors exist as metabotropic receptors designated as P2Y receptors (P2Y) and ionotropic receptors (ionic channels) designated as P2X receptors. P2Y receptors are also metabotropic G-protein coupled receptors and they exist in eight isoforms: P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, P2Y14 (Burnstock 2006).

1.3 Ligand-gated P2X receptor channels

P2X receptors are ligand gated ionic channels. Ligand gated ionic channels are activated trough increasing the probability of channel opening by the rising concentration of a ligand present in the extracellular environment which binds to a channel (Colquhoun 1998). All ligand gated channels demonstrate the ligand saturation kinetics, which means that when maximum channel activation is elicited, further addition of the ligand does not increase the channel response. Because of this, the parameter which characterizes the ligand potency for the channel activity is the dose of the ligand which induces half-maximum amplitude of the response (designated as EC_{50}). The amplitude of the response corresponding to maximal channel activity elicited by the supramaximal ligand dose is designated as I_{max}. Although classification of ligand-gated ion channels is based upon the major natural agonist of the channel, there are four structural superfamilies of ligand-gated ion channels: transient receptor potential channels, pentameric cys-loop receptors (nicotinic acetylcholine, γ-aminobutyric acid and glycine gated receptors), tetrameric glutamate-gated channels and trimeric ATP-gated receptors. ATP-gated receptors are P2X receptor-channels (Nicke, Baumert et al. 1998) which were cloned in the mid 1990s. The corresponding proteins consist of 379 (P2X6) to 595 (P2X7) amino acid residues. The sequence homology between the receptors in the rat (*Rattus norvegicus*) is ranging from 26 to 47%. The sequence alignment of the rat P2X1-7 receptors is demonstrated in figure 1.2. Every subunit consists of two transmembrane domains (TM1 and TM2) connected by a large extracellular loop which folds into an ectodomain (Newbolt, Stoop

et al. 1998). Within the P2X2 ectodomain, there are four glycosylation sites at N153, N184, N210, and N300 (Torres, Egan et al. 1998; Rettinger, Aschrafi et al. 2000; Hu, Senkler et al. 2002). The ectodomain also contains ten cysteines forming five disulphide bridges which are conserved across the mammalian P2X family (Clyne, Wang et al. 2002; Ennion and Evans 2002). Three subunits make a functional channel which exists in homo or hetero trimeric forms (Nicke, Kerschensteiner et al. 2005).

Figure 1.2 Amino acid sequence alignment of seven rat P2X receptor isoforms and zebra fish P2X4 as generated by UniProt server. (http://www.uniprot.org/align) The transmembrane domains are designated in green and conserved cystein residues are

shaded in yellow. The amino acid residues comprising the extracellular vestibule are shaded in blue.

1.4 Physiology of P2X receptors and importance of P2X4 subtype

P2X receptors are widely distributed (Table 1.1) and have proven to be important for many physiologica functions, including the modulation of myocardial rythmicity (Vassort 2001), long and short term nociception (Chizh and Illes 2001), regulation of vascular tone as well as mediating ejaculation (Burnstock 2013). Their role is investigated in the process of neurotransmitter release (Evans, Derkach et al. 1992), neuromodulation (Khakh and North 2012) and regulation of excitability of pituitary cells (Zemkova, Balik et al. 2008; Zemkova, Kucka et al. 2010). P2X2 and P2X3 subunits are commonly found co-expressed in sensory neurons where they mediate fast pain initiation and in certain cases neuropathic pain (North 2003; North 2004; North and Verkhratsky 2006).

We used rP2X4 receptor as a model to study molecular properties of P2X receptor because this receptor subtype is highly expressed in neuroendocrine cells, including anterior pituitary cells and neurons of hypothalamic supraoptic nuclei where it controls electrical excitability and hormone secretion (Vávra, Balik et al. 2008; Vavra, Bhattacharya et al. 2011). P2X4 receptor cDNAs were independently isolated by five different groups of investigators from various rat tissues and have been isolated from human, mouse, chick, *Xenopus*, schistostoma and zebrafish (North 2002; North 2002). The P2X4 subunit is expressed widely and in highest concentration throughout the CNS and PNS, yet, the receptors have also been localized in lung, brochial epithelium, thymus, bladder, salivary gland, adrenal gland, and vas deferens (Ralevic and Burnstock 1998; Ralevic and Burnstock 1998; Virginio, MacKenzie et al. 1999; Virginio, MacKenzie et al. 1999; Khakh 2001; Khakh, Burnstock et al. 2001). Although not yet investigated entirely, the high expression of P2X4 receptorin the CNS has yielded discourse regarding the theoretical possibility of a role for the receptors in synaptic modulation via pore-dilation-induced increases in Ca^{2+} uptake or the uptake of larger regulatory molecules (Virginio, MacKenzie et al. 1999). Another potentially important role of brain-localized P2X4 receptors is in the CNS effects of ethanol, due to the observation that ethanol displays an inhibitory effect on P2X4 receptors (Xiong, Hu et al. 2005), and that ATP-activated current is inhibited in rat hippocampal neurons within the range of pharmacological concentrations of ethanol (Li, Xiong et al. 2000). Including P2X4

receptor, various P2X have been implicated in the transmission or modulation of nociceptive signals through the spinal cord. Recently, the role of P2X4 receptor was confirmed in tactile allodynia, the hypersensitivity of nociceptive transmission in injured primary sensory neurons, as the inhibition of P2X4 response, or blockade of p38 mitogen-activated protein kinase, resulted in an inhibition of the hypersensitive nociceptive signaling (Figure 1.1.), suggesting P2X4 receptor as a possible therapeutic target for tactile allodynia (Inoue, Tsuda et al. 2004; Inoue, Tsuda et al. 2004).

The P2X4 receptor has been implicated to play a role in the parasympathetic activation of salivary gland acinar cells, resulting in saliva production and excretion via an increase in the levels of intracellular Ca^{2+} (Tenneti, Gibbons et al. 1998; Brown, Bruce et al. 2004). The increase of the second messenger cAMP results in increased saliva production via an increase in Ca^{2+} release from intracellular stores, yet, an increase in P2X4-dependent Ca^{2+} uptake indicates the role of synaptic ATP release in the parasympathetic regulation of saliva release (Brown, Bruce et al. 2004). Other identified physiological roles of P2X4 receptors include a decrease in the permeability of human cervical epithelial cells, indicating an ATP-dependent regulation of fertility and the potential for a role in contraception (Gorodeski 2002), and the transmission of shear-stress signals causing transcriptional regulation of endothelial cell proteins (Yamamoto, Korenaga et al. 2000).

1.5 Pharmacology of P2X receptors

The distinct P2X receptor subtypes are functionally differentiated by comparisons in calcium conductivity, sensitivity to agonists, antagonists, and allosteric modulators, and the rate of desensitization (North 2002). For example, the P2X1 receptor is highly sensitive to ATP and is also activated by αβ-methylene adenosine-5`-triphosphate and 2-methylthio-adenosine triphosphate (Ralevic and Burnstock 1998). The P2X1 receptor can be differentiated from the P2X3 receptor by sensitivity to βγ-methylene adenosine-5`-triphosphate, where a 30-fold smaller concentration is required to activate P2X1 than P2X3 (Ralevic and Burnstock 1998). Another effective agonist is 2',3'-O-(benzoyl-4-benzoyl)-adenosine triphosphate, which acts as a P2X7 receptor-selective agonist

(Bianchi, Lynch et al. 1999; Roberts and Evans 2004). Table 1.2 summarizes the works on the estimation of ATP potency to open the channel (EC_{50} values) for seven P2X receptors. Note that although other P2X receptors activate to ATP concentration in the low μM range, ATP activates the P2X7 receptor with extremely low potency, the EC_{50} falling into the 100 μM range

Isoform	EC_{50} [μM]	Reference
P2X1	<1	(Valera, Hussy et al. 1994; Evans, Lewis et al. 1995)
P2X2	0.5-8	(Evans, Lewis et al. 1995; King, Neary et al. 1996; King, Wildman et al. 1997; Bianchi, Lynch et al. 1999; Lynch, Touma et al. 1999; Neelands, Burgard et al. 2003)
P2X3	0.5-2	(Robertson, Rae et al. 1996; Garcia-Guzman, Soto et al. 1997; Bianchi, Lynch et al. 1999; Neelands, Burgard et al. 2003)
P2X4	3-20	(Bo, Zhang et al. 1995; Buell, Lewis et al. 1996; Seguela, Haghighi et al. 1996; Soto, Garcia-Guzman et al. 1996; Miller, Michel et al. 1998; Khakh, Proctor et al. 1999)
P2X5	2-16	(Collo, North et al. 1996; Garcia-Guzman, Soto et al. 1996; Ruppelt, Ma et al. 2001; Bo, Jiang et al. 2003)
P2X6	~0.5	(Brake, Wagenbach et al. 1994; Evans, Lewis et al. 1995; Jones, Chessell et al. 2000; Jones, Vial et al. 2004)
P2X7	80-200	(Bo, Zhang et al. 1995; Evans, Lewis et al. 1995; Collo, North et al. 1996; Bianchi, Lynch et al. 1999)

Table 1.2 The summary of ATP potency to induce a current response in seven isoforms of the rat P2X receptor.

The further experimental work in pharmacology was driven towards the finding of ATP mimicking compounds which compete for binding to the ATP binding site and which could be used in the research and characterization of *in vivo* P2X receptors (North and Surprenant 2000; North 2002). These ATP analogues function as either agonists (positive modulators) or antagonists (negative modulators) of P2X receptors. Figure 1.3. demonstrates the ATP-like structures of several well characterized ATP mimetic compounds.

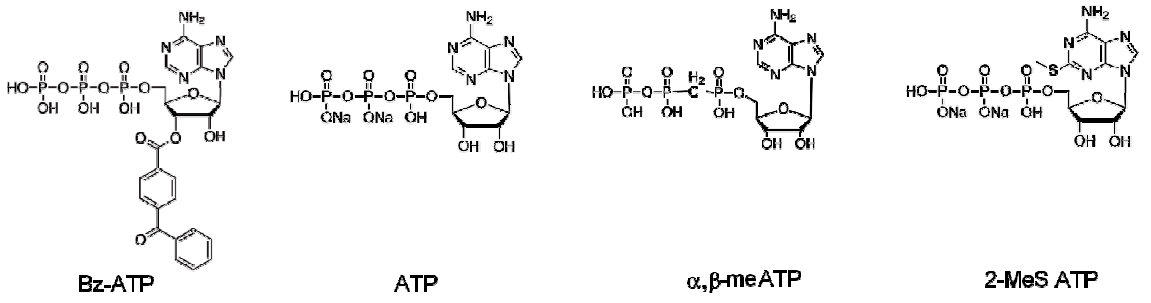

Figure 1.3 The formulas of pharmacologicaly active, widely used ATP analogues. These compounds are used for pharmacological dissection of particular P2X isoforms in native systems e.g 2′(3′)-O-(4-Benzoylbenzoyl) adenosine 5′-triphosphate (BzATP) is used as agonist for P2X7 receptor, α,β-methyleneadenosine 5′-triphosphate (α,β-meATP) for P2X3 receptor and 2-methythio adenosine-triphosphate (2-MeS ATP) which is not highly isoform selective.

The most widely used antagonists of P2X receptors are suramin and pyridoxal phosphate-6-azophenyl-2`,4`-disulphonic acid (PPADS) which are not isoform-selective (Figure 1.4). The P2X4 receptor is the least sensitive to these compounds (Soto, Garcia-Guzman et al. 1996; Soto, Lambrecht et al. 1999). Brilian Blue G and calmidazolium are used for antagonizing P2X7 receptors (Jiang, Mackenzie et al. 2000; Cervetto, Mazzotta et al. 2012). KN-62 is used for antagonizing human isoform of P2X7 (Chessell, Michel et al. 1998). One of the most specific drugs used to characterize P2X receptors is ivermectin (IVM), positive allosteric modulator of P2X4 subtype (Jelinkova et al, 2006; Khakh et al, 1999). This compound is widely used in veterinary medicine as an anthelminthic drug (Mohebbipour, Saleh et al. 2012), it potentiates exclusively the P2X4 receptor together with its sensitization and prolongation of receptor deactivation (Bennett, Williams et al. 1988; Khakh, Proctor et al. 1999). It has also been shown to increase the P2X4 receptor expression at the cell surface (Toulme, Soto et al. 2006). The molecular determinants of IVM binding to P2X4 receptor are found within transmembrane domains (Silberberg, Li et al. 2007; Jelinkova, Vavra et al. 2008). Since IVM up-regulates gating of the channel, mutants which inhibit gating of P2X4 receptor channels are rescued by IVM application (Priel and Silberberg 2004; Jelinkova, Yan et al. 2006). In this thesis, IVM is used as a tool to differentiate the deleterious effects of either disrupted ATP binding or abolished gating of P2X4 channel. The deactivation of the receptor in the presence of IVM is

shown to correlate negatively and linearly to the sensitivity of the receptor to ATP (Zemkova, He et al. 2004; Zemkova, Yan et al. 2007). P2X2 receptors have characteristic pH dependence. By reducing pH of extracellular solution by just one unit (from 7,4 to 6,5) the receptor sensitivity is increased approximately 4-fold (EC_{50} from 5μM to 1,2μM). In the case of increase of pH, the receptor sensitivity is reduced (King, Neary et al. 1996; King, Wildman et al. 1997). In other isoforms the pH sensitivity is the opposite (Gever, Cockayne et al. 2006).

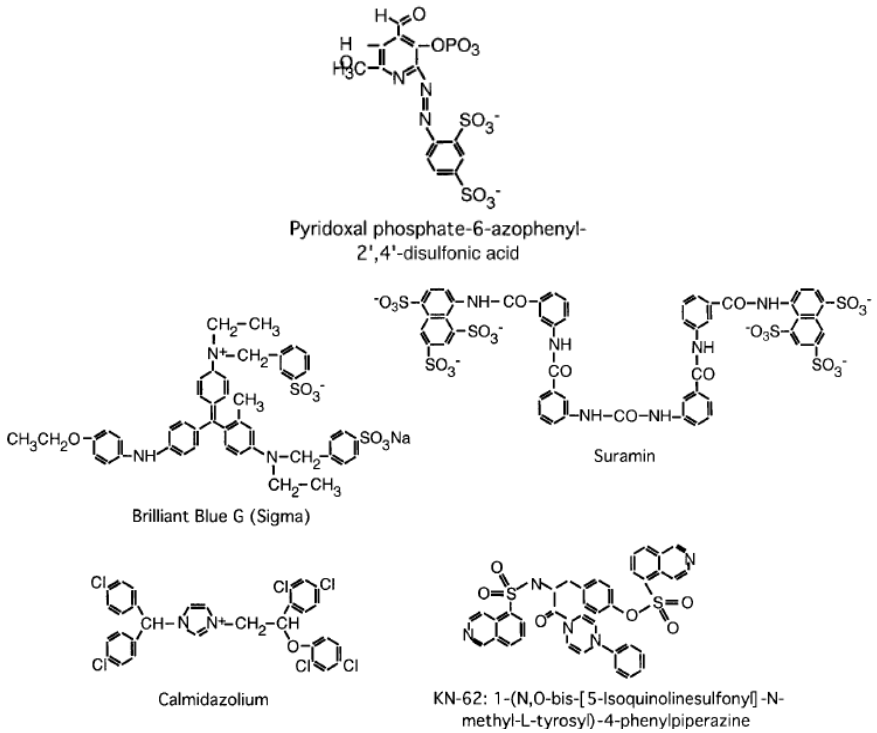

Figure 1.4 The structural formulas of several synthetic P2X antagonists which have no structural similarity to ATP. Taken from North 2002.

Several studies demonstrate the modulation of P2X receptors by heavy metal ions. Extracellular histidine residues have been recognized as important for zinc and copper allosteric potentiation of P2X2 receptor. The potentiation of P2X2 currents is also proven in the case of mercury, nickel, palladium, cobalt, cadmium and platinum ions (Clyne, LaPointe et al. 2002; Lorca, Coddou et al. 2005). In contrast, mercury induces strong inhibition and nickel, manganese and gallium did not alter the function of the P2X4 receptor (Coddou, Lorca et al. 2005). It has been also shown that phosphoinositol-3-phosphate and phosphoinositol-2-phosphate augment the receptor functions by direct

binding to transmembrane domain of P2X4 (Bernier, Ase et al. 2008). Direct potentiation of P2X1 is also confirmed in the case of phosphatidylinositol 4.5-bisphosphate (Bernier, Ase et al. 2008). Pregnanolon is shown to inhibit P2X4 and alfaxolone is potentiating the receptor function, at the same time 17β-estradiol does not affect the receptor function (Codocedo, Rodriguez et al. 2009).

1.6 Gating and conductance of P2X receptor channels

The patterns of activation of P2X receptors differ among homotrimeric isoforms in relation to maximum current amplitude and desensitization velocity. (Figure 1.6) The permeability data for ions were initially available from calculating relative permeability from Goldman-Hodgkin-Katz equation. All P2X receptors are permeable to sodium, potassium, and calcium ions (Ding and Sachs 1999) but the permeability to Ca^{2+} varies widely (Evans, Lewis et al. 1996; Soto, Garcia-Guzman et al. 1996; Virginio, North et al. 1998) and may be an important determinant of the physiological role of P2X receptors (North 2002). A few also show significant chloride permeability, especially P2X5 (Thomas and Hume 1990; Ruppelt, Ma et al. 2001; Bo, Jiang et al. 2003). Furthermore, some P2X receptors also display a time-dependent modulation of ion selectivity and develop a new open state that permits relatively large cations and anions to traverse the pore (Cockcroft and Gomperts 1979; Steinberg, Newman et al. 1987; Tatham and Lindau 1990). This phenomenon is called pore dilatation and it will be discussed in detail. The first detailed characterization of P2X currents was performed in P2X2 isoform by single channel recording (Figure 1.5). The results of this study indicate that P2X receptors are flickering conductance channels where unitary conductance is very difficult to obtain because of very fast events of channel opening and closure which exceed the bandwidth of the recording equipment (Bean 1990; Wong, Burnstock et al. 2000). In excised membrane patches, the P2X2 demonstrates very fast rundown (Ding and Sachs 1999).

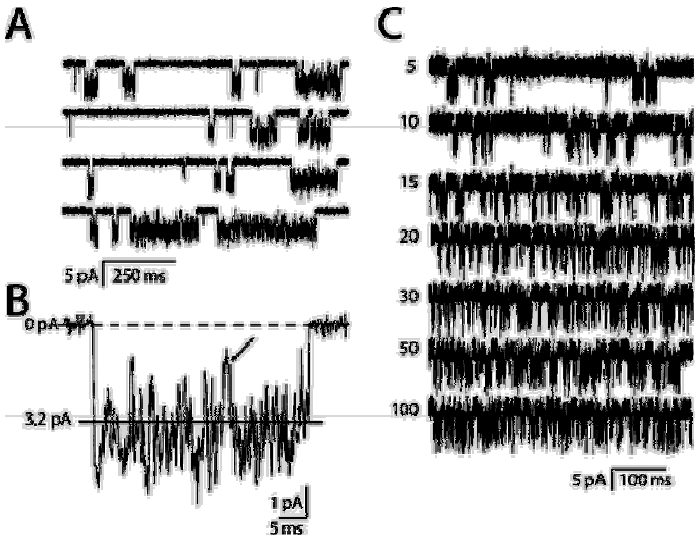

Figure 1.5 Single channel currents recorded from outside-out patches of membrane containing recombinant P2X2 receptors. (A) The channel opens in bursts in the presence of 1.5 μM ATP. (B) Each burst contains many poorly defined openings and closings. Subconductance levels may be present but are difficult to resolve (arrow). (C) The open channel probability is a function of the concentration of ATP (taken from Egan 2006.)

The inability to accurately measure the peak amplitudes of single channel currents, because of channel flickering, made a precise determination of conductance levels impossible. Instead, the average chord conductance measured at a holding potential of −100 mV was estimated from the Gaussian fit of the open component of the all-points histogram. Calculated in this way, the mean conductance was 32 pS (Ding and Sachs 1999). Other groups report single channel conductance that ranges from about 10–60 pS depending on membrane holding potential, identity of the subunit under investigation, and the composition of the extracellular solution (Krishtal, Marchenko et al. 1988; Nakazawa and Hess 1993; Evans 1996; Khakh, Humphrey et al. 1997; Zhou and Hume 1998; Wong, Burnstock et al. 2000; Whitlock, Burnstock et al. 2001). Apart from ATP gating, P2X2 channel demonstrates voltage-gating, making the discussion of P2X single channel currents more difficult (Fujiwara, Keceli et al. 2009; Keceli and Kubo 2009; Kubo, Fujiwara et al. 2009).

1.7 Desensitization of P2X receptors

Desensitization is the process of loosing channel activity in the actual presence of the ligand. This is physiologically very important molecular adaptation to excessive ligand activation and prolonged ligand presence in the extracellular environment. The process of desensitization is usually dose dependent, where greater doses of the ligand promote faster desensitization. Desensitization is characterized by the rate of current decrease in the continuous presence of agonist, which is calculated from the time constant(s) obtained by monoexponential or biexponential fitting of current decay. Sometimes the desensitization follows a biphasic decay indicating that it is a two-step process and it is facilitated trough the intermediary state. The desensitization kinetics of the P2X receptors vary widely (Figure 1.6). The P2X1 receptor, which is highly sensitive to ATP, desensitizes most rapidly within 300 ms and it does not resensitize even after long-term ATP washout (North and Surprenant 2000; North 2002). The P2X3 receptor desensitizes rapidly but after washout its amplitude could be 100% recovered (Sokolova, Skorinkin et al. 2004; Sokolova, Skorinkin et al. 2006). P2X6 is inactive in homomeric form because its trimeric assembly is inhibited (Ormond, Barrera et al. 2006) and the receptor is retained in the endoplasmic reticulum; it is proven to be a trafficking affected isoform (Bobanovic, Royle et al. 2002). Rat P2X5 is poorly responsive to ATP and it is slow desensitizing (tens of seconds) and the P2X2 and P2X4 receptors are desensitized in moderate rate (seconds). The P2X7 receptor doesn't desensitize and moreover demonstrates the pore dilatation property (Figure 1.6) (Virginio, MacKenzie et al. 1999). The mechanism of desensitization is little understood essentially because electrophysiological approaches including recording of single channels are limited by fast transitions into long living non-conducting states. During ultra-fast and short agonist application the unbinding rate of agonist (deactivation) and receptor desensitization are undistinguishable (Khmyz, Maximyuk et al. 2008). Although mutagenesis approaches already indicated multiple determinants of desensitization (Koshimizu, Koshimizu et al. 1999; Fabbretti, Sokolova et al. 2004; Fountain and North 2006), they were unable to provide essential insights into the mechanism of this phenomenon. A study in P2X3 suggests that molecular determinants of P2X receptor desensitization might be aromatic residues of the first transmembrane domain. When the conserved aromatic residues Y42

in P2X4 or Y37 in P2X3 are substituted with alanine residues the desensitization kinetics is prolonged (Jindrichova, Vavra et al. 2009; Jindrichova, Khafizov et al. 2010). It has been also suggested that the desensitization is the consequence of interactions at the interface between subunits. The negatively charged residue D266 was shown to promote the desensitization process (Fabbretti, Sokolova et al. 2004). Considering this, desensitization is a process which involves many amino acid residues within the P2X receptor molecule.

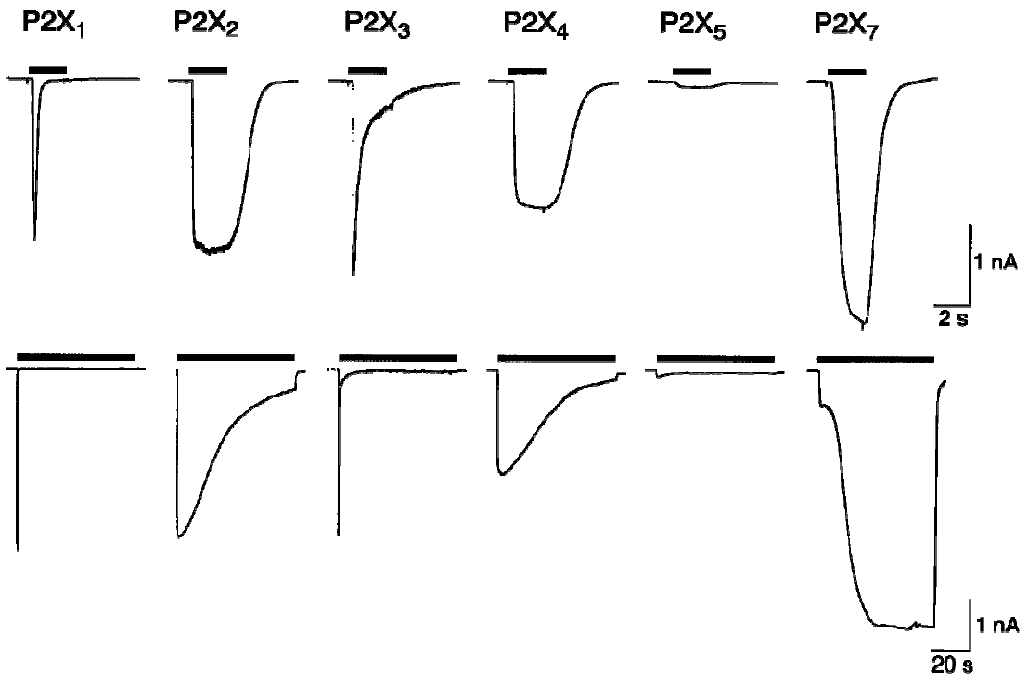

Figure 1.6 Comparison of desensitization patterns of the rat P2X receptors. Time courses of membrane current induced by application of supramaximal agonist concentration for 2 s (upper row) and 60 s (lower row) are shown. These patterns demonstrate that P2X1 and P2X3 exhibit faster desensitization, with a consideration that P2X3 can be fully re-sensitized and P2X1 not. The P2X4 and P2X2 have moderate desensitization rates and the P2X5 has significantly lower amplitude so that its desensitization cannot be determined. The P2X7 current shows the absence of desensitization but prominent pore dilatation after long-term agonist application. Application lines demonstrate 100 μM ATP, in the case of P2X7 100 μM BzATP. The P2X6 receptor homomer is nonfunctional (therefore not shown here). Taken from North 2002.

1.8 Pore dilatation of P2X receptors

Pore dilatation is a biophysical property of P2X receptors, representing the second, additional, open state of higher conductance (Cockcroft and Gomperts 1979; Steinberg, Newman et al. 1987; Tatham and Lindau 1990). It is a process of widening of the pore of the receptor channel to such extent that even a large molecule of a buffer such as HEPES or non-charged molecules can traverse the pore of P2X channel (Virginio, MacKenzie et al. 1999). Dilation can be studied electrophysiologically as a shift in reversal potential when large cation such as N-methyl-D-glucamine ($NMDG^+$) was added to the extracellular environment to substitute for the sodium ions (Figure 1.7). Upon dilatation in the presence of agonist and $NMDG^+$, the ramp protocol has demonstrated a shift in reversal potential (Yan, Li et al. 2008).

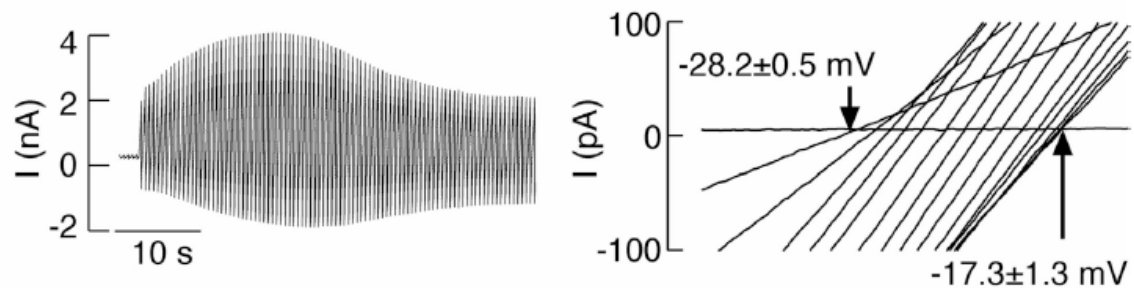

Figure 1.7. Voltage ramp protocol from -80mM to +80mV in the presence of NMDG+ upon long term (30s) ATP application demonstrates the rightward shift of reversal potential indicating the loss of selectivity and increase of $NMDG^+$ conductance. Taken from Yan 2008.

Pore dilatation is also confirmed by additional imaging studies when ATP-induced pore enlargement has resulted in increased permeability to a 4-[(3-methyl-2(3H)-benzoxazolylidene) methyl]-1-[3-(trimethylammonio)propyl] quinolinium diiodide or YO-PRO-1. YO-PRO-1 is a macrolid fluorescent dye which was detected in the cellular interior after prolonged application of P2X agonists (Virginio, MacKenzie et al. 1999). The molecular determinants of this process are yet unknown. Pore dilatation occurs in physiological conditions in case of P2X7 receptor after repetitive or long-term application of ATP (Yan, Li et al. 2008). Pore dilatation is also demonstrated in P2X5 and P2X2 receptor heteromers (Compan, Ulmann et al.). In the case of P2X4 receptor,

pore dilatation is a process which occurs in the absence of calcium ions (Shinozaki, Sumitomo et al. 2009). Pore dilatation was also studied by using atomic force microscopy (Shinozaki, Sumitomo et al. 2009) that revealed macromolecular rearrangements during the dilatation process of the rat P2X4 receptor (Figure 1.8). The minimum pore size of the dilated state of the P2X7 is estimated from the diameter of the largest polyethylene glycol that blocks the NMDG+ current and YO-PRO-1 uptake and found to be about 30–50 Å (Virginio et al. 1999).

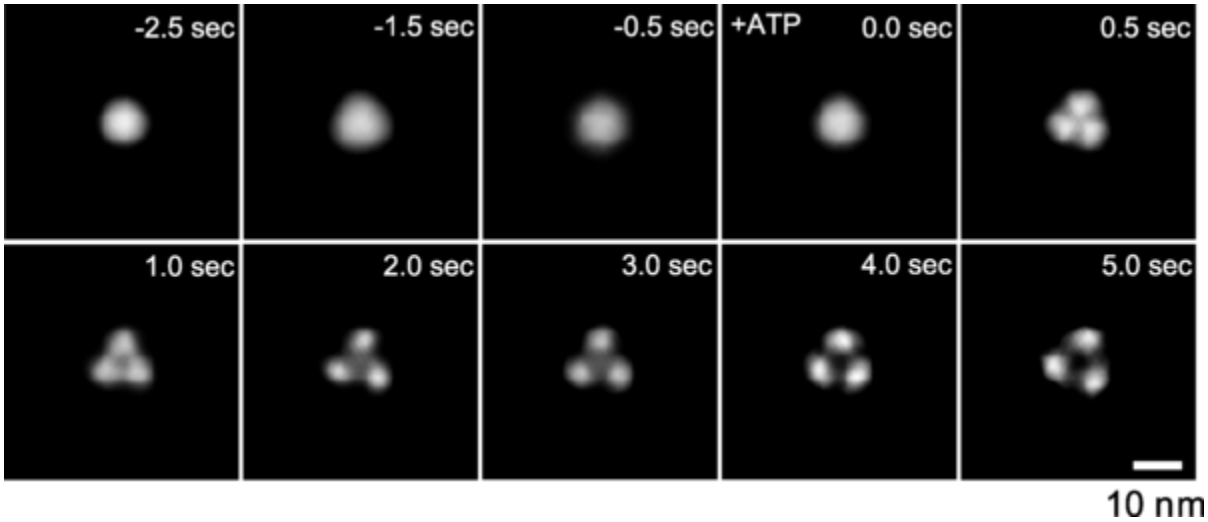

Figure 1.8 The atomic force microscopy images of the P2X4 receptors during ATP action. ATP induces two types of structural changes in P2X4 in the absence of calcium ions. After 2s or 3s of ATP action the channel opening and pore widening is identified but additional pore widening is seen after 5s which corresponds to pore dilatation process. Taken from Shinozaki 2009.

1.9 Heteromeric P2X receptors

The functional endogenously expressed P2X receptors are usually found in heteromeric form, meaning that the trimer composition is achieved by combining different isoforms of the receptor. The heteromerization is a process which *in vivo* occurs in nonstechiometric manner where combining of isoforms is governed on the level of gene expression (Zhao and Ennion 2006; Zhou, Luo et al. 2009). On the basis of co-immunoprecipitation the existence of several heteromeric forms has been postulated: P2X1/P2X3, P2X1/P2X6, P2X2/P2X5, P2X3/P2X5, P2X4/P2X5 a P2X5/P2X6 (Torres, Egan et al. 1999). The influence of composition of the heteromer on its activity was

investigated in recombinant receptors by employing co-transfection or concatamer construction. Extensive work using co-transfection has demonstrated the functionality and specificity of P2X1/P2X2, P2X1/P2X4, P2X1/P2X5, P2X2/P2X3, P2X2/P2X6 and P2X4/P2X6 heteromeric receptors (Lewis, Neidhart et al. 1995; Le, Babinski et al. 1998; Nicke, Baumert et al. 1998; Le, Boue-Grabot et al. 1999; King, Townsend-Nicholson et al. 2000; Brown, Townsend-Nicholson et al. 2002). Different heteromers can be also distinguished by their rate of desensitization and by different pharmacological properties (Surprenant, Schneider et al. 2000; Patel, Khakh et al. 2001; Rettinger, Braun et al. 2005).

1.10 Structure of P2X receptor

1.10.1 The experimental problem of solving the structure of P2X receptor

The structure of membrane proteins is difficult to study for a number of reasons. Because of high in-membrane anisotropy due to constrained molecular movement, NMR techniques are highly limited and of very low use for obtaining the structural data. X-ray crystallography has offered limited but yet significant understanding regarding the native structural folding of membrane proteins and it is applied with certain modifications to give information on structure-function relationship. The surface of membrane proteins is relatively hydrophobic and they can only be extracted from the cell membrane using detergents. They are also often flexible and unstable outside membrane environment. This leads to challenges at all methodological levels, including expression, solubilisation, purification, crystallization, data collection and structure solution which must be solved in such way to offer reliable results. Unlike soluble proteins, usually solubilized with buffers, hydrophobic membrane proteins need a detergent to solubilize and form adequate crystals for the diffraction analysis. Membrane proteins are extracted from the host cell membrane by the addition of detergents, which cover the hydrophobic surface of the protein, allowing solubilization. The choice of detergent is thus the most important step of the purification process. Usually a series of detergents are tested and the detergent that

extracts the largest quantity of soluble, active, homogeneous and stable protein is used, provided that the cost of the detergent is not limiting. The detergent dodecyl maltoside is often used to extract membrane proteins from the lipid bilayer as it is relatively cheap and can give stably solubilised membrane proteins (Prive 2007). Protein can subsequently be transferred into a variety of different detergents for crystallization trials.

1.10.2 Experimental strategy in solving P2X receptor structure

The P2X4 receptor is the first ligand-gated ion channel which crystal structure in both closed and open state is solved. Toshimitsu Kawate from the Goaux´s group has performed a functional expression, solubilization, purification and crystallization of zebra fish P2X4 receptor (zfP2X4 receptor) at a resolution of 3.1 Å. (Figure 1.9) (Kawate, Michel et al. 2009). The constructs used for crystallization were N- and C- termini cut until the minimal yet functional construct was obtained (ΔP2X4-A). The experimental strategy was further modification of ΔP2X4-A surface amino acid residues in order to prevent molecular heterogeneity resulting in differential glycosylation as well as prevention of non-native disulfide bond formation. To facilitate crystallization process single point mutations were made at positions C51F, N78K, N187R to make the surface of the molecule more charged and to promote crystallization in the presence of detergents. These modifications resulted in forming ΔP2X4-B receptor form. The ΔP2X4-A receptor used for crystallization had I_{max} 50-fold lower and the ΔP2X4-B receptor 100-fold lower than WT zfP2X4 receptor. Further research performed by Hattori from the Goaux group has resulted in polypeptide construct of zfP2X4 which has crystallized in both open and closed state (Hattori and Gouaux 2012). Recent crystal structures of zfP2X4 receptor with and without bound ATP, ΔP2X4-C and ΔP2X4-B2 apo, were solved at a resolution of 2.8 Å and 2.9 Å, respectively (Hattori and Gouaux 2012). The used construct ΔP2X4-C had restored C51 residue and several residues at the carboxy terminus in comparison to ΔP2X4-B. The crystallized ΔP2X4-C had similar gating and ATP binding properties as WT zfP2X4 receptor channel and its structure was solved at higher resolution of 2.8 Å (Figure 1.10 and 1.11). Since both of these structures,

ΔP2X4-C and ΔP2X4-B apo, were significantly altered in order to facilitate crystallization process, it is an open question how these structures can be correlated to widely experimentally used rat and human P2X4 receptors and other receptor isoforms.

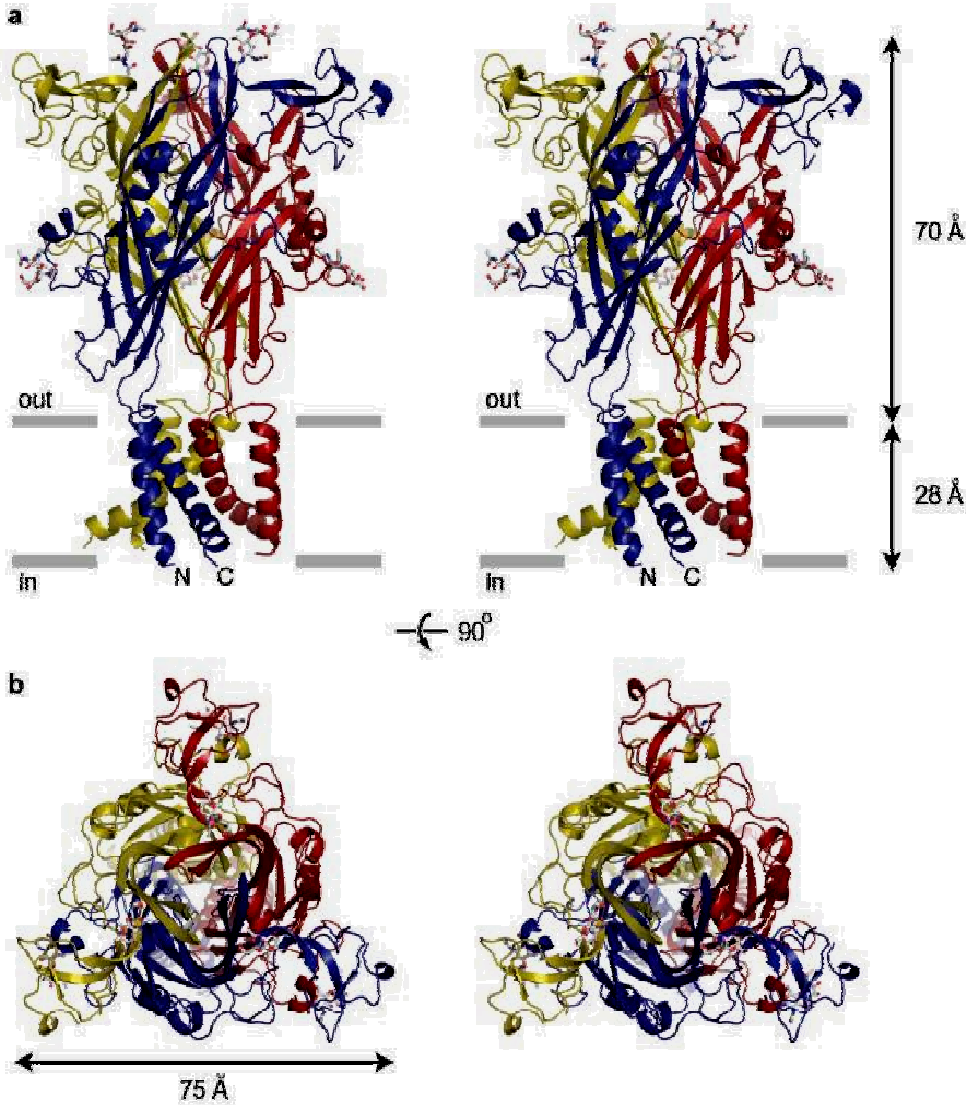

Figure 1,9 The stereoimages represent the trimeric organization of P2X receptor. (a) Lateral view of the receptor which consists of three subunits each one is made of two transmembrane domains linked by a large extracellular loop. (b) Top view reveals trigonal symmetry. Taken from Kawate 2009.

1.10.3 P2X receptor architecture

The homotrimeric receptor was found to have a chalice-shape, with large extracellular domain of 70 Å away from the membrane (Figure 1.9). Each subunit resembles a dolphin- like structure with a tale of a dolphin representing a transmembrane domains. The head of the dolphin represents a structurally stable form which contains three disulphide bonds. The left flipper and dorsal fin are involved in intersubunit contact while the body of the dolphin forms intramolecular vestibules (Figure 1.10 and Figure 1.11). The solved structures have demonstrated the existence of five disulphide bonds between conserved cysteine residues. Three bonds are present in the head domain (C119-C168, C129-C152, C135-C162; ΔzfP2X4.1 numbering), one bond is present in dorsal fin (C220-C230) and one bond is situated in lower part of the dolphin body very near to the postulated access portals for the ions. The disulphide bonding was an issue that was addressed already before the actual structure of zfP2X4 was available. Mutagenesis and electrophysiological study in human P2X1 receptor revealed the existence of disulphide bonding within all 10 cysteine residues (C117-C165, C126-C149, C132-C159, C217-C227 and C261-C270) (Ennion and Evans 2002). Another study done before confirmed only the connection between C115-C164, C214-C224 and C258-C266 in rat P2X2 receptor (Clyne, Wang et al. 2002). These studies did not provide explanations of the mechanisms by which the receptor function is altered by disruption of disulfide bonds and also failed to dissect the role of individual non-bound cysteine residues in the P2X receptor functions.

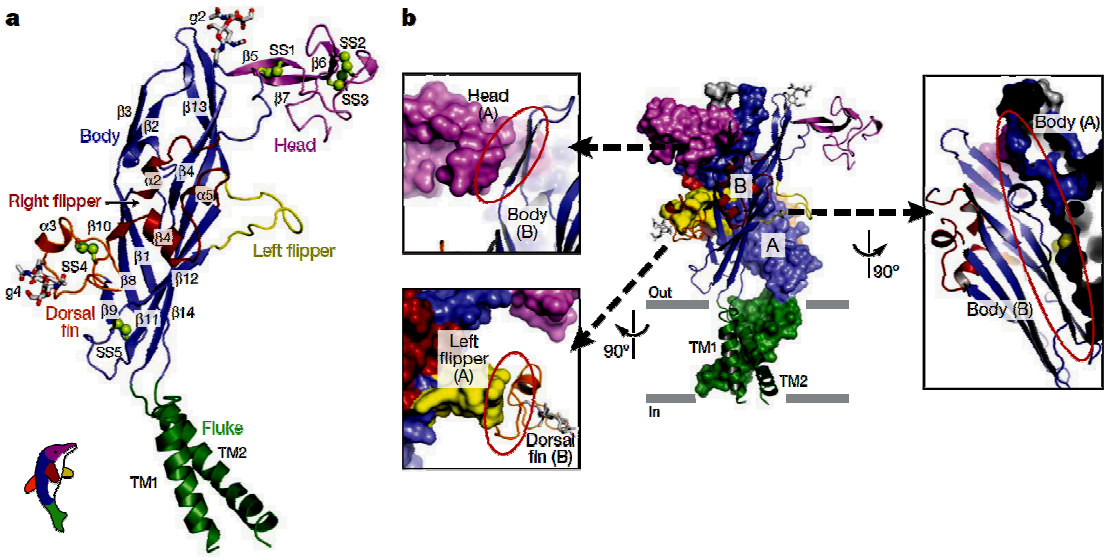

Figure 1.10 Dolphin-like architecture of zfP2X4 receptor. (a) The representation of a dolphin and zfP2X4 receptor structure reveals similarities green-tail, blue-body, yellow-left flipper, red-right flipper, orange dorsal fin and head group in mangeta. Particuar β-sheets (β1-14), gycosylation sites (g1-4) and disulfide bonds (SS1-5) are marked. (b) The postulated intersubunit interactions are maintained between left flipper and dorsal fin of adjacent subunits, head group and body segment of adjacent subunits and body-body interaction. Taken from Kawate 2009.

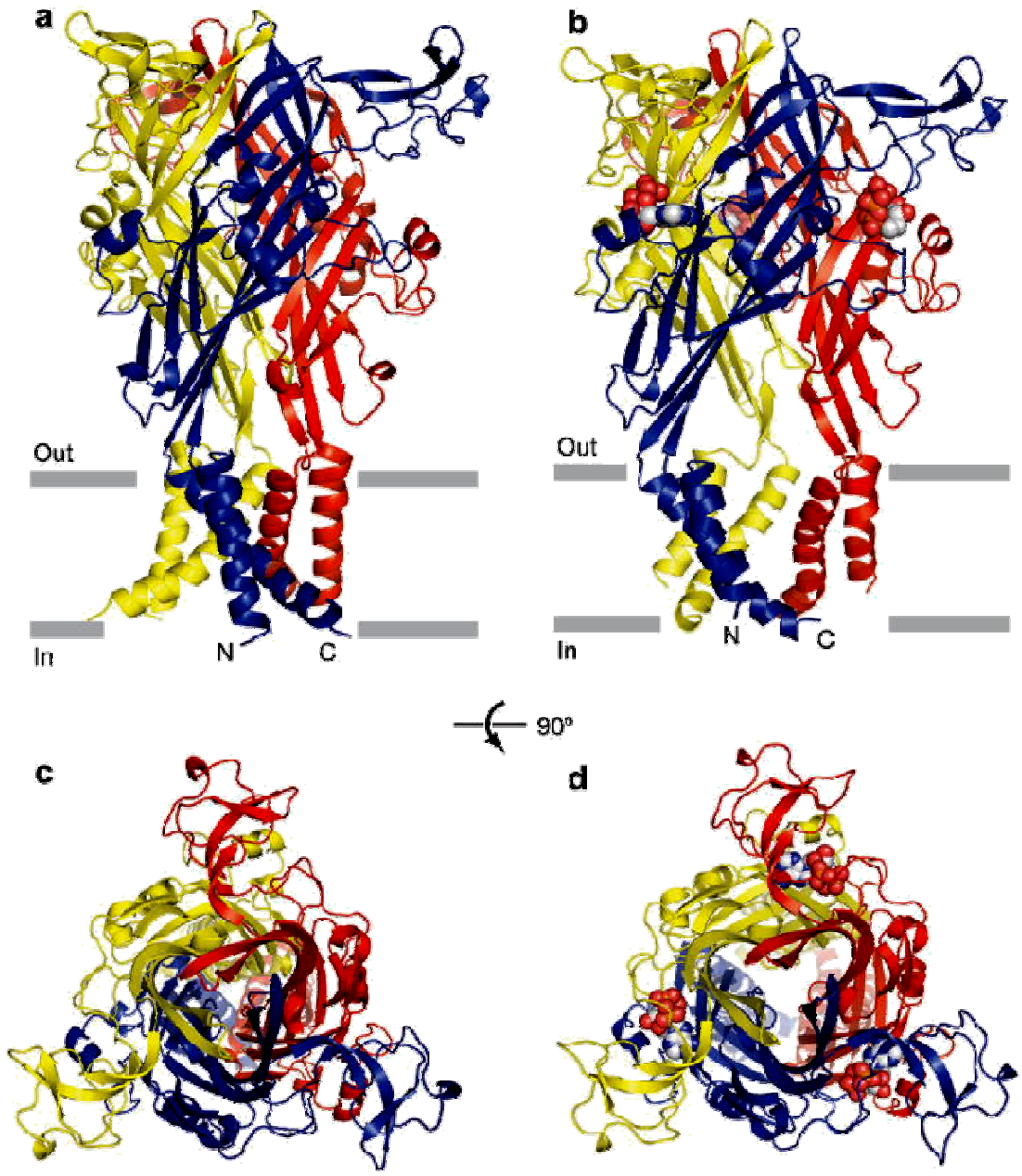

Figure 1.11 zfP2X4 receptor structure in open with bonded ATP (right) and closed (left) state reveals trimeric architecture, the widening of lateral fenestration just above the outer leaflet of the lipid bilayer and expansion of TM domains (a and b). ATP binding is facilitated on the interface between subunits in between the head group and dorsal fin (b and d). The widening of the pore is depicted along the central axes. (c and d) ATP molecule is presented in space filled model in right panel. From Hattori and Gouaux 2012.

The structure of zfP2X4 receptor also revealed the existence of extracellular vestibule with three lateral fenestrations trough which the ion entrance was initially postulated (Kawate, Michel et al. 2009) and later confirmed in functional studies. The ion access was confirmed to be facilitated by negatively charged amino acid residues E56 and D58 at the entrance point to the fenestration in human P2X4 (Kracun, Chaptal et al. 2010; Kawate, Robertson et al. 2011; Samways, Khakh et al. 2011). Furthermore, cysteine-scanning mutagenesis of P2X1 residues from E52 to G96 showed that all vestibule residues mutants are functional (Allsopp, El Ajouz et al. 2011), which is not a case of P2X2 (Jiang, Rassendren et al. 2000; Kawate, Robertson et al. 2011). The role of other residues, for example conserved (G328, K329, F330; P2X4 numbering) and positively charged amino acid residues in that region (K52, K326 and K329; P2X4 numbering) is still unclear. Extensive research in P2X2 receptor suggests that ion passes towards amino acid residues of the upper part of TM2 (I332, T336, T339) and several residues of TM1 (D15, F44, V48, Q37) after the entrance to the vestibule. These amino acids are responsible for ion permeation, but precise mechanism was not proposed (Stoop, Thomas et al. 1999; Jiang, Rassendren et al. 2001; Li, Chang et al. 2008; Kracun, Chaptal et al. 2010). The charged E51 residue in P2X4 was investigated in detail and confirmed that it is important for the permeation of calcium ion (Samways and Egan 2007; Samways, Khakh et al. 2012). The region of extracellular vestibule was also investigated in terms of facilitation of channel activation after agonist binding and it has been concluded that the region above TM2, which encompasses the residues from K313 to I333, is important for the signal transduction between ATP binding site and the pore (Yan, Liang et al. 2006).

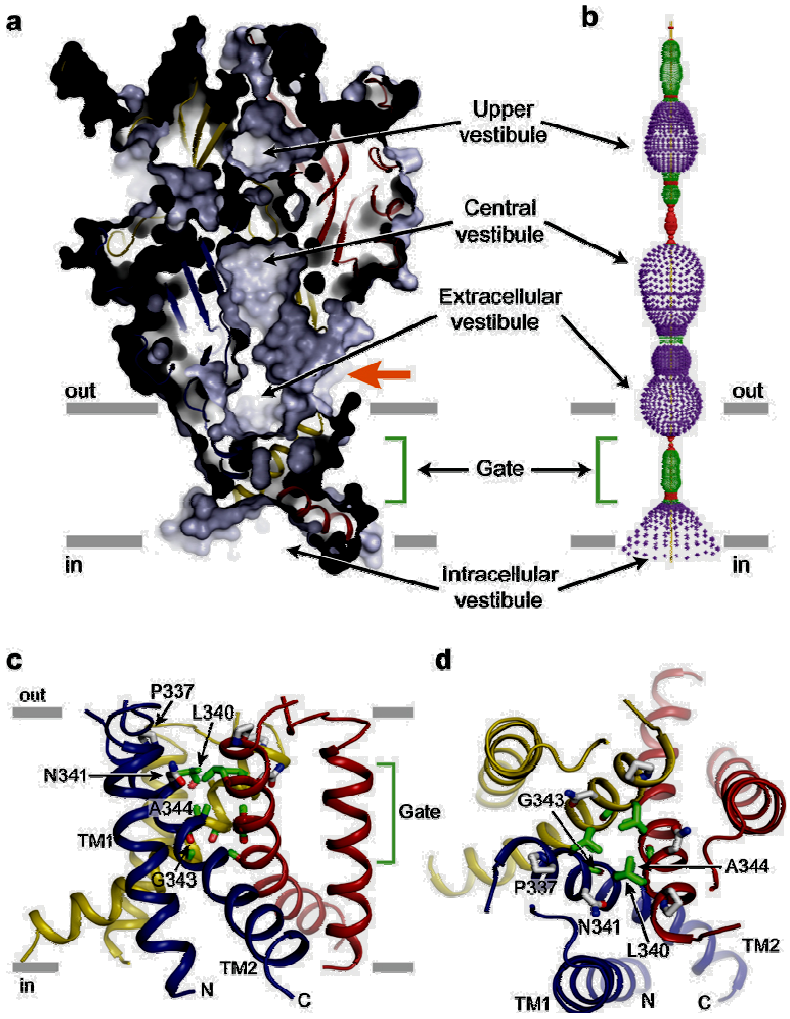

Figure 1.12 Surface representation of zfP2X4 receptor. (a and b) The structure reveals the existence of four intramolecular vestibules designated as upper, central, extracellular and intracellular vestibule. The activation of the receptor is postulated to lead towards widening of vestibules, (c and d) The architecture of the pore of closed zfP2X4 channel reveals the functionally important amino acid residues for gating and conductance. Taken from Kawate 2009.

1.10.4 Ligand selectivity and ligand binding mechanism of P2X receptor

ATP binding was the enigmatic problem throughout the years of P2X research, because the ATP binding motif that resemble to any of already solved structures of ATP binding proteins (canonical ATP binding) and tertiary homology was impossible to find. Different isoforms most surely differ in the affinity of ligand binding site, but a study on the actual

affinity has never been undertaken. The only data existing are the data evaluating the electrophysiologicaly investigated potency of ATP to activate the channel (Table 1.2). The work of Hattori has shown the structure of P2X receptor in apo-closed state and ATP-bound open state which gave a perfect structural insight to the mechanism of ATP binding (Hattori and Gouaux 2012). Most of conserved amino acid residues involved in ATP binding and identified earlier by means of single point mutagenesis and functional studies were confirmed by crystallization (Figure 1.13). These residues in zfP2X4 are: K70, K72, F188, T189, N296, F297, R298 and K316 and they form the ATP binding pocket of P2X receptor being responsible for phosphate group binding and hydrophobic interactions with purine ring (Ennion, Hagan et al. 2000; Jiang, Rassendren et al. 2000; Marquez-Klaka, Rettinger et al. 2007; Roberts and Evans 2007; Zemkova, Yan et al. 2007; Roberts, Digby et al. 2008; Bodnar, Wang et al. 2011). Further study confirmed that the binding of ATP occurs at the interface between adjacent subunits assuming that ATP stabilizes the P2X trimer (Marquez-Klaka, Rettinger et al. 2007). The investigation of the influence of Zn^{2+} ions on P2X receptor led to a conclusion that histidine residues near ATP binding site play role in binding the Zn^{2+} and thus facilitating ATP binding (Clyne, LaPointe et al. 2002; Nagaya, Tittle et al. 2005; Friday and Hume 2008).

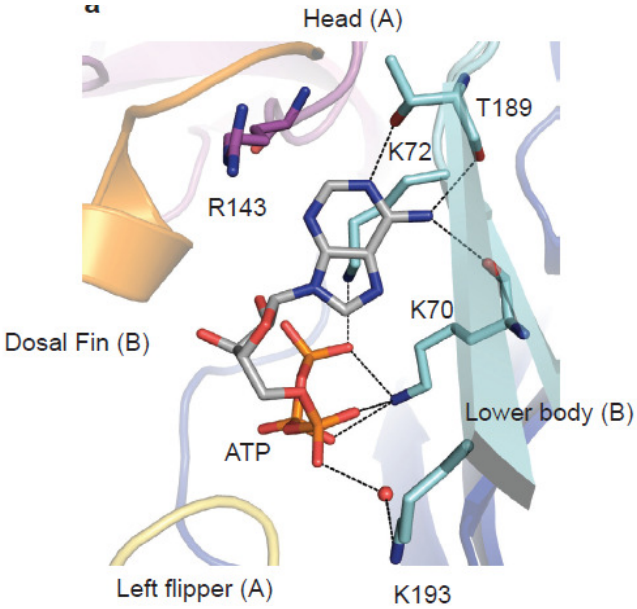

Figure 1.13 Structural considerations of ATP recognition within the binding pocket. The ATP molecule in bound state of zfP2X4 receptor is stabilized by polar interactions between adenine ring N-atoms and T189. The triphosphate group is stabilized by K70, K72, K193 and R143. Taken from Hattori 2012.

P2X receptors preferentially recognize ATP while cytidine-5′-triphosphate (CTP) has at most a weak effect on receptor activity (Soto, Garcia-Guzman et al. 1996; Garcia-Guzman, Soto et al. 1997; Garcia-Guzman, Stuhmer et al. 1997) and guanosine-5′-triphosphate (GTP) and uridine-5′-triphosphate (UTP) do not activate P2X receptors (Gever, Cockayne et al. 2006). CTP and ATP contain similar amidine functional groups within their base ring structures and on the basis of base superposition, CTP can form hydrogen bonds between the base N4 atom and the carbonyl oxygen atoms of K70 and possibly T189 (Figure 1.14). Because the citidine base is smaller than adenine, the N3 atom is too far from the side chain of Thr189 to form a hydrogen bond which stabilizes binding. The smaller cytidine base also is unable to fill entirely the agonist binding pocket and full hydrophobic interactions may not come to happen. This results in inability of CTP to activate P2X receptors because of lacking conformational movement. GTP and UTP have similar hydrogen bonding groups on their base rings but there is still no optimal number of hydrogen bonding interactions for proper binding with carbonyl oxygen atoms of Lys70 and Thr189, thus providing a chemical explanation for why GTP and UTP bind with low affinity to P2X receptors (Figure 1.14).

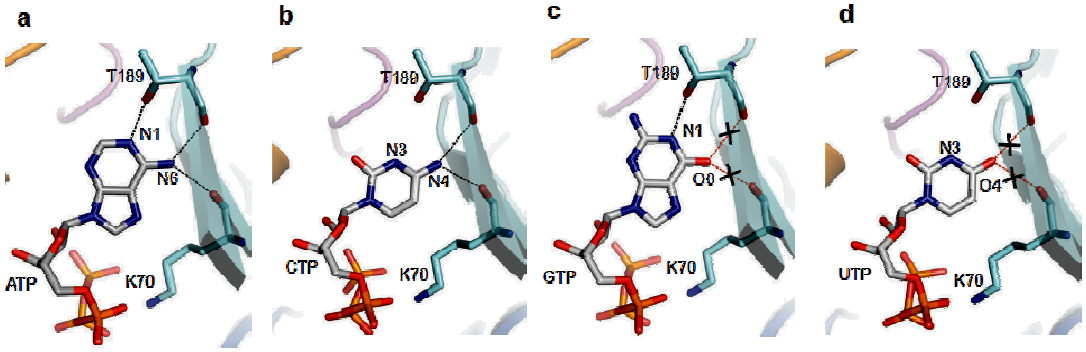

Figure 1.14 The structural hypotheses of nucleotide recognition selectivity. (a) Nucleoside triphosphates and residues involved in ATP binding are labeled and shown in stick representation. CTP (b), GTP (c) and UTP (d) are superimposed on the ATP binding site, and shown in sticks. Black dashed lines indicate possible hydrogen bonding interactions (<3.3 Å). Red dashed lines with black crosses indicate non-complimentary hydrogen bonding partners despite reasonable distances for hydrogen bonding (<3.3 Å). Taken from Hattori 2012.

1.10.5 The structural insights of P2X channel activation

The work of Hattori has (Hattori and Gouaux 2012) has proposed the series of movements in zfP2X4 molecule induced by ligand binding which lead to channel opening (Figure 1.15). It has been suggested that ATP binding promotes the closure of the cleft by the mechanism of induced fitting, between the head and dorsal fin domain. This is done in order to stabilize the ATP molecule via hydrophobic interactions, whereas ATP pushes out the left flipper from the ATP binding pocket (Figure 1.15 A and B). As a consequence the left flipper and dorsal fin which are interacting change their conformation and induce the outward flexing movement of the lower part of body segment which leads to widening of the extracellular vestibule. This widening is supposed to be crucial for up-taking the ion and further rotation of body segment by ~8° (Figure 1.15 C). This movement is responsible for the concomitant rotation of TM1 and TM2 by ~10° and ~55° , respectively, around axis perpendicular to the membrane lipid bilayer plane which represents the iris-like movement of TM1 and TM2 (Figure 1.15 D). The helices move away from the central axis by ~3 Å promoting the expansion of the narrowest pore segment. Molecular dynamics simulation of these movements was presented in other work, but the passage of ion was not simulated due to excessive need of computational strength (Du, Dong et al. 2012). Further experimental studies in rat P2X2 receptor proposed the mechanism of ATP binding by salt bridge switching from R290/E167 when channel occupies closed state to R290/ATP when channel is open and ATP bound (Hausmann, Gunther et al. 2013).

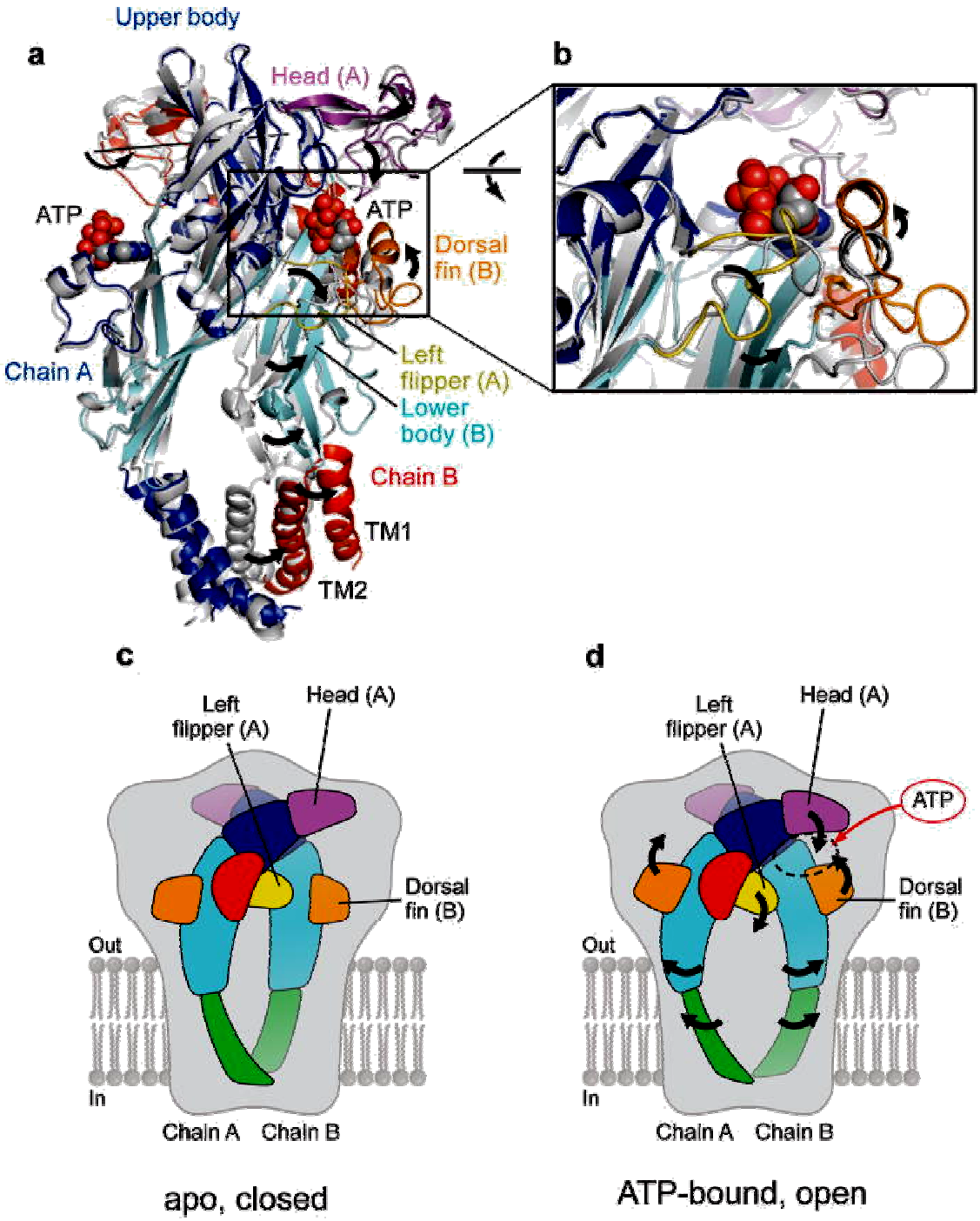

Figure 1.15 The proposed structural mechanism of P2X receptor activation. (a) Dorsal fin and head group polypeptide segments are involved in the (b) induced fitting during ATP binding. This induced fitting leads to the rotation of body segments (blue) and disruption of interaction between dorsal fin and left flipper (c and d). As a consequence of this the TM domains (green) change angle in relation to axis perpendicular to membrane and induce a iris-like motion leading to channel opening (c and d).

2. AIMS

The aim of this thesis is to gain the knowledge on several structure/function related aspects of the rat P2X4 (rP2X4) receptor. We used this receptor as a model because in neuroendocrine cells the P2X4 subtype is involved in control of neurotransmitter and hormone secretion by extracellular ATP. The crystal structure of P2X4 receptor subtype was already solved; both in closed and ATP-bound open state, and consequently the details about ATP binding site and the pathway for ions from outside to the pore are known. Combination of open and closed state also allowed proposing a mechanism of channel opening: ATP binding promotes cleft closure in an intersubunit-binding site, outward flexing of the lower body domain, expansion of the extracellular vestibule and increasing the separation of TMs. Nonetheless, the molecular and neurochemical mechanisms underlying these effects are far from being fully understood and likely involve multiple conserved residues and various non-conserved regions. The aim of this work is to understand the role of conserved cysteine bridges and extracellular vestibule residues in the P2X receptor function.

The specific tasks of this work are to:

1. Identify disulfide bridges in the ectodomain that are important for the function of rP2X4 receptor and provide mechanistic explanations of possible changes induced by disruption of these bonds. Additional task is to dissect between the functional importance of particular cysteine residues and disulphide bonds for the receptor function.

2. Find amino acid residues of the extracellular vestibule which are crucial for channel gating, signal transduction or vestibule widening and elucidate the role of identified amino acid residues by exploring the mechanisms of their interaction with their microenvironment in channel closed and open state.

3. MATERIALS AND METHODS

3.1 Experimental concept

Structure-function relationships in rP2X4 receptor were investigated by estimating the activity of WT P2X4 receptor and its artificially engineered mutants. Gene for rP2X4 receptor was subcloned into a pIRES vector containing gene for enhanced green fluorescent protein (EGFP) and kanamycin resistance gene which was important for further selection. The P2X4 receptor DNA cloned into vector was used as a template for generating single point and double point mutants in a nucleotide mismatch PCR reaction at a wanted specific site for amino acid substitution. Mutational process was verified by nucleotide sequencing and upon verification the DNA was amplified trough transformation of bacteria and subsequent isolation of amplified vectors from bacterial culture. Amplified vectors containing P2X4 mutant gene were used for either lipid or polymer based transfection of human embryonic kidney 293T (HEK293T) cells which are competent for functional expression of P2X4 receptor. Transfected HEK293T cells were investigated electrophysiologicaly by recording of ATP-induced current using patch clamp technique. Electrophysiological data were analyzed after digitalization, and topology of hits were discussed in terms of published crystallographic data.

3.2 Single point mutant preparation

3.2.1. Principle

cDNAs encoding the sequences of rP2X4 receptor and mutated subunits were subcloned into the pIRES2-EGFP vector (Clontech, Mountain View, CA, USA). Subcloned WT-P2X4 genes were provided to our laboratory by the courtesy of Dr. Stanko S. Stojilkovic. To generate the mutants, oligonucleotides (synthesized by VBC-Genomics, Vienna, Austria and Sigma Chemical Company, USA) containing specific point mutations were

introduced into the rP2X4/pIRES2-EGFP template using highly processive and highly accurate PfU Ultra DNA polymerase (Fermentas international Inc, USA). PCR products were used for transformation of TOP10 bacterial cell line by means of heat shock procedure and after transformation the bacteria were plated onto kanamycin LB agar for the selection of the bacteria expressing P2X4 mutant. After selection process, the transformants were sub-cultured in liquid LB medium and High-Speed Plasmid Mini Kit (Geneaid, Shijr City, Taipei County, Taiwan) was used to isolate the plasmids for transfection. Dye terminator cycle sequencing (ABI PRISM 3100, Applied Biosystems, Foster City, CA) was used to identify and verify the presence of the mutations. The sequencing was performed by the DNA Sequencing Laboratory, Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague.

3.2.2. PCR reaction

The template used for PCR reaction is a rP2X4 receptor gene in its WT form (rp2x4-wt). The forms which were intended for the fluorescent analysis of expression were prepared by using the EGFP tagged mutant (egfp-rp2x4-wt). The PCR primers were constructed in a length of 33-36 base pairs with a mutation on a site for a desired change in the amino acid type. The primers were purchased from VBC-Biotech, Vienna, Austria. The reaction mixture content is summarized in a table.

The component	Volume [µl]
10x *PfuUltra* buffer	5
template (300 ng/µl)	1
dNTP mixture (50 mM)	1.2
primer DOWN (100 pm/µl)	2
primer UP (100 pm/µl)	2
Deionized water	37.8
PfuUltra DNA polymerase	1

The PCR tube with a mix was vortex mixed and centrifugated in order to make the reaction mixture homogenated. It was than placed into the PCR Mastercycler (Eppendorf, Hamburg, Germany). The PCR protocol was the following:

Step	t[^{o}C]	t [min]
1. Denaturation	95	1
2. Annealing	55	1
3. Elongation	68	10
The steps from 1 to 3 were repeated 19 times		
4.Final elongation	68	12

After the termination of the PCR 1 µl of Dpn I enzyme was added to the mix in order to eliminate the template DNA. The tube was incubated at 37^{o}C for one hour. The mix was used for the transformation of the competent *E.coli* TOP10 cell line.

3.2.3. Transformation of bacteria

Chemicals

Liquid Luria-Bretani medium: 1% trypton *(ICN Biomedicals, Aurora, Ohio, USA)*, 0,5% yeast extract *(Serva)*, 1% NaCl (*Sigma-Aldrich)*. The medium pH was adjusted to 7.4 and sterilized in autoclave for 20 min at 121^{o}C. The medium was kept in refrigerator at 4°C.

Luria-Bretani agar with kanamycin: 1% trypton *(ICN Biomedicals, Aurora, Ohio, USA)*, 0,5% yeast extract *(Serva)*, 1% NaCl (*Sigma-Aldrich)*, 1,5-2,0% agar *(Difco, Detroit, USA).* The suspension was prepared in deionized water. The medium pH was adjusted to 7.4 and sterilized in autoclave for 20 min at 121^{o}C. After cooling to approximately 50^{o}C the kanamycin was added to the medium in a dose of 30 µg/ml. The medium was dispensed onto Petri dishes and kept in refrigerator (4°C).

The transformation is performed by applying a heat-shock to the mix of a PCR product and TOP10 competent *E.coli* culture. 50 µL of competent cells were mixed in vortex with 10 µL of PCR product and it was incubated on ice for 20-30 minutes. During that time, 1.5 ml of liquid Luria-Brtani medium was preheated in a thermoblock (Eppendorf, Hamburg, Germany). After 20-30 minutes on ice, the transformation mix was inserted into a thermoblock for 45s after which the mix was returned to the ice. In 2-3 minutes on ice, 170 µl of liquid Luria-Bretani medium was added to the mix and the incubation was continued at 37°C during 1 hour in the rocking thermoblock at 350 revolutions per minute. After the incubation, the mix was plated onto a kanamycin (30 µg/ml) Luria-Bretani agar plates and incubated in the incubator for 16-20 hours at 37^{o}C. After the growth of kanamycin resistant colonies, the single colonies were suspended into 5 ml of liquid LB mediun with added kanamycin (30 µg/ml). The liquid culture was incubated in 200 revolutions per minute mixer at 37 ^{o}C for 12-16 hours. The cell suspension was ready for the isolation of plasmids and DNA concentration measurement.

3.2.4. Plasmid isolation and DNA characterization

The isolation of plasmids was performed by using JETquick Plasmid Miniprep Kit (Genomed, Löhne, Germany). The cellular suspension in Luria-Bretani medium was centrifugated and the pellet was collected for further manipulation. The liquid was resuspended in 250 µl of G1 solution and 250 µl of G2 solution was added. The content of the test tube was gently mixed and 350 µl of G3 solution was added. The mixture was again gently stirred until achieving the homogeneity and centrifuged at 13000 revolutions per minute at room temperature for 10 minutes. The supernatant was applied to the silica column and centrifuged in column for 1 minute, at 13000 revolutions per minute. The column was than added 500 µl of GX solution and after its elimination by centrifuging (1 min, 13000 rpm) 500 µl more of G4 solution was added. The column was again centrifuged and at the end 60 µl of TE buffer preheated at 70°C in thermomixer was added. The final centrifugation was done and in this final volume of TE eluted DNA, the concentration of DNA was measured. The DNA concentration was measured by using NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc.).

3.3 Cultivation and transfection of HEK293T cells

Chemicals

Dulbecco Modified Eagle's Medium (Gibco)
Fetal Bovine Serum (Sigma-Aldrich)
Penicillin/Streptomycin in solution (Gibco)
Versene (Gibco)
Trypsin (Sigma-Aldrich)
Jet Prime Kit (PolyPlus, Ikreich, France)
Lipofectamine 2000 (Invitrogen,Carlsbad,CA)
Opti-Mem Medium (Gibco)

To express the recombinant channels, we used HEK 293T cells (American Type Culture Collection, Rockville, MD, USA) grown in Dulbecco modified Eagle's medium supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin in a humidified 5% CO2 atmosphere at 37°C. The cells were passaged by 1 min trypsin 0.25 mg/ml treatment followed by an addition of 5 ml of fresh DMEM medium and centrifugation at 500 rpm in a laboratory centrifuge. The cells were resuspended in 5 ml of fresh DMEM medium supplemented with 10% of FBS and penicillin/streptomycin and 100 μl of this suspension was plated onto the single plastic dishes. Alternatively, the cells were plated onto glass impregnated by poly-L-lysine by plating of 40 μl of cell suspension per glass. After reaching 80% of confluence the cells were transfected. Transfection was done using 2 μg of either WT or mutant receptor DNA with 2 μl of jetPRIME™ reagent in 2 ml of Dulbecco modified Eagle's medium, according to manufacturer's instructions (PolyPlus-transfection,Illkirch, France). Alternatively transfection was done by Lipofectamine 2000 (Invitrogen). For each culture dish the transfection mixture was prepared containing: 1 μl of DNA, 2 μl of Lipofectamine 2000 and 2 ml of Opti-MEM medium. The transfection mixture was

incubated for 20 minutes and the medium over the grown cells was substituted by the transfection mixture for 6 hours. After six hours the transfection mixture over the cells was discarded and fresh portion of cell medium was added. The transfection efficiency was identified by monitoring the fluorescence of EGFP using the Olympus IX71 inverted fluorescent microscope.

3.4 In situ fluorescent analysis of P2X receptor expression

Chemicals

10x phosphate buffered saline (1l deionized water, 1.37M NaCl, 27 mM KCl, 15 mM KH_2PO_4, 80 mM Na_2HPO_4)
Parafomaldehyde 2% solution in phosphate buffered saline
Vectashield (VectorLabs)

Transfected cells on poly-L-lysine glasses were first washed two times with phosphate buffered saline and than fixed with the solution of paraformaldehyde 2%. The incubation was lasting 15 minutes on ice. The solution was eliminated and the cells were washed two more times with phosphate buffered saline. The cells were further incubated on ice during 5 minutes and than been exposed to a growing concentration of ethanol (70%, 80% and than 95%) every solution for one minute. After drying the glass with the cells were inverted to a glass surface with a small drop of a Vectashield medium. The localization of EGFP marked receptors were estimated trough laser scanning confocal microscopy. Used excitation laser wavelength is 488 nm. The images were captured by using inverted microscope Leica SP2 AOBS (Leica Microsystems, Wetzlar, Germany) with 63X power and additional 2X zoom.

3.5 Western blot expression analysis of P2X receptor

Principle

To determine the amount of rP2X4 mutants that were membrane-bound, all membrane proteins were labeled by biotin using the Pierce® Cell Surface Protein Isolation Kit, according to the manufacturer's instructions (Thermo Scientific, Rockford, USA). Total protein samples were collected after cell lysis and centrifugation of cell debris. The membrane protein fraction was isolated from cell lysate by affinity column binding to avidin, followed by washing and elution in sodium-dodecyl sulphate sample buffer. Both fractions were subjected to sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemlli, transferred to polyvinyl-difluoride membrane, immunoblotted with an anti-rP2X4 receptor monoclonal antibody (Alomone Labs, Ltd. Israel) and detected using horseradish peroxidase coupled to anti-rabbit Fc-IgG antibody in a luminol assay. The luminescent signals were captured using a Luminescent Image Analyzer LAS-1000plus (Fuji Photo Film Co., Ltd. Japan).

3.6 Electrophysiological recording

Chemicals

Potassium Chloride (Sigma-Aldrich)
Calcium Chloride (Sigma-Aldrich)
Magnesium Chloride (Sigma-Aldrich)
Ethylene glycol-bis (2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA; Sigma-Aldrich)
4-(2-hidroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Sigma-Aldrich)
Sodium Hydroxide (Sigma-Aldrich)
Sodium Chloride (Sigma-Aldrich)
Cesium Chloride

Cesium Hydroxide
Potassium Hydroxide (Sigma-Aldrich)
Glucose (Sigma-Aldrich)
Hydrochloric acid (Sigma-Aldrich)
Adenosine -5´-triphosphate (Sigma-Aldrich)
Ivermectin (Sigma-Aldrich)

Patch electrodes were filled with intracellular solution containing (dissolved in deionized water, in mM): 154 CsCl, 11 EGTA. pH was adjusted to 7,4 with 1M CsOH solution. The osmolarity was 306-311 mOsm, was determined by vapor pressure osmometer (Model VAPRO 5520; Wescor, Logan, UT). The intracellular solution was stored in 1 ml aliquots at -80^{o}C and kept on ice during experiments. During recording cells were bathed and drugs were dissolved in extracellular solution containing (dissolved in deionized water, in mM):142 NaCl, 3 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 Glucose, 10 HEPES. pH was adjusted to 7,4 with 1M NaOH and the osmolarity was 290-300 mOsm. The pH was consistently checked in stored solution and if needed readjusted to pH 7,4. Cells were stimulated with ATP which was diluted in extracellular solution from the stock solutions at concentration of 100 mM. Ivermectin was dissolved in dimethyl-sulfoxide and stored in stock solutions of 20 mM at 5^{o}C and diluted to required concentrations in extracellular solution. A fresh stock solution of IVM was prepared at least once in a month.

Recordings

Electrophysiological recordings were performed on transfected HEK293T cells at room temperature using whole-cell patch-clamp recording techniques. ATP-induced currents were recorded from whole cells clamped to -60 mV using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Union City, CA). The recordings were done at a sampling rate of 2 kHz and Bessel filtered at 1 kHz. The data was captured by using the Digidata 1322A and pClamp9 software package. Glass electrodes were pulled using a Flaming Brown horizontal electrode puller (Model P-97; Sutter Instruments, Novato, CA) and polished by using a heat polisher (Model MF-830; Narishige, Tokyo, Japan) to a final

electrode resistance of 3-5 MΩ. As recording electrode Ag/AgCl wire was used and stored in sodium-hypochlorite, the reference electrode was Ag/AgCl pellet. Cells chosen for recording were similar in size and displayed similar properties, in terms of surrounding cells, cell shape and cytoplasmic granulation. During the experiments, the cell culture was continuously perfused with an extracellular solution at a flow rate of 2 ml/minute. The patch electrodes were filled with a cesium containing intracellular solution. The microelectrode tip was lowered into the solution and brought to the cell surface slowly to achieve first cell-attached configuration and than giga-seal configuration. This was done by using a motorized micromanipulator (Model MP-285; Sutter instruments, Novato, CA). Pressure and suction tube was connected to the side-porter of the pipette holder before the experiment and this system was used to apply the puls-pressure suction and achieve whole-cell configuration. Unless otherwise stated, ATP was applied for 2-10 s at different concentrations to evoke inward currents and one or two responses were recorded from one cell to prevent desensitization of the receptors. In some experiments, whole cell currents were measured by the preincubation of the cells with 3 μM IVM for 2-6 min. The control and ATP-containing solutions and IVM were applied via rapid (exchange time ~30-40 ms) superfusion system (RSC-200, BIOLOGIC, Claix, France). To estimate the concentration producing 50% of the maximal response (EC_{50}), the responses from different cells were pooled. Data points are presented as mean ± SEM from 5–35 cells.

3.7 Analysis of electrophysiological data

The concentration-response data points were fitted by a three-parameter logistic equation using a non-linear curve-fitting program that derives the EC_{50} values and Hill coefficient values of the produced curves. The equation is

$$y=I_{max}/[1+(EC_{50}/x)^{h}]$$

where y is the amplitude of the current evoked by ATP, I_{max} is the maximum current amplitude induced by 100 μM ATP, EC_{50} is the agonist concentration producing 50% of

the maximal response, *h* is the Hill coefficient which in all cases was fixed to a value of 1.3 (obtained by fitting of dose-response curve for WT-P2X4 receptor), and *x* is the concentration of ATP (SigmaPlot 2000 v9.01; SPSS Inc., Chicago, IL). The kinetics of current decay induced by the washout of agonists, deactivation, were fitted by a single exponential function,

$$Y=Aexp^{(-t/\tau_{off})}$$

where A is the current amplitude, and τ_{off} is the deactivation time constant. The current decay was fitted using Clampfit 10 Axon Instruments). All numerical values in the text are reported as the mean ± SEM. Significant differences ($p < 0.01$ and $p < 0.05$) between means were determined using SigmaStat 2000 v9.01 by employing Student's t-test or ANOVA.

3.8 Molecular model representations

All graphical representations of the protein structure were prepared using PyMOL software (DeLano Scientific LLC, USA), and the models were extracted from the Brookhaven Protein Data Bank under accession number 4DW1 for the zfP2X4 receptor in the ATP-bound open state and 4DW0 for receptor in the apo-closed state.

4. RESULTS

4.1 The role of disulphide bonding in activity and expression of rP2X4 receptor

The disulphidic bonding in rP2X4 receptor was investigated by estimating the activity of a series of single point and double point mutants. Double alanine or double threonine mutants were generated by introducing either two alanine or two threonines on the positions of each disulphide pair. In this way the specific role of disulphide bond was investigated. Single cystein residues were further substituted with alanine or threonine one by one in order to elucidate the role of particular cystein residues in rP2X4 receptor function and their importance in particular disulphide bond. Before estimating the WT and mutant receptor activity, the functional expression was checked by tagging the receptor with enhanced green fluorescent protein (EGFP).

4.1.1 The expression of WT and alanine mutants of cysteine residues of rP2X4 receptor

To study the functional roles of ten conserved cysteine residues forming disulfide bonds in the ectodomain of the rP2X4 receptor, these residues were substituted with alanine (A-mutants) or threonine (T-mutants), two residues of similar size to cysteine. Even tough the geometry of serine would be more appropriate for substitution threonine was chosen because it has similar size and polarity to cysteine. The WT and A-, T-mutants were expressed in HEK293T cells. At 24 h after transfection, their expression pattern was analyzed by laser scanning confocal microscopy.

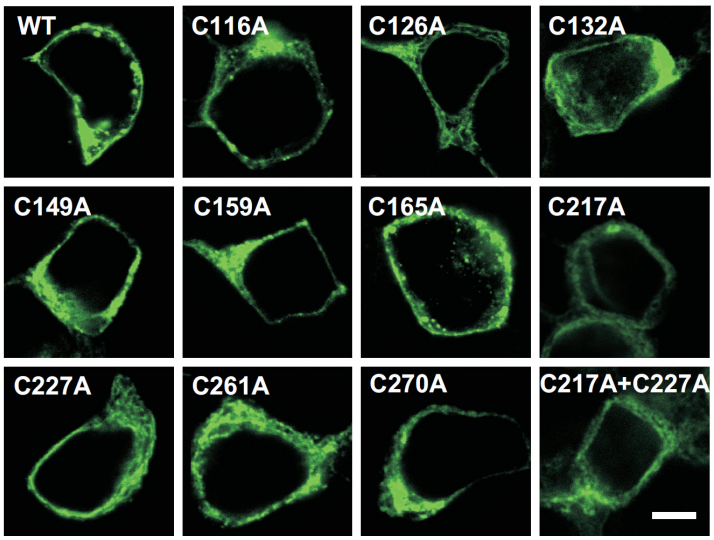

Figure 4.1 Expression and function of wild type (WT) and mutant rP2X4 receptor in the plasma membrane. The expression pattern of the WT receptor, ten conserved cysteine-to-alanine single mutants, and the C217A+C227A double mutant of the rP2X4 receptor in HEK293T cells.

Figure 4.1 shows no obvious difference in the localization of EGFP-tagged WT and A-mutants of the rP2X4 receptor as well as no difference in intensity of the signal suggesting that the expression was normal. The EGFP fluorescence was predominantly localized in the membrane region in the case of WT and all mutants (Figure 4.1).

4.1.2 The function of double substituted cysteine mutants

In experiments where both cysteines were substituted with threonines, the influence of disruption of SS bonds on receptor function was investigated. In this work, positive allosteric modulator ivermectin was used as a tool to estimate the effect of the mutation on receptor-channel binding/gating domain. Significant IVM rescue effect could indicate that mutants dysfunction is gating related. In the cells expressing the WT receptor, ivermectin treatment caused three types of changes in current response: it increased the sensitivity of the receptors to ATP by approximately eight-fold, as indicated by a leftward shift in the EC_{50} value; it augmented I_{max} by 1.7-fold; and it slowed the deactivation kinetics of the receptor by about 60-fold, as estimated by τ_{off} (Figure 4.2).

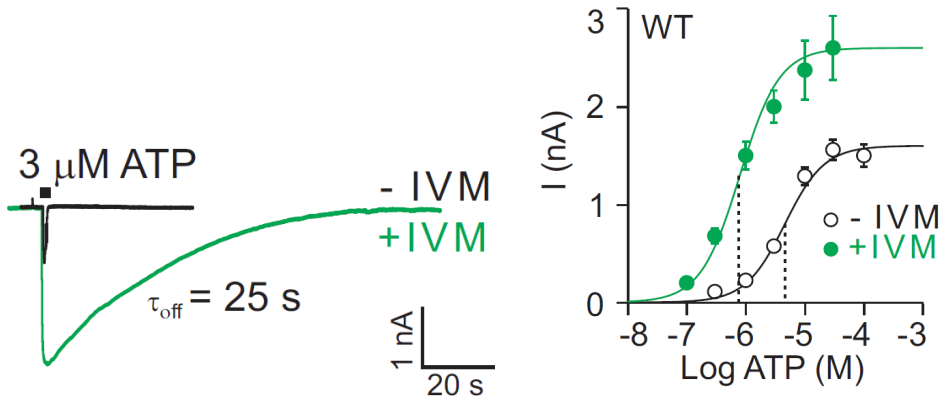

Figure 4.2 The effects of ivermectin (IVM) on ATP-induced current in HEK293T cells expressing P2X4 receptor-WT. Concentration dependence of ATP on the peak current amplitude in the absence (open symbols) and presence (closed symbols) of 3 μM IVM. (right) The vertical dotted lines represent the mean EC_{50} values. On left, a sample recording showing the effects of IVM on the peak amplitude and deactivation time (τ_{off}) of current induced by stimulation with 3 μM ATP (2-s pulse) is shown. The τ_{off} value is calculated in the presence of IVM.

The SS1 (C116-C165) double threonine mutant showed significantly lower ATP potency to open the channel, with its EC_{50} value increased 12-fold compared to that of P2X4 receptor-WT (Figure 4.3 and Table 4.1). Rapid channel deactivation was detected in the presence of IVM, but no effect on I_{max} was detected (Figure 4.4 A and Table 4.1).

The SS2 (C126-C149) double threonine mutant also showed a rightward shift in the ATP potency (EC_{50} value increased 14-fold) but exhibited a significant decrease in I_{max}, which was partly rescued with ivermectin treatment (Figure 4.3). The IVM-induced increase in the maximum current for this mutant was 3-fold higher, than in cells expressing the WT receptor (1.7-fold).

The replacement of the SS3 (C139-C159) cysteines with threonines did not affect receptor function in any observed parameter (Figure 4.3 and Table 4.1).

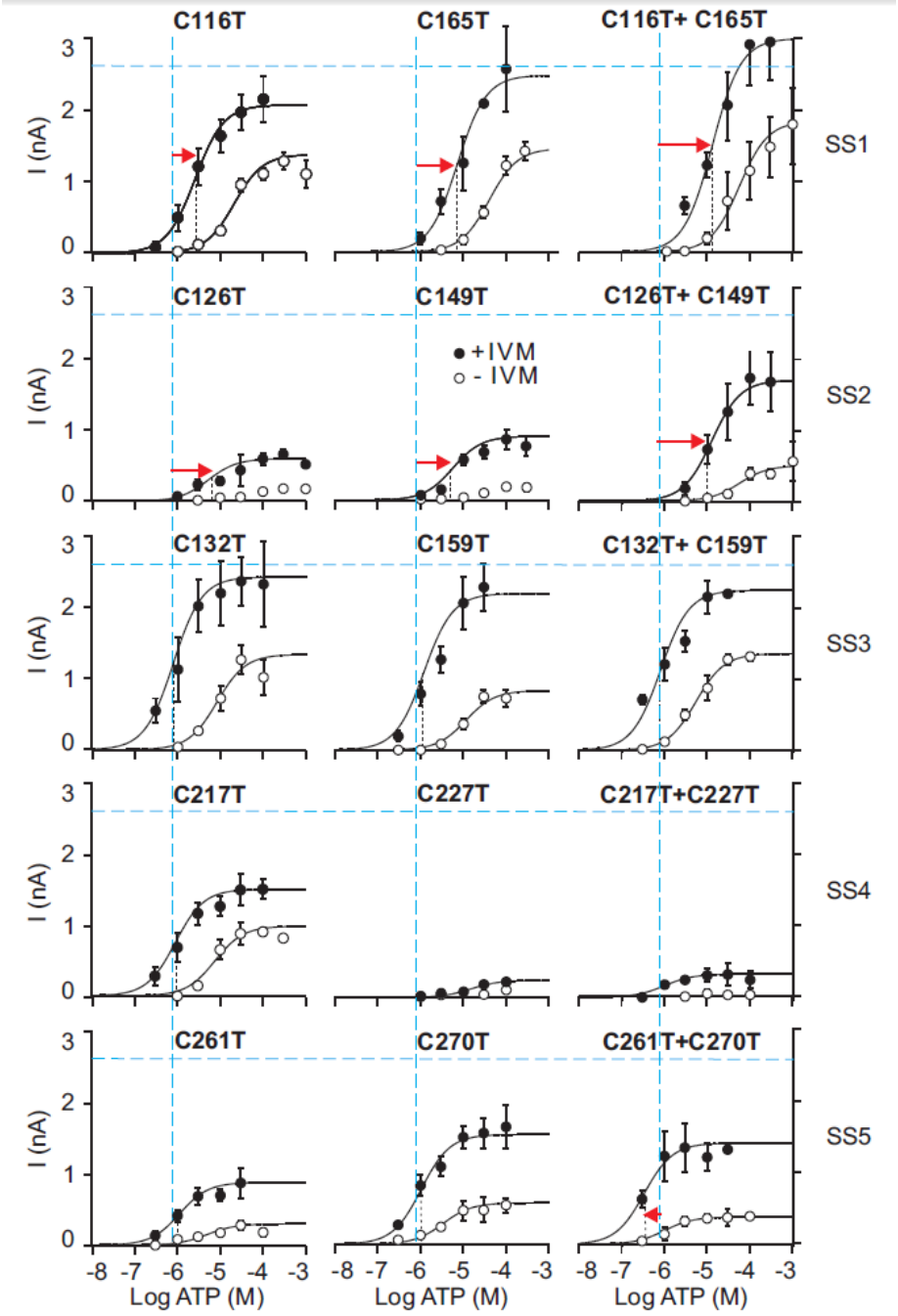

Figure 4.3 ATP dose-response curves for single and double threonine mutants of P2X4 receptor. Concentration dependence of ATP on the peak current amplitude responses in single-point mutants (left and middle panels) and double mutants (right panels) for five disulfide bonds is shown in the absence (open circles) and presence (closed circles) of 3 μM IVM. Each row shows paired cysteine mutants that form disulfide bonds (SS1-5). Vertical and horizontal dashed lines (in blue) indicate the EC_{50} and I_{max} values, respectively, for P2X4 receptor-WT in the presence of 3 μM IVM. The vertical dotted lines (in black) represent the mean EC_{50} values for T-mutants in the presence of IVM and significant shifts (red arrows).

Bond	P2X4 receptor	-IVM EC_{50} [μM]	+IVM EC_{50} [μM]	-IVM I_{max} [nA]	+IVM I_{max} [nA]	+IVM τ_{off} [s]
	WT	4.6±0.5	0.8±0.2	1.5±0.2	2.6±0.2	22±0.6
SS1	C116T+C165T	57±19*	13±4.4*	1.8±0.4	3.0±0.5	4.6±0.5*
SS2	C126T+C149T	65±24*	10+2.4*	0.6±0.3*	1.7±0.4*	7.4±0.6*
SS3	C132T+C159T	5.3±0.8	0.8±0.3	1.3±0.1	2.2±0.4	22.4±1.3
SS4	C217T+C227T	n. d.	n. d.	0.2±0.03*	0.3±0.1*	7.7±1.8*
SS5	C261T+C270T	1.7±0.8	0.3±0.1*	0.4±0.1*	1.4±0.4*	26±1.5

Table 4.1 Characterization of threonine double-point mutants of cysteine pairs SS1-SS5 in the rP2X4 receptor ectodomain. The EC_{50} values represent the ATP concentration producing a half-maximum effect, whereas 100-300 μM ATP was used to estimate I_{max} in both the presence and absence of ivermectin (IVM). The deactivation time constant (τ_{off}) was derived from mono-exponential fitting of the current decay after washout of 3 or 10 μM ATP in the presence of 3 μM IVM. n. d., these values could not be determined. $^{*}p < 0.01$ between mutant and wild type (WT) receptor.

Double mutation of the SS4 (C217-C227) cysteines resulted in a highly non-functional channel for which the EC_{50} value could not be determined, and the current amplitude was not augmented by IVM. This indicates the critical importance of either the SS4 bond or the individual cysteine residues in rP2X4 receptor function. SS4 is situated in the lower part of ectodomain and the second part of this thesis is focused on exploring the functional role of polypeptide segment which links ectodomain with transmembrane domains and comprises the extracellular vestibule. Similar to the SS1 and SS2 double mutants, the deactivation time constant estimated after the removal of agonist in the presence of IVM could be measured and it was significantly reduced in the SS4 double mutant (Table 4.1).

The SS5 (C261-C270) double mutant also responded with low peak current amplitude. After IVM treatment, however, the current value was partly rescued. The increase in I_{max} for this mutant was significantly higher (3.5-fold increase) than for the WT receptor, and the EC_{50} value was shifted slightly leftward (Figure 4.3 and Table 4.1).

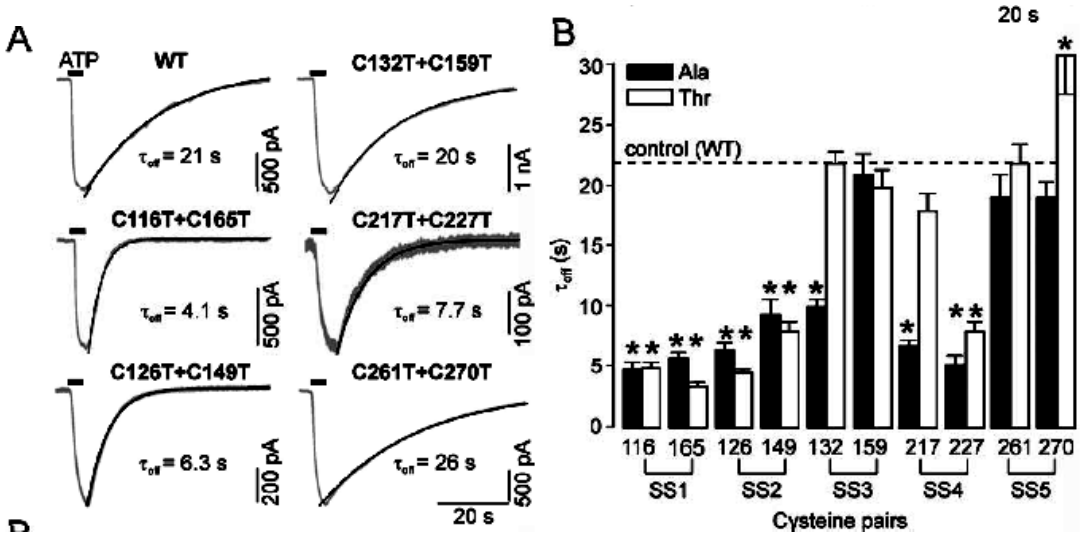

Figure 4.4 Effects of the disruption of disulfide bonds on the deactivation kinetics of P2X4 receptor in the presence of ivermectin. (A) Sample recordings of ectodomain cysteine double mutants showing differences in the current decay after washout of ATP. All traces were recorded 4-6 min after the application of 3 μM IVM, and a non-desensitizing concentration of ATP (1-3 μM for SS1-3 and SS5 and 30 μM for SS4) was used for stimulation. The time of ATP application is indicated by horizontal bars. Numbers below the traces indicate the deactivation time constant (τ_{off}) values. (B) Comparison of IVM effects on the deactivation time constant (τ_{off}) of A- (closed bars) and T- (open bars) single-point mutants of cysteine residues forming the disulfide bonds SS1-5. $^{*}p < 0.01$ between mutant and wild type (WT) receptor.

4.1.3 The function of substituted single cysteine residues

To evaluate the functional roles of individual cysteine residues in particular pairs all residues were replaced one by one by either threonine or alanine. As for the double SS1 mutants, single A- and T-mutants of cysteine 116 and 165 residues resulted in a significant decrease in ATP potency to activate the rP2X4 receptor, in both the presence and absence of IVM, without affecting I_{max} (Table 4.2). These changes were accompanied by faster receptor deactivation in the presence of IVM, after removal of agonist, indicating that disruption of SS1 was solely responsible for the observed decrease in receptor sensitivity to ATP (Figure 4.4 B). For three single point SS2 mutants, C126T, C149A and C149T, the I_{max} amplitude was much lower compared with the SS2 double and C126A single mutants, while the EC_{50} values could not be determined in the absence of IVM (Table 4.2). In the presence of IVM, the A- and T-mutants of cysteines 126 and

149 mimicked the effects of the double SS2 mutant on ATP sensitivity; a rightward shift in the EC_{50} was accompanied by faster deactivation, and the fold-increase of I_{max} current for all single mutants was higher (3- to 7-fold increase) than for the WT receptor (Figure 4.4 B). Receptor function was not obviously affected by the C132T, C159A and C159T mutation, confirming that disruption of SS3 bond alone does not significantly affect P2X4 receptor function. Furthermore, the C132A mutation resulted in the channel responding to ATP with a significantly smaller current, a rightward shift in the EC_{50} and faster deactivation time (Figure 4.4 B). These effects probably reflect the introduction of alanine at this position rather than liberating the C159, because the C132T mutant was fully functional, although non-native disulphide bonding with C159 could not be excluded.

Bonds	P2X4 receptor	-IVM EC_{50} [μM]	+IVM EC_{50} [μM]	-IVM I_{max} [nA]	+IVM I_{max} [nA]	+IVM τ_{off} [s]
	WT	4.6±0.5	0.8±0.2	1.5±0.2	2.6±0.2	22±0.6
SS1	C116T	21±4.1*	2.5±0.4*	1.3±0.1	2.3±0.4	4.9±0.5*
	C116A	27±6.5*	5.9±1.6*	1.0±0.1	2.2±0.3	4.7±0.7*
	C165T	45±3.8*	9.2±2.7*	1.6±0.2	2.6±0.4	3.4±0.3*
	C165A	42±8.9*	5.6±1.8*	1.5±0.2	2.3±0.6	5.7±0.5*
SS2	C126T	n. d.	5.3±1.7*	0.2±0.02*	0.6±0.1*	4.5±0.3*
	C126A	24±5.6*	3.7±1.0*	0.6±0.1*	2.0±0.5	6.4±0.6*
	C149T	n. d.	5.6±2.6*	0.2±0.07*	0.9±0.1*	8.0±0.8*
	C149A	n. d.	3.1±0.9*	0.1±0.02*	0.7±0.1*	9.3±1.3*
SS3	C132T	8.2±2.1	0.9±0.1	1.3±0.2	2.4±0.4	22±1.0
	C132A	14±4.1	1.8±0.5*	0.3±0.1*	0.8±0.2*	10±0.6*
	C159T	12±4.1	1.2±0.5	0.8±0.1*	2.3±0.3	20±1.5
	C159A	5.1±1.4	0.9±0.4	0.9±0.2	1.7±0.4	20±1.7
SS4	C217T	7.2±2.9	0.9±0.1	0.9±0.2	1.5±0.2*	18±1.5
	C217A	n. d.	n. d.	0.1±0.05*	0.3±0.1*	6.7±0.5*
	C227T	n. d.	n. d.	0.1±0.05*	0.2±0.05*	4.9±0.9 *
	C227A	n. d.	n. d.	0.1±0.04*	0.3±.0.1*	6.3±1.1*
SS5	C261T	n. d.	1.1±0.4	0.3±0.1*	0.9±0.2*	22±1.6
	C261A	5.5±1.7	0.8±0.4	1.6±0.3	2.7±0.7	19±2.0
	C270T	3.4±1.8	0.9±0.3	0.5±0.2*	1.6±0.2*	31±3.2*
	C270A	n. d.	1.0±0.2	0.2±0.03*	1.1±0.2*	19±1.4

Table 4.2 Characterization of alanine (A) and threonine (T) single mutants of ten cysteine residues forming disulfide bonds SS1-5 in the rP2X4 receptor ectodomain. Arginine (R) variants of cysteines 217 and 227 were also tested. Each receptor was examined in the presence (+IVM) or absence (-IVM) of 3 μM IVM. n.d., these values could not be determined; n. f., nonfunctional receptor. $^{*}p < 0.01$ between mutant and WT receptor.

The devastating effects of replacement of the SS4 cysteine pair with threonine were completely mimicked by C217A, C227A and C227T mutations; these mutants showed a low response to ATP or were nonfunctional, and receptor function could not be rescued by IVM (Table 4.2). In contrast, the peak current amplitude and ATP sensitivity was not significantly affected by replacing cysteine 217 with threonine. We further generated C217L, C217E, C217R and C227R mutants. Three mutants were non-functional and the peak current amplitude and ATP sensitivity were not significantly affected when cysteine 217 was replaced with arginine (the EC_{50} value was 9.2±2.3 μM and 1.9±0.8 μM, the I_{max} was 1.2±0.3 nA and 2.7±0.4 nA before and after IVM treatment, respectively). In six of the eight SS4 single-point mutants, I_{max} was significantly reduced. This reduction probably did not reflect inefficient trafficking of the mutants to the plasma membrane because double point A-mutants of SS4 were present in the membrane. As for the double SS4 mutant, the deactivation kinetics, but not the EC_{50} value, could be determined for C217A, C227A and C227T mutants in the presence of IVM. Their low τ_{off} value indicated that these mutants have reduced sensitivity to ATP (Table 4.2). The deactivation kinetics of the C217T and C217R mutant was comparable to those of WT-P2X4 receptor. A decrease in the peak current amplitude observed in cells expressing the SS5 double mutant was mimicked by single point mutants C261T, C270T and C270A (Table 4.2), and the rescue effect of IVM on I_{max} was also higher (3- to 7-fold increase) than for the WT receptor (1.7-fold increase). No changes in the sensitivity to ATP were observed in the single SS5 mutants, and the deactivation time constant of the C270T mutant was higher compared to the WT receptor (Figure 4.4 B). In contrast, the C261A mutant was fully functional.

4.2 The role of amino acid residues comprising the extracellular vestibule in rP2X4 receptor functions

Since both SS4 and SS5 disulphide bonds, which disruption strongly influenced receptor function, are in the lower body of the P2X4 receptor ectodomain, further analysis was focused on the importance of the region which links the transmembrane domains with the ectodomain, the extracellular vestibule..

4.2.1 The functional identification of amino acid residues comprising extracellular vestibule of rP2X4 receptor

To study the functional importance of the amino acid residues of the extracellular vestibule and lateral fenestrations of the rP2X4 receptor, the scanning mutagenesis of amino acid residues V47–V61 and F324-N338 was done. Both alanine- and cysteine-scanning mutagenesis were performed to provide an adequate comparison to the work performed previously (Stoop, Thomas et al. 1999; Jiang, Rassendren et al. 2001; Jiang, Kim et al. 2003; Li, Chang et al. 2008; Kawate, Robertson et al. 2011; Samways, Khakh et al. 2011). WT and mutant receptors were expressed in HEK293T cells, and ATP-induced currents were measured 24-48 h after transfection using the whole-cell patch clamp method. The results are summarized in Fig. 1 and Tables 1 and 2 as the mean ± SEM for EC_{50} and I_{max} evoked by 100 μM ATP.

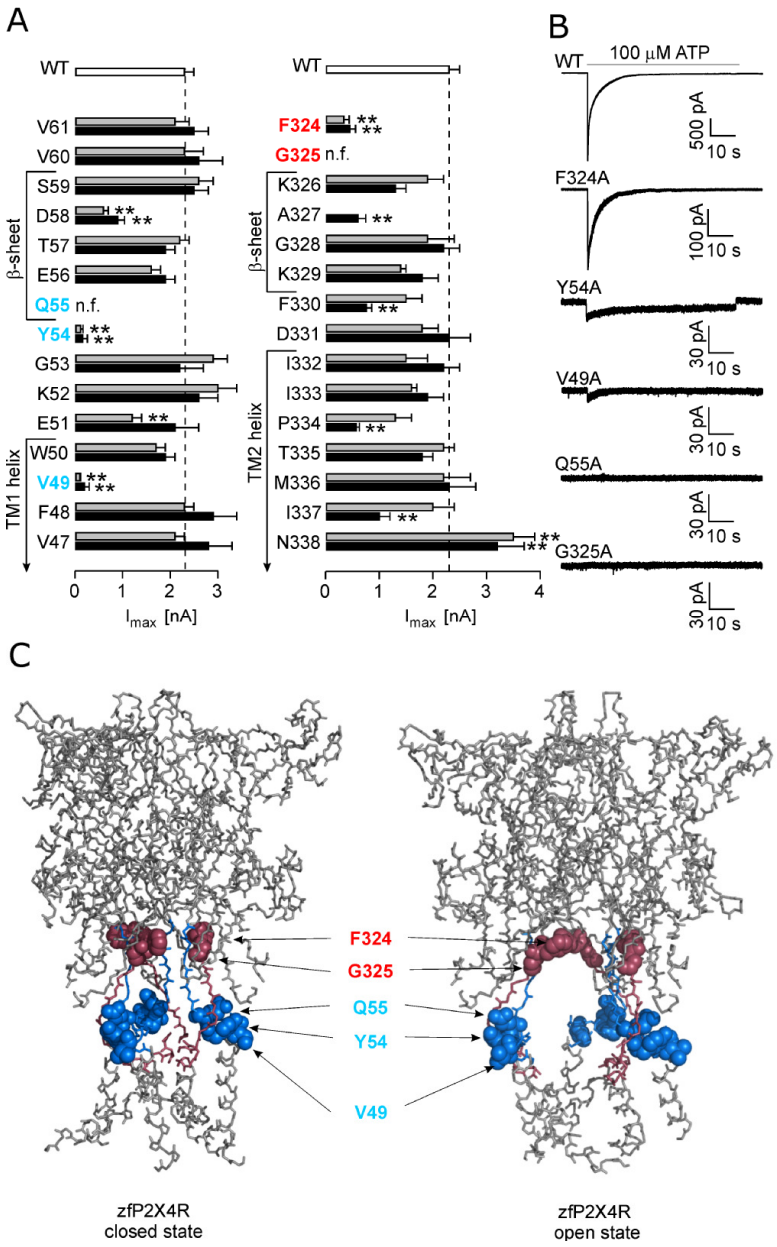

Figure 4.5 Effect of alanine and cysteine point mutations on maximum current amplitude. (A) Alanine and cysteine scanning mutagenesis of residues V47-V61 and F324-N338 that contain the upper parts of the TM1 and TM2 helices and the β-sheets in the open state. The maximum amplitude of the ATP-induced currents (I_{max}) in the wild type (WT; white bars) and cysteine (dark bars) and alanine (gray bars) mutant receptors. The receptors most affected had mutations at positions V49, Y54, Q55 (blue), F324 and G325 (red). (B) The 100 μM ATP-induced currents by the WT and low-responsive rP2X4 receptor alanine mutants. (C) The topology of low-active residue mutants in the zfP2X4 receptor in the apo-closed state (left) and ATP-bound open state (right); the mutated regions containing the upper parts of the TM1 and TM2 are shown in blue and red, respectively; affected mutated residues (rP2X4 numbering) are shown in red and blue spheres.

P2X4	EC_{50}[μM]	I_{max} [nA]
WT	2.3±0.4	2.3±0.2
F324A	1.1±0.8	0.34±0.10**
F324C	0.6±0.9	0.27±0.05**
F324L	2.9±1.1	0.9±0.1**
F324Y	4.5±1.7	0.8±0.1**
F324W	8.9±2.7	2.7±0.5
G325A	n.f	<0.1**
G325C	n.f	< 0.1**
G325P	3.0±1.3	1.7±0.2
K326A	1.7±0.6	1.9±0.3
K326C	2.4±0.6	1.3±0.2**
A327C	1.7±0.6	0.6± 0.2**
G328A	2.6±0.9	1.9± 0.5
G328C	2.7±0.6	2.2± 0.3
K329A	4.9± 1.5	1.4±0.1
K329C	4.0±1.2	1.8±0.3
F330A	3.2±1.2	1.5±0.3
F330C	4.0±1.2	0.8± 0.1**
D331A	1.5±0.2	1.8±0.3
D331C	2.1±0.2	2.3±0.4
I332A	1.3±0.3	1.5±0.4
I332C	1.6±0.3	2.2±0.3
I333A	2.9±0.8	1.6±0.1
I333C	2.8±1.1	1.9±0.3
P334A	1.0±0.3*	1.3±0.3
P334C	1.4±0.4	0.6±0.06**
T335A	1.8±0.6	2.2±0.2
T335C	2.6±0.5	1.8±0.2
M336A	2.5±0.3	2.2±0.5
M336C	1.2±0.2	2.3±0.5
I337A	2.9±1.3	2.0±0.4
I337C	4.1±0.6	1.0±0.2**
N338A	2.0±0.2	3.5±0.4**
N338C	1.1±0.2	3.2±0.5**

P2X4	EC_{50}[μM]	I_{max} [nA]
WT	2.3±0.4	2.3±0.2
V47A	5.2±0.7*	2.1±0.2
V47C	2.3±0.7	2.8±0.5
F48A	2.4±0.3	2.3±0.2
F48C	2.0±0.9	2.9±0.5
V49A	n.d.	0.04±0.01**
V49C	n.d	0.21±0.13**
V49D	1.6±0.4	1.7±0.3
V49W	2.7±0.4	2.3±0.3
V49L	1.6±0.4	3.1±0.4
W50A	3.6±0.3	1.9 ±0.2
W50C	4.4±0.8	2.2±0.4
E51A	1.6±0.6	1.2±0.2**
E51C	3.5±0.5	2.1±0.5
K52A	3.1±1.1	3.0±0.4
K52C	2.4±0.6	2.6±0.4
G53A	2.8±0.6	2.9±0.3
G53C	3.8±0.5	2.2±0.5
Y54A	n.d.	0.12±0.06**
Y54C	n.d.	0.16±0.10**
Y54W	6.3±2.6	1.6±0.2
Y54F	2.7±0.5	2.4±0.5
Y54L	n.f.	< 0.1**
Q55A	n.f.	< 0.1**
Q55C	n.f	< 0.1**
Q55T	n.f.	< 0.1**
Q55N	n.f.	< 0.1**
Q55E	n.d.	0.16±0.05**
Q55K	n.f.	< 0.1**
E56A	2.0±0.9	1.6±0.2
E56C	3.9± 0.7	1.9±0.2
T57A	1.9±0.6	2.2±0.3
T57C	2.0±0.3	1.9±0.2
D58A	3.2±1.3	0.6±0.1**
D58C	2.3±0.9	0.9±0.1**
S59A	2.9±1.3	2.6±0.4
S59C	2.0±0.5	2.5±0.3
V60A	2.4±0.6	2.3±0.4
V60C	2.1±0.8	2.6±0.5
V61A	2.9±0.4	2.1±0.3
V61C	3.7±1.5	2.5 ±0.3

Table 4.3 Alanine- and cysteine-scanning mutagenesis of the V47-V61 rP2X4 receptor segment and F324-N338 segment. In this table the potency is expressed as the ATP concentration producing 50% of the maximal response (EC_{50}), and the efficacy as the maximum induced current (I_{max}) in response to 100 μM ATP. n.d., not determined; n.f., nonfunctional; ** $p < 0.01$ between mutant and WT; $^{*}p < 0.05$ between mutant and WT.

Compared to the cells expressing WT receptors, the E51A, D58A, D58C, A327C, F330C, P334C, I337C, N338A and N338C mutants exhibited significant changes in the I_{max} values (Figure 4.3A). The V47A mutant also exhibited significant change in the EC_{50} value for ATP compared to WT receptor (Table 4.3). Because the I_{max} values for the I337A, P334A, F330A, and E51C mutants and the EC_{50} value for the V47C mutant were comparable to WT receptor, and E51A, D58A, D58C, A327C, F330C, and I337C mutants were characterized previously so these mutants were not studied further.

Substitution of five residues (V49, Y54, Q55, F324 and G325) with both alanine and cysteine resulted in a low functional or non-functional receptors, that responded with I_{max} amplitudes less than 20% of that observed in the WT receptor (Figure 4.5 A and B) and their EC_{50} could not be determined, except for F324 mutants (Table 4.3). The V49, Y54 and Q55 residues proximal to the TM1 are situated at the bottom of the lateral ion access portal. In contrast, the F324 and G325 residues are found at the top segment of the lateral fenestration, at the level of conjunction between the central and extracellular vestibule. In further work, the focus was driven on these five residues.

4.2.2 Role of V49 residue in receptor trafficking

The unique position of V49, Y54, Q55, F324 and G325 residues in the rP2X4 receptor could indicate their relevance in gating but could also reflect altered trafficking of mutant receptors. To test the second hypothesis, the quantitative Western blot analysis was performed by using membrane fractions and total protein derived from HEK293T cells expressing alanine mutant and WT receptors.

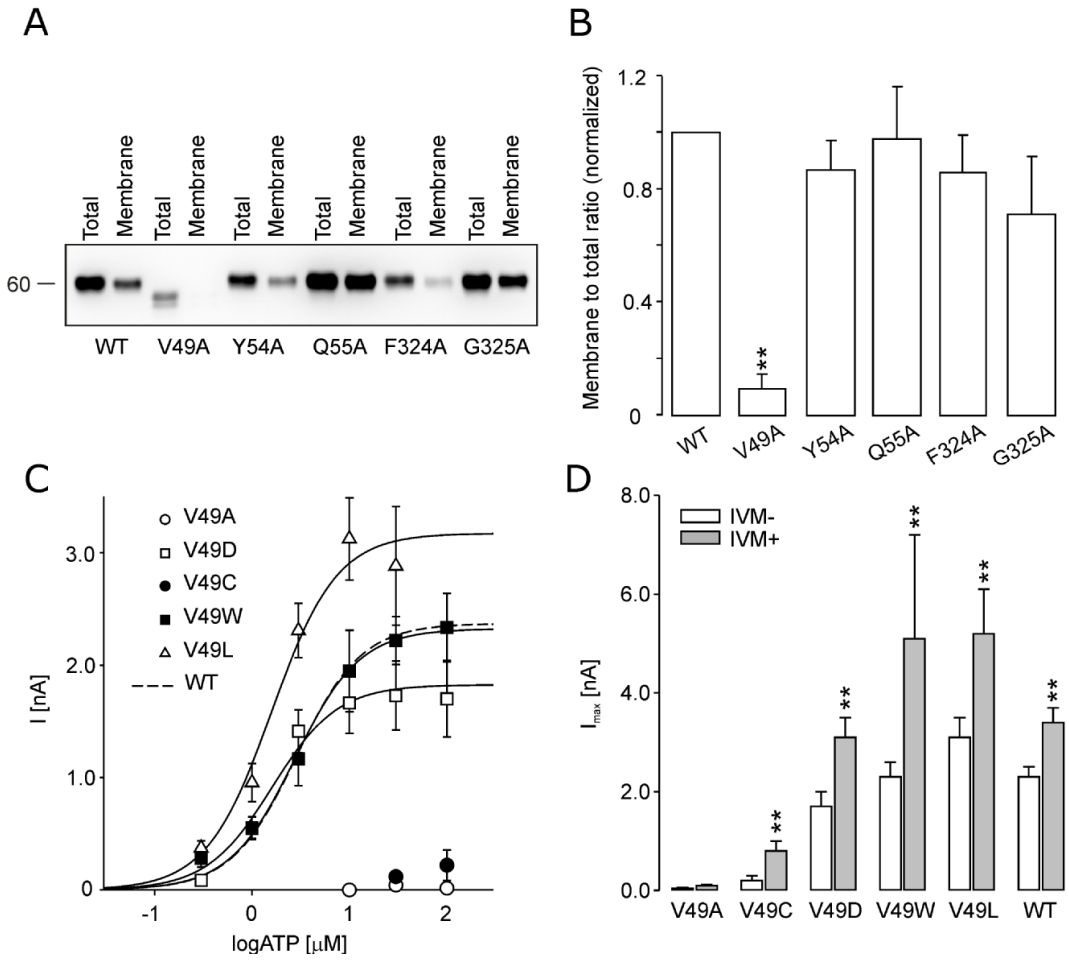

Figure 4.6 The membrane expression of the low-responsive alanine mutants and functional characterization of the V49-rP2X4 receptor mutants. (A) Western blots showing the expression pattern of the rP2X4 receptor-WT and V49A, Y54A, Q55A, F324A and G325A mutants. (B) Densitometry quantification of the membrane/ total ratio for five low-active mutants. The data are expressed as the mean ± SEM of 4 Western blot images. (C) The ATP concentration response curves of the WT and V49 mutant receptors. (D) The augmentation of the maximum current amplitude of the WT and V49 mutants by ivermectin (IVM). The I_{max} was determined prior to the IVM application (white bars) and 2-6 min after application of 3 μM IVM (gray bars). In this and following figures, ** $p < 0.01$ between the presence (IVM+) and absence (IVM-) of ivermectin..

The V49A mutant showed low or almost no expression in the plasma membrane fraction, whereas other mutants were present in the plasma membrane in variable quantities (Figure 4.6 A and B). Thus, for the V49A mutant, the loss of receptor function reflects the lack of its expression at the plasma membrane. In contrast to the V49A and V49C mutants, substitution of the valine at position 49 with another non-polar (V49L,

V49W) residue or a negatively charged residue of similar size (V49D) did not significantly alter the receptor function compared with WT (Figure. 4.6 C and Table 4.3). Furthermore, 2-6 min of preincubation with 3 μM IVM augmented the I_{max} values for these mutants 1.7- to 2.2-fold, that was a comparable with 1.5-fold augmentation observed in cells expressing the WT receptor (Figure 4.6 D). Together, these results indicate that trafficking but not gating of the rP2X4 channel depends on the V49 residue.

4.2.3 Involvement of Y54 and Q55 residues in channel gating

The Y54 alanine and cysteine mutants exhibited very low (I_{max} < 0.2 nA) activity. Contrary to the WT and V49A-P2X4 receptor mutant, the current amplitude of Y54A and Y54C mutants was augmented by IVM 5.4- and 5.8-fold, respectively. These results indicate that substitution of Y54 with alanine and cysteine led to a rightward shift in the potency of ATP for mutants, and that the functionality of these receptors was partially restored by IVM, which increases the frequency of channel openings (Priel and Silberberg 2004) and sensitizes P2X4 receptor to ATP (Khakh, Proctor et al. 1999). The activity of rP2X4 receptor was fully rescued by introducing phenylalanine and tryptophan, but not leucine (I_{max} < 0.1 nA), mutations to Y54, further indicating that an aromatic residue at position 54 is essential for the receptor to function (Figure 4.7 A and B Table 4.3).

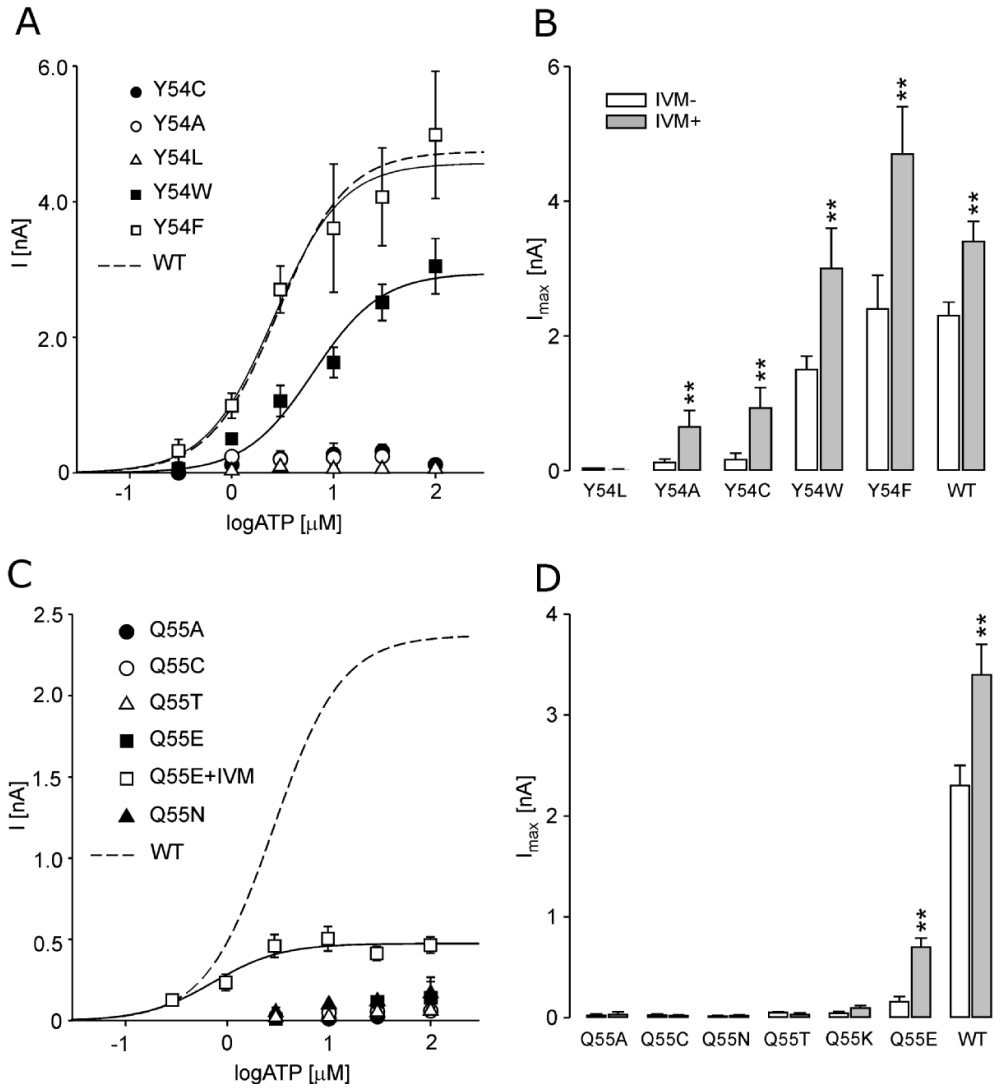

Figure 4.7 The effects of the Y54 and Q55 mutations on rP2X4 receptor function. (A) The ATP-concentration dependence curves for the WT and Y54 substitution mutants. (B) The I_{max} values for the WT and Y54 substitution mutants in the presence (gray bars) and absence (white bars) of IVM. (C) The ATP dose response curve for the Q55 mutants. (D) The I_{max} values of the Q55 mutants measured in the absence (white bars) and in the presence (gray bars) of IVM.

The Q55A and Q55C mutants were non-functional and IVM treatment was ineffective. Introducing amino acids of similar structure and chemical properties at Q55 (Q55N, Q55E, Q55T and Q55K) did not rescue the receptor function. The function of Q55E was partially restored by the treatment with IVM (4.4-fold augmentation), whereas IVM was ineffective with other mutants. Thus, it is reasonable to conclude that substitutions of conserved residues Y54 and Q55 above TM1 with alanine or cysteine

cause a stronger rightward shift in the potency of ATP for P2X4 receptor than mutation of V49-P2X4 receptor residue.

4.2.4. Characterization of the F324 and G325 mutants

The F324A and F324C mutants exhibited significantly reduced currents (I_{max} < 0.4 nA), and preincubation with IVM increased the current amplitude by 1.7- and 2.9-fold, respectively, comparable to that observed with the WT receptor (Figure 4.8 A and B). The EC_{50} value and time course of F324A current were also similar to the WT receptor (Figure 4.9 A). The mutation of F324 to another non-polar (F324L, F32W) residue or a polar aromatic (F324Y) residue partially or fully rescued the receptor function (Figure 4.8 A and B Table 4.3). For the F324 mutants, there was linear correlation between the log EC_{50} values and the hydrophobicity (r=0.46) and the volume (r=0.86) of the amino acid side chain substituent (Figure 4.9 A and B). These data suggest that hydrophobicity and the size, rather than aromaticity, at position 324 are important for the proper receptor function.

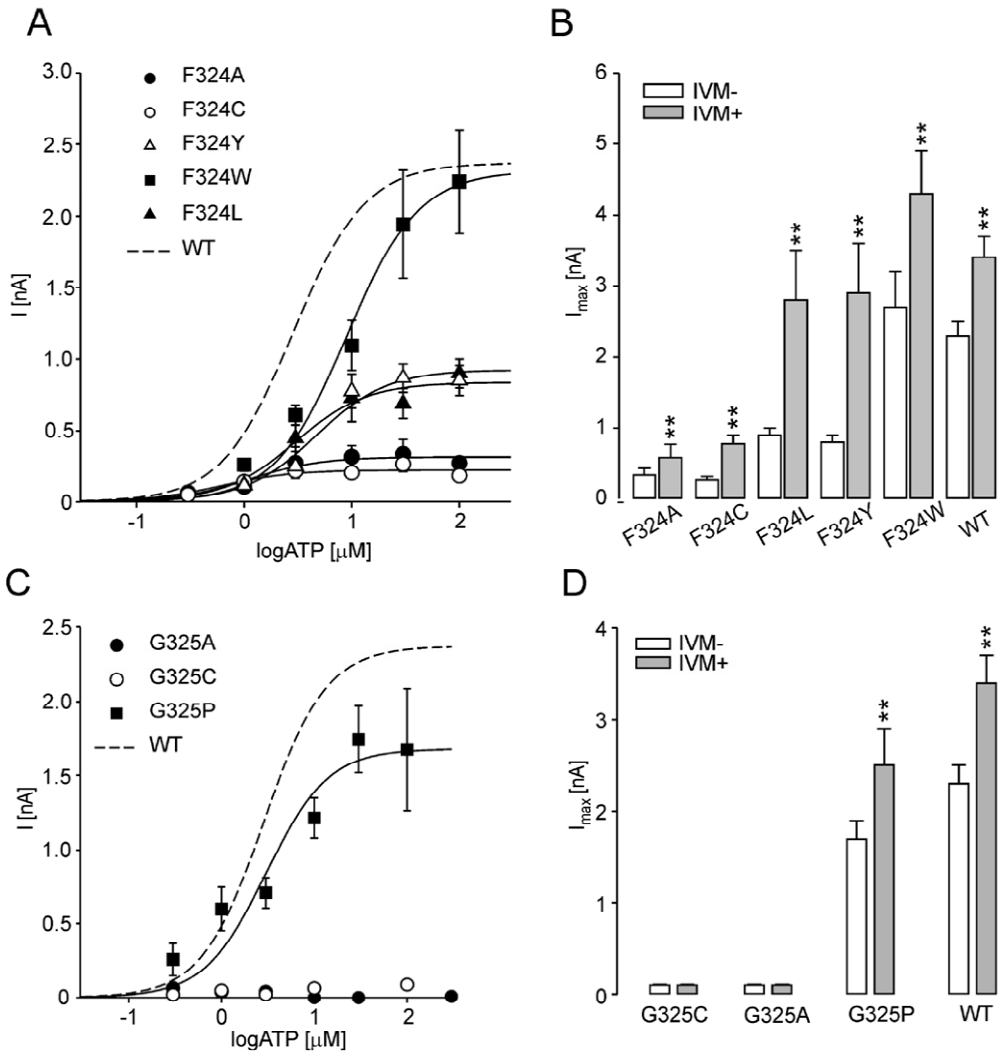

Figure 4.8 The effects of the F324 and G325 mutations on rP2X4 receptor function. (A) The ATP-concentration response curves for the single point mutants at position F324. (B) The I_{max} values of the F324 mutants measured in the absence (white bars) and presence (gray bars) of IVM. (C) The concentration-response curve for the rP2X4-G325P (symbols) and WT receptor (dotted line). (D) The I_{max} values of G325 mutants measured in the absence (white bars) and in the presence (gray bars) of IVM.

The G325A and G325C mutants were not active in the absence and presence of IVM. The G325P mutant had an EC_{50} and I_{max} value comparable to the WT receptor. Furthermore, this mutant showed a comparable degree of IVM-induced I_{max} augmentation to the WT receptor (Figure 4.8 C and D). The G325 and F324 residues are located in the breakage point of the β-sheet in P2X4 receptor open state. These data indicate that when the receptor undergoes activation-induced conformational changes, the G325 could operate as a hinge (Figure 4.9 C).

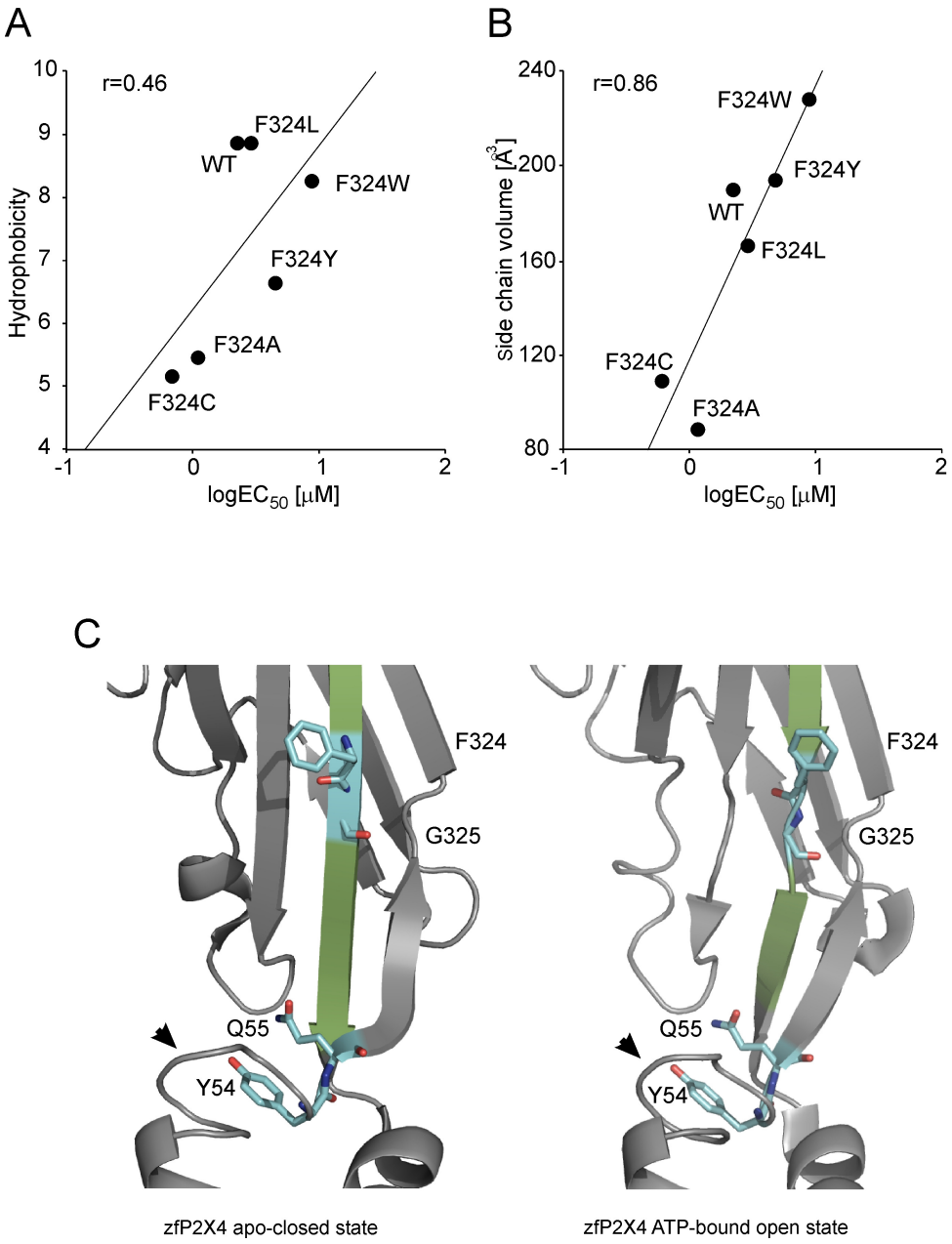

Figure 4.9 The effect of hydrophobicity and the size at position 324 on ATP potency and the localization of the F324 and G325 residues in the rP2X4 receptor molecule. (A and B) The correlation between the EC_{50} values with the hydrophobic effect (A) and the change in side chain residue volume (B). (C) Both the F324 and G325 residues are within the β-sheet connecting the ATP binding site and the pore (in green) in the zfP2X4 apo-closed state (left) and outside the β-sheet in the ATP-bound open state (right); rP2X4 numbering. Notice the stable position of Y54 and Q55, and conserved protein fold above TM1 (arrowhead) both in the closed and open state.

5. DISCUSSION

The first aim of this study was to understand the nature of SS bonding and the role of SS bonding in agonist binding and channel gating by using rP2X4 receptor as a receptor model. Previous work on the topic investigated P2X1 and P2X2 receptor subtypes. The bonding order was first identified in P2X2 receptor within SS1, SS4, and SS5 pairs (Clyne, Wang et al. 2002), another study focused on P2X1, receptor identifying the pairing order of all five SS pairs (Ennion and Evans 2002). The existence of these disulfide bonds was later confirmed by solving the crystal structure of zfP2X4 receptor (Kawate, Michel et al. 2009; Hattori and Gouaux 2012). The role of extracellular cysteine residues in the inhibitory effects of ethanol on the P2X4 receptor function was also available (Yi, Liu et al. 2009).

In this thesis, the effects of SS1-5 bond disruption and the effects of substitution of particular cysteine residues contained within the bond were separately investigated. The results show that disruption of the SS1, SS2 and SS4 bonds by substituting both cysteines with threonine generated receptors less sensitive to ATP. All four SS1 single mutants, three of four SS2 single mutants and six of eight SS4 single mutants showed similar effect. SS4 cysteine residues generated the most profound changes in the receptor function. For SS4 double mutant and six single mutants, the EC_{50} values could not be determined, but the amplitude of response was sufficient to estimate deactivation time constant in the presence of IVM. Because there is a significant correlation between the EC_{50} values for ATP and the rate of receptor deactivation in the presence of IVM (Zemkova, Yan et al. 2007), it is reasonable to conclude that these SS4 mutants are also less sensitive to ATP. This conclusion is in general agreement with data shown by Evans' and Hume's groups. Cysteine-rich head domain of P2X1 receptor was recently shown to play role in activation and desensitization of P2X1 receptor yet the role of particular cysteins remained unclear (Lorinczi, Bhargava et al. 2012). The P2X1-C217A and the P2X1-C227A mutants were fully functional but were the least sensitive among ten A-mutants (Ennion and Evans 2002). The P2X2-C224A mutant expressed in oocytes was not functional, whereas the C214A mutant showed decreased sensitivity to ATP. Furthermore, the I_{max} value for both SS4 mutants was significantly reduced or

undetectable when P2X2 receptor was expressed in HEK293T cells (Clyne, Wang et al. 2002).

The double SS5 mutant and three of four single P2X4 receptor mutants show low I_{max} and EC_{50} values closer to that of the WT receptor. Their rates of deactivation in the presence of IVM were also comparable to those observed in controls, clearly indicating that their sensitivity for ATP is not affected. The sensitivity of the SS5 A-mutants of P2X1, P2X2 was also not affected and I_{max} values were significantly lower. It has been suggested previously that trafficking of P2X1 to the plasma membrane is reduced by disruption of the SS5 bond, accounting for the smaller I_{max} value (Ennion and Evans 2002). The EGFP-tagged A-mutants of the SS5 cysteines, however, were localized to the membrane region. Together, these results indicate that the lower I_{max} values for four of the five SS5 mutants could reflect their relevance for channel gating and suggesting that these mutants show a preference for the closed state of the channel.

The difference in the results with SS5-P2X1 and P2X4 mutants probably indicates receptor specificity in the requirement of the SS bonds for particular functions. In agreement with this, in P2X1 none of these bonds individually is essential for receptor function, and several double mutants affecting the SS2 and SS3 bonds were functional (Ennion and Evans 2002). For P2X2, however, the SS1-4 bonds are individually needed for proper receptor function (Ennion and Evans 2002). In contrast, the P2X4 receptor function was not affected in double SS3 mutant nor in three of four single mutants, clearly indicating that SS3 bond is not critical for P2X4 receptor function. The expression system may also play a role in receptor function. For example, in contrast to results presented in this thesis, each of ten P2X4 receptor mutants expressed in oocytes generated a robust inward current (>50 % of the WT receptor), and the EC_{50} values of the C132A and C217A mutants were comparable to P2X4 receptor-WT (Yi, Liu et al. 2009). In addition, the P2X2-C258A mutant was practically non-functional when expressed in HEK293T cells (I_{max} about 4% of that observed in the WT receptor) but generated a larger current (about 25% of that observed in the WT receptor) when expressed in oocytes (Clyne, Wang et al. 2002).

The results presented in this thesis also showed that not all single residue SS mutants behaved similarly and comparably to the double mutants. For example, a

decrease in ATP sensitivity and I_{max} in cells expressing the C132A mutant is observed, whereas the receptor function was not affected by double SS3 mutants, as well as three single mutants: C132T, C159A, and C159T. Similarly, the P2X4 receptor function was not affected when the C217 was replaced with threonine or arginine. Also, mutant C261A showed properties comparable to the WT receptor, in contrast to double and three single SS5 mutants. These results indicate that individual cysteines forming the bond or their substitutes might also play a role in receptor function independently of the loss in bonding, probably by generating new interactions that might impair and/or preserve the structure of the channel necessary for agonist binding and/or channel gating. Consistent with this conclusion, the SS4 bond is absent in the simple eukaryote *Ostreococcus*, and the SS4 and SS5 bonds are absent in *Dictyostelium* (Jarvis and Khakh 2009; Surprenant and North 2009); however, a high concentration of agonist is required for their activation. Thus, it is reasonable to conclude that formation of the SS4 and SS5 bonds was an important step in the evolution of P2X proteins.

The crystal structure of zebrafish P2X4 receptor (Kawate, Michel et al. 2009; Hattori and Gouaux 2012) provides some rationale for the specific roles of the SS1-5 disulfide bonds in P2X function. It shows that a long turn of 13 amino acids starting from cysteine 165 (in green) could inhibit ATP binding by covering the ligand binding site if liberated by disruption of the SS1 bond. Disruption of the SS2 could also change the shape or size of the ATP binding pocket, as well as the head-to-body interface. A long turn of 14 amino acids, the dorsal fin, preceding cysteine 217 could play a similar role in the opposite half of the predicted ATP binding site if liberated by disruption of the SS4 bond. The impairing effect of the SS4 double mutant on receptor function could be explained by changes in the left-flipper-to-dorsal-fin interface affecting the interaction between receptor subunits and subsequent signal transduction from the ATP binding site to the pore. This interface is one of three major subunit-subunit contacts suggested to be important for receptor function (Kawate, Michel et al. 2009). The SS5 bond is located relatively far from the putative ATP binding site but close to the extracellular vestibule above the TM domains, supporting its role in channel gating and proper signal transduction from the ATP-binding site to the pore.

The second aim of this study was to elucidate the functional role of extracellular vestibule, again using rP2X4 receptor as a receptor model. By employing alanine- and cysteine-scanning mutagenesis of two sequences that contribute to formation of extracellular vestibules and lateral fenestrations, the results of this thesis show that a majority (53%) of the individual substitutions of residues V47-V61 and F324-N338 with alanine and cysteine did not significantly affect the function of rP2X4 receptor. Among them, the F48, K52, K326, G328, I332, I333 and T335 residues are well conserved among the mammalian subunits. Previous studies have shown a role of V47, I337 and N338 residues in P2X2 channel gating and pore formation (Rassendren, Buell et al. 1997; Jiang, Rassendren et al. 2001; Li, Migita et al. 2004; Khakh and Egan 2005; Cao, Broomhead et al. 2009). In P2X1, the A327 amino acid residue may also contribute to the P2X1 channel gating (Roberts and Evans 2007). Charged residues E56 and D58 are important for forming an access pathway for the ion entrance (Samways, Khakh et al. 2011) and residue E51 contributes to the high Ca^{2+} permeability of P2X4 receptor (Egan and Khakh 2004; Samways and Egan 2007; Samways, Khakh et al. 2012). Further studies are needed to characterize the potential role of F330 and P334 residues in the receptor's function.

The research described in this thesis has identified five amino acid residues from the extracellular vestibule that are critical for rP2X4 receptor function: V49, Y54, Q55, F324 and G325. These residues are also present in zfP2X4 receptor and among the mammalian P2X receptors, Y54, Q55 and G325 are fully conserved, V49 is present in five receptors, and F324 is non-conserved across P2X subtypes. In general, the loss of receptor function by substituting these residues with alanine and cysteine could reflect the altered trafficking of mutants to the plasma membrane or the loss of responsiveness to ATP, whereas their possible participation in ATP binding is highly unlikely in the light of recent crystallographic data of zfP2X4 receptor with ATP bound (Hattori and Gouaux 2012). Here it is confirmed that trafficking of receptors was significantly affected by mutation of V49 but not other residues. In addition, the V49D mutant is fully functional, although repulsive forces may form between the carboxyl side chain of aspartate and the negatively charged phospholipids. Similarly, the V49W mutation did not alter the receptor's function (Silberberg, Chang et al. 2005), although bulky amino acids could

accumulate in this part of the helix (F48-W49-W50) resulting in helix destabilization. The V50-P2X2 receptor mutant is also functional (Li, Migita et al. 2004; Khakh and Egan 2005). These results indicate that V49 residue plays a role in trafficking of the rP2X4 receptor, rather than destabilizing anchoring of the channel inside membrane or influencing channel gating.

The previous studies performed on P2X2 also showed that the Y54C and Q55C mutants (rP2X4 numbering) did not form functional channels (Jiang, Rassendren et al. 2001; Kawate, Robertson et al. 2011), but both studies provided no explanation for these effects. Cysteine-scanning mutagenesis of residues E52-G96 in human P2X1 receptor showed that all extracellular vestibule mutants are functional; therefore, there are subtype differences in the ability of the receptor to tolerate Q55 and Y54 mutations (Roberts and Evans 2006; Allsopp, El Ajouz et al. 2011). The other polar or charged mutations at Q55 position were not able to rescue the receptor function, while the Y54F and Y54W mutants responded to ATP, indicating the crucial relevance of aromatic residue at the position 54. It is surprising that the Q55N mutant was entirely nonfunctional in spite of significant structural similarity between the Gln and Asn side chains. The Y54A/C-P2X4 receptor function is partially rescued by IVM as well as the Q55E-P2X4 receptor function, whereas Q55A, Q55C, Q55N Q55T, and Q55K mutants were IVM insensitive. Because IVM causes a leftward shift in the sensitivity of receptors to ATP (Khakh, Proctor et al. 1999), it is reasonable to suggest that the Y54 residue contributes to gating. It is speculated that this is also the case for Q55 residue, but that the loss of responsiveness to ATP is more severe and could not be rescued by IVM with exception of Q55E. A recently published crystal structure of zfP2X4 receptor (Hattori and Gouaux 2012) showed that the Q55 residue forms hydrogen bonds with the N262 (zfP2X4 has aspartic acid in corresponding position) or D264 residues from the same subunit. Interestingly, an unchanging structural fold is present within 7Å of the Q55 and Y54 residues when the channel is either open or closed (Hattori and Gouaux 2012). Our results indicate that even the slightest change in the size and geometry of Q55 residue disrupts this conserved peptide fold and disrupts receptor function. Therefore, we conclude that the structure fold proximal to the Q55 residue must be maintained for the channel to be functional in either open or closed conformational state. In a study

addressing the molecular dynamics simulations of the P2X4 receptor, the Y54 residue was also hypothesized to form a hydrogen bond with the D264 residue, implying that this interaction is important for channel opening (Du, Dong et al. 2012). The results given in this thesis suggest that, if such interaction exists, it is not functionally important because while Y54F mutant lacks a hydroxyl group, it forms a functional receptor. It is further shown that stacking interactions are most likely important for the functionality of the Y54 mutants because an aromatic residue is crucial at this position for receptor function. The possible interaction partners of Y54 are F48 from TM1 and F330 from the TM2 of the same subunit. In agreement with this hypothesis, the F330C mutant exhibited a significantly reduced I_{max} current. Therefore, the conclusion is that Q55 and Y54 could play a crucial role in the mutual axial orientation of the TM1 and TM2 helices to a position that is functionally important for channel gating.

The G325 residue of rP2X4 receptor is identified to be functionally important because both G325A and G325C were not responsive to ATP. The topology of G325 in the open state of zfP2X4 receptor reveals that the β-sheet above TM2, connecting ATP binding domain with the channel pore, is disrupted in the position of G325, unlike closed state where the linear integrity of this β-sheet is maintained (Hattori and Gouaux 2012). The rescue effect of G325P mutant clearly shows the importance of this region because proline makes angular disruption and brakeage of β-sheet above TM2. These results indicate that angular brakeage of polypeptide at the level of G325 is crucial prerequisite for open-close state transition and that G325 could be a flexible hinge, which might be crucial for the twisting of the β-sheets and extracellular vestibule widening after receptor activation. Although the G325 residue is conserved along the P2X receptor isoforms, its critical functional importance is shown only in rP2X4 receptor. In the P2X1, the G321C mutant (equivalent to G325C in P2X4) was normal (Digby, Roberts et al. 2005) but exhibited a modified response to ATP in the presence of MTS reagents (Roberts and Evans 2007). Other study with rP2X2 also showed that H319C and G320C receptor mutants (analogous to F324C and G325C) are fully functional (Rassendren, Buell et al. 1997; Kawate, Robertson et al. 2011). Cysteine and alanine mutants of F324 residue in P2X4 receptor had reduced I_{max}, which was found also by others (Popova, Asatryan et al. 2010), but were partially rescued by introducing F324L, F324Y, and F324W mutations.

The correlation between the EC_{50} value and hydrophobicity for a particular F324 substituent indicates that the size of the residue and the ability to form hydrophobic interactions are important for receptor function. This intersubunit interaction could stabilize the closed state. Both the F324 and G325 residues are located in a non-structuralized region, if the β-sheet proximal to the TM2 is disrupted upon opening (Hattori and Gouaux 2012). Therefore, the F324 mutation may pose as obstruction to vestibule enlargement.

6. CONCLUSION

By using the molecular biology and electrophysiology techniques several questions on structure/function relationships in the rP2X4 receptor have been addressed. This doctoral thesis elucidated the importance of conserved ectodomain cysteines and disulphide bondings (SS1-5) in the receptor functions and identified amino acids in the extracellular vestibule which are important for channel gating, vestibule enlargement and receptor expression in the plasma membrane.

1. The disruption of SS3 bond failed to change function of P2X4 receptor while disruption of SS4 and SS5 bonds have profoundly affected the P2X4 receptor, indicating that these bond are important for receptor function.
2. SS1 and SS2, and to some extent SS4, bond disruption produced changes in receptor sensitivity to ATP which suggests that it may disrupt the integrity of the ATP binding site. SS5 bond is important for maintaining the integrity of channel gate or signal transduction from the ATP binding site to the pore.
3. The extensive mutagenesis of residues forming the extracellular vestibule resulted in identifying the crucial residues for the functions of the channel: V49, Y54, Q55, F324 and G325. Only the residue V49 has been found to be important for channel trafficking from the endoplasmic reticulum to the plasma membrane. The functioning of the remaining four residues has been investigated in details.
4. F324 and G325 residues were found to be important for widening of the extracellular vestibule as a crucial prerequisite for channel opening. F324 may also pose as a residue which is involved in filling the vestibule lumen and obstruction of vestibule enlargement.
5. Adjacent Q55 and Y54 residues are found to be important for gating of the channel, most probably by structurally constraining the transmembrane domains towards the conserved axial orientation.

LITERATURE

Abbracchio, M. P. and G. Burnstock (1994). "Purinoceptors: are there families of P2X and P2Y purinoceptors?" Pharmacol Ther **64**(3): 445-75.

Allard, B., M. Turcotte, et al. (2012). "CD73-generated adenosine: orchestrating the tumor-stroma interplay to promote cancer growth." J Biomed Biotechnol **2012**: 485156.

Allsopp, R. C., S. El Ajouz, et al. (2011). "Cysteine scanning mutagenesis (residues Glu52-Gly96) of the human P2X1 receptor for ATP: mapping agonist binding and channel gating." J Biol Chem **286**(33): 29207-17.

Baldwin, S. A., P. R. Beal, et al. (2004). "The equilibrative nucleoside transporter family, SLC29." Pflugers Arch **447**(5): 735-43.

Bean, B. P. (1990). "ATP-activated channels in rat and bullfrog sensory neurons: concentration dependence and kinetics." J Neurosci **10**(1): 1-10.

Bennett, J. L., J. F. Williams, et al. (1988). "Pharmacology of ivermectin." Parasitol Today **4**(8): 226-8.

Bernier, L. P., A. R. Ase, et al. (2008). "Phosphoinositides regulate P2X4 ATP-gated channels through direct interactions." J Neurosci **28**(48): 12938-45.

Bernier, L. P., A. R. Ase, et al. (2008). "Direct modulation of P2X1 receptor-channels by the lipid phosphatidylinositol 4,5-bisphosphate." Mol Pharmacol **74**(3): 785-92.

Bianchi, B. R., K. J. Lynch, et al. (1999). "Pharmacological characterization of recombinant human and rat P2X receptor subtypes." Eur J Pharmacol **376**(1-2): 127-38.

Bo, X., L. H. Jiang, et al. (2003). "Pharmacological and biophysical properties of the human P2X5 receptor." Mol Pharmacol **63**(6): 1407-16.

Bo, X., Y. Zhang, et al. (1995). "A P2X purinoceptor cDNA conferring a novel pharmacological profile." FEBS Lett **375**(1-2): 129-33.

Bobanovic, L. K., S. J. Royle, et al. (2002). "P2X receptor trafficking in neurons is subunit specific." J Neurosci **22**(12): 4814-24.

Bodnar, M., H. Wang, et al. (2011). "Amino acid residues constituting the agonist binding site of the human P2X3 receptor." J Biol Chem **286**(4): 2739-49.

Bonan, C. D. (2012). "Ectonucleotidases and nucleotide/nucleoside transporters as pharmacological targets for neurological disorders." CNS Neurol Disord Drug Targets **11**(6): 739-50.

Brake, A. J., M. J. Wagenbach, et al. (1994). "New structural motif for ligand-gated ion channels defined by an ionotropic ATP receptor." Nature **371**(6497): 519-23.

Brown, D. A., J. I. Bruce, et al. (2004). "cAMP potentiates ATP-evoked calcium signaling in human parotid acinar cells." J Biol Chem **279**(38): 39485-94.

Brown, S. G., A. Townsend-Nicholson, et al. (2002). "Heteromultimeric P2X(1/2) receptors show a novel sensitivity to extracellular pH." J Pharmacol Exp Ther **300**(2): 673-80.

Buell, G., C. Lewis, et al. (1996). "An antagonist-insensitive P2X receptor expressed in epithelia and brain." Embo J **15**(1): 55-62.

Burnstock, G. (1977). "The purinergic nerve hypothesis." Ciba Found Symp: 295-314.

Burnstock, G. (1986). "Purines and cotransmitters in adrenergic and cholinergic neurones." Prog Brain Res **68**: 193-203.
Burnstock, G. (2006). "Purinergic signalling." Br J Pharmacol **147 Suppl 1**: S172-81.
Burnstock, G. (2006). "Purinergic signalling--an overview." Novartis Found Symp **276**: 26-48; discussion 48-57, 275-81.
Burnstock, G. (2008). "Purinergic signalling and disorders of the central nervous system." Nat Rev Drug Discov **7**(7): 575-90.
Burnstock, G. (2011). "Introductory overview of purinergic signalling." Front Biosci (Elite Ed) **3**: 896-900.
Burnstock, G. (2013). "Introduction to purinergic signalling in the brain." Adv Exp Med Biol **986**: 1-12.
Cao, L., H. E. Broomhead, et al. (2009). "Polar residues in the second transmembrane domain of the rat P2X2 receptor that affect spontaneous gating, unitary conductance, and rectification." J Neurosci **29**(45): 14257-64.
Cervetto, C., M. C. Mazzotta, et al. (2012). "Calmidazolium selectively inhibits exocytotic glutamate release evoked by P2X7 receptor activation." Neurochem Int **60**(8): 768-72.
Che, M., D. F. Ortiz, et al. (1995). "Primary structure and functional expression of a cDNA encoding the bile canalicular, purine-specific Na(+)-nucleoside cotransporter." J Biol Chem **270**(23): 13596-9.
Chessell, I. P., A. D. Michel, et al. (1998). "Effects of antagonists at the human recombinant P2X7 receptor." Br J Pharmacol **124**(6): 1314-20.
Chizh, B. A. and P. Illes (2001). "P2X receptors and nociception." Pharmacol Rev **53**(4): 553-68.
Choi, J. S. and A. J. Berdis (2012). "Nucleoside transporters: biological insights and therapeutic applications." Future Med Chem **4**(11): 1461-78.
Clyne, J. D., L. D. LaPointe, et al. (2002). "The role of histidine residues in modulation of the rat P2X(2) purinoceptor by zinc and pH." J Physiol **539**(Pt 2): 347-59.
Clyne, J. D., L. F. Wang, et al. (2002). "Mutational analysis of the conserved cysteines of the rat P2X2 purinoceptor." J Neurosci **22**(10): 3873-80.
Cockcroft, S. and B. D. Gomperts (1979). "ATP induces nucleotide permeability in rat mast cells." Nature **279**(5713): 541-2.
Coddou, C., R. A. Lorca, et al. (2005). "Heavy metals modulate the activity of the purinergic P2X4 receptor." Toxicol Appl Pharmacol **202**(2): 121-31.
Codocedo, J. F., F. E. Rodriguez, et al. (2009). "Neurosteroids differentially modulate P2X ATP-gated channels through non-genomic interactions." J Neurochem **110**(2): 734-44.
Collo, G., R. A. North, et al. (1996). "Cloning OF P2X5 and P2X6 receptors and the distribution and properties of an extended family of ATP-gated ion channels." J Neurosci **16**(8): 2495-507.
Colquhoun, D. (1998). "Binding, gating, affinity and efficacy: the interpretation of structure-activity relationships for agonists and of the effects of mutating receptors." Br J Pharmacol **125**(5): 924-47.
Compan, V., L. Ulmann, et al. "P2X2 and P2X5 subunits define a new heteromeric receptor with P2X7-like properties." J Neurosci **32**(12): 4284-96.

Deaglio, S. and S. C. Robson (2011). "Ectonucleotidases as regulators of purinergic signaling in thrombosis, inflammation, and immunity." Adv Pharmacol **61**: 301-32.
Digby, H. R., J. A. Roberts, et al. (2005). "Contribution of conserved glycine residues to ATP action at human P2X1 receptors: mutagenesis indicates that the glycine at position 250 is important for channel function." J Neurochem **95**(6): 1746-54.
Ding, S. and F. Sachs (1999). "Ion permeation and block of P2X(2) purinoceptors: single channel recordings." J Membr Biol **172**(3): 215-23.
Ding, S. and F. Sachs (1999). "Single channel properties of P2X2 purinoceptors." J Gen Physiol **113**(5): 695-720.
Drury, A. N. and A. Szent-Gyorgyi (1929). "The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart." J Physiol **68**(3): 213-37.
Du, J., H. Dong, et al. (2012). "Gating mechanism of a P2X4 receptor developed from normal mode analysis and molecular dynamics simulations." Proc Natl Acad Sci U S A **109**(11): 4140-5.
Egan, T. M. and B. S. Khakh (2004). "Contribution of calcium ions to P2X channel responses." J Neurosci **24**(13): 3413-20.
Ennion, S., S. Hagan, et al. (2000). "The role of positively charged amino acids in ATP recognition by human P2X(1) receptors." J Biol Chem **275**(38): 29361-7.
Ennion, S. J. and R. J. Evans (2002). "Conserved cysteine residues in the extracellular loop of the human P2X(1) receptor form disulfide bonds and are involved in receptor trafficking to the cell surface." Mol Pharmacol **61**(2): 303-11.
Evans, R. J. (1996). "Single channel properties of ATP-gated cation channels (P2X receptors) heterologously expressed in Chinese hamster ovary cells." Neurosci Lett **212**(3): 212-4.
Evans, R. J., V. Derkach, et al. (1992). "ATP mediates fast synaptic transmission in mammalian neurons." Nature **357**(6378): 503-5.
Evans, R. J., C. Lewis, et al. (1995). "Pharmacological characterization of heterologously expressed ATP-gated cation channels (P2x purinoceptors)." Mol Pharmacol **48**(2): 178-83.
Evans, R. J., C. Lewis, et al. (1996). "Ionic permeability of, and divalent cation effects on, two ATP-gated cation channels (P2X receptors) expressed in mammalian cells." J Physiol **497**(Pt 2): 413-22.
Fabbretti, E., E. Sokolova, et al. (2004). "Identification of negative residues in the P2X3 ATP receptor ectodomain as structural determinants for desensitization and the Ca2+ sensing modulatory sites." J Biol Chem **279**: 53109-53115.
Fountain, S. J. and R. A. North (2006). "A C-terminal lysine that controls human P2X4 receptor desensitization." J Biol Chem **281**(22): 15044-9.
Friday, S. C. and R. I. Hume (2008). "Contribution of extracellular negatively charged residues to ATP action and zinc modulation of rat P2X2 receptors." J Neurochem **105**(4): 1264-75.
Fujiwara, Y., B. Keceli, et al. (2009). "Voltage- and [ATP]-dependent gating of the P2X(2) ATP receptor channel." J Gen Physiol **133**(1): 93-109.

Garcia-Guzman, M., F. Soto, et al. (1997). "Characterization of recombinant human P2X4 receptor reveals pharmacological differences to the rat homologue." Mol Pharmacol **51**(1): 109-18.
Garcia-Guzman, M., F. Soto, et al. (1996). "Molecular cloning and functional expression of a novel rat heart P2X purinoceptor." FEBS Lett **388**(2-3): 123-7.
Garcia-Guzman, M., W. Stuhmer, et al. (1997). "Molecular characterization and pharmacological properties of the human P2X3 purinoceptor." Brain Res Mol Brain Res **47**(1-2): 59-66.
Gever, J. R., D. A. Cockayne, et al. (2006). "Pharmacology of P2X channels." Pflugers Arch **452**(5): 513-37.
Gorodeski, G. I. (2002). "Expression, regulation, and function of P2X(4) purinergic receptor in human cervical epithelial cells." Am J Physiol Cell Physiol **282**(1): C84-93.
Green, H. N. and H. B. Stoner (1950). "Present Status of Adenine Nucleotides in Bodily Response to Injury." Br Med J **1**(4657): 805-9.
Hattori, M. and E. Gouaux (2012). "Molecular mechanism of ATP binding and ion channel activation in P2X receptors." Nature **485**(7397): 207-12.
Hausmann, R., J. Gunther, et al. (2013). "Salt Bridge Switching from Arg290/Glu167 to Arg290/ATP Promotes the Closed-to-Open Transition of the P2X2 Receptor." Mol Pharmacol **83**(1): 73-84.
Hu, B., C. Senkler, et al. (2002). "P2X4 receptor is a glycosylated cardiac receptor mediating a positive inotropic response to ATP." J Biol Chem **277**(18): 15752-7.
Huang, Q. Q., S. Y. Yao, et al. (1994). "Cloning and functional expression of a complementary DNA encoding a mammalian nucleoside transport protein." J Biol Chem **269**(27): 17757-60.
Inoue, K., M. Tsuda, et al. (2004). "ATP- and adenosine-mediated signaling in the central nervous system: chronic pain and microglia: involvement of the ATP receptor P2X4." J Pharmacol Sci **94**(2): 112-4.
Inoue, K., M. Tsuda, et al. (2004). "Chronic pain and microglia: the role of ATP." Novartis Found Symp **261**: 55-64; discussion 64-7, 149-54.
Jarvis, M. F. and B. S. Khakh (2009). "ATP-gated P2X cation-channels." Neuropharmacology **56**(1): 208-15.
Jelinkova, I., V. Vavra, et al. (2008). "Identification of P2X(4) receptor transmembrane residues contributing to channel gating and interaction with ivermectin." Pflugers Arch **456**(5): 939-50.
Jelinkova, I., Z. Yan, et al. (2006). "Identification of P2X(4) receptor-specific residues contributing to the ivermectin effects on channel deactivation." Biochem Biophys Res Commun **349**(2): 619-25.
Jiang, L. H., M. Kim, et al. (2003). "Subunit arrangement in P2X receptors." J Neurosci **23**(26): 8903-10.
Jiang, L. H., A. B. Mackenzie, et al. (2000). "Brilliant blue G selectively blocks ATP-gated rat P2X(7) receptors." Mol Pharmacol **58**(1): 82-8.
Jiang, L. H., F. Rassendren, et al. (2001). "Amino acid residues involved in gating identified in the first membrane-spanning domain of the rat P2X(2) receptor." J Biol Chem **276**(18): 14902-8.

Jiang, L. H., F. Rassendren, et al. (2000). "Identification of amino acid residues contributing to the ATP-binding site of a purinergic P2X receptor." J Biol Chem **275**(44): 34190-6.
Jindrichova, M., K. Khafizov, et al. (2010). "Highly conserved tyrosine 37 in the first transmembrane domain of ATP-gated P2X3 receptor is critical for maintaining rapid desensitization." JBC submitted.
Jindrichova, M., V. Vavra, et al. (2009). "Functional relevance of aromatic residues in the first transmembrane domain of P2X receptors." J Neurochem **109**(3): 923-34.
Jones, C. A., I. P. Chessell, et al. (2000). "Functional characterization of the P2X(4) receptor orthologues." Br J Pharmacol **129**(2): 388-94.
Jones, C. A., C. Vial, et al. (2004). "Functional regulation of P2X6 receptors by N-linked glycosylation: identification of a novel alpha beta-methylene ATP-sensitive phenotype." Mol Pharmacol **65**(4): 979-85.
Kawate, T., J. C. Michel, et al. (2009). "Crystal structure of the ATP-gated P2X(4) ion channel in the closed state." Nature **460**(7255): 592-8.
Kawate, T., J. L. Robertson, et al. (2011). "Ion access pathway to the transmembrane pore in P2X receptor channels." J Gen Physiol **137**(6): 579-90.
Keceli, B. and Y. Kubo (2009). "Functional and structural identification of amino acid residues of the P2X2 receptor channel critical for the voltage- and [ATP]-dependent gating." J Physiol **587**(Pt 24): 5801-18.
Khakh, B. S. (2001). "Molecular physiology of P2X receptors and ATP signalling at synapses." Nat Rev Neurosci **2**(3): 165-74.
Khakh, B. S., G. Burnstock, et al. (2001). "International union of pharmacology. XXIV. Current status of the nomenclature and properties of P2X receptors and their subunits." Pharmacol Rev **53**(1): 107-18.
Khakh, B. S. and T. M. Egan (2005). "Contribution of transmembrane regions to ATP-gated P2X2 channel permeability dynamics." J Biol Chem **280**(18): 6118-6129.
Khakh, B. S., P. P. Humphrey, et al. (1997). "ATP-gated cation channels (P2X purinoceptors) in trigeminal mesencephalic nucleus neurons of the rat." J Physiol **498 (Pt 3)**: 709-15.
Khakh, B. S. and R. A. North (2012). "Neuromodulation by extracellular ATP and P2X receptors in the CNS." Neuron **76**(1): 51-69.
Khakh, B. S., W. R. Proctor, et al. (1999). "Allosteric control of gating and kinetics at P2X(4) receptor channels." J Neurosci **19**(17): 7289-99.
Khmyz, V., O. Maximyuk, et al. (2008). "P2X3 receptor gating near normal body temperature." Pflugers Arch **456**(2): 339-47.
King, B. F., J. T. Neary, et al. (1996). "P2 purinoceptors in rat cortical astrocytes: expression, calcium-imaging and signalling studies." Neuroscience **74**(4): 1187-96.
King, B. F., A. Townsend-Nicholson, et al. (2000). "Coexpression of rat P2X2 and P2X6 subunits in Xenopus oocytes." J Neurosci **20**(13): 4871-7.
King, B. F., S. S. Wildman, et al. (1997). "Effects of extracellular pH on agonism and antagonism at a recombinant P2X2 receptor." Br J Pharmacol **121**(7): 1445-53.
Koshimizu, T., M. Koshimizu, et al. (1999). "Contributions of the C-terminal domain to the control of P2X receptor desensitization." J Biol Chem **274**(53): 37651-7.

Kracun, S., V. Chaptal, et al. (2010). "Gated access to the pore of a P2X receptor: structural implications for closed-open transitions." J Biol Chem **285**(13): 10110-21.
Krishtal, O. A., S. M. Marchenko, et al. (1988). "Cationic channels activated by extracellular ATP in rat sensory neurons." Neuroscience **27**(3): 995-1000.
Kubo, Y., Y. Fujiwara, et al. (2009). "Dynamic aspects of functional regulation of the ATP receptor channel P2X(2)." J Physiol **587**(Pt 22): 5317-24.
Le, K. T., K. Babinski, et al. (1998). "Central P2X4 and P2X6 channel subunits coassemble into a novel heteromeric ATP receptor." J Neurosci **18**(18): 7152-9.
Le, K. T., E. Boue-Grabot, et al. (1999). "Functional and biochemical evidence for heteromeric ATP-gated channels composed of P2X1 and P2X5 subunits." J Biol Chem **274**(22): 15415-9.
Lewis, C., S. Neidhart, et al. (1995). "Coexpression of P2X2 and P2X3 receptor subunits can account for ATP-gated currents in sensory neurons." Nature **377**(6548): 432-5.
Li, C., K. Xiong, et al. (2000). "Ethanol inhibition of adenosine 5'-triphosphate-activated current in freshly isolated adult rat hippocampal CA1 neurons." Neurosci Lett **295**(3): 77-80.
Li, M., T. H. Chang, et al. (2008). "Gating the pore of P2X receptor channels." Nat Neurosci **11**(8): 883-7.
Li, Z., K. Migita, et al. (2004). "Gain and loss of channel function by alanine substitutions in the transmembrane segments of the rat ATP-gated P2X2 receptor." J Neurosci **24**(33): 7378-86.
Longhi, M. S., S. C. Robson, et al. (2011). "Biological functions of ecto-enzymes in regulating extracellular adenosine levels in neoplastic and inflammatory disease states." J Mol Med (Berl).
Lorca, R. A., C. Coddou, et al. (2005). "Extracellular histidine residues identify common structural determinants in the copper/zinc P2X2 receptor modulation." J Neurochem **95**(2): 499-512.
Lorinczi, E., Y. Bhargava, et al. (2012). "Involvement of the cysteine-rich head domain in activation and desensitization of the P2X1 receptor." Proc Natl Acad Sci U S A **109**(28): 11396-401.
Lynch, K. J., E. Touma, et al. (1999). "Molecular and functional characterization of human P2X(2) receptors." Mol Pharmacol **56**(6): 1171-81.
Maenhaut, C., J. Van Sande, et al. (1990). "RDC8 codes for an adenosine A2 receptor with physiological constitutive activity." Biochem Biophys Res Commun **173**(3): 1169-78.
Marquez-Klaka, B., J. Rettinger, et al. (2007). "Identification of an intersubunit cross-link between substituted cysteine residues located in the putative ATP binding site of the P2X1 receptor." J Neurosci **27**(6): 1456-66.
Miller, K. J., A. D. Michel, et al. (1998). "Cibacron blue allosterically modulates the rat P2X4 receptor." Neuropharmacology **37**(12): 1579-86.
Mohebbipour, A., P. Saleh, et al. (2012). "Treatment of Scabies: Comparison of Ivermectin vs. Lindane Lotion 1%." Acta Dermatovenerol Croat **20**(4): 251-5.
Molina-Arcas, M., F. J. Casado, et al. (2009). "Nucleoside transporter proteins." Curr Vasc Pharmacol **7**(4): 426-34.

Molina-Arcas, M., L. Trigueros-Motos, et al. (2008). "Physiological and pharmacological roles of nucleoside transporter proteins." Nucleosides Nucleotides Nucleic Acids **27**(6): 769-78.
Moro, S., Z. G. Gao, et al. (2006). "Progress in the pursuit of therapeutic adenosine receptor antagonists." Med Res Rev **26**(2): 131-59.
Nagaya, N., R. K. Tittle, et al. (2005). "An intersubunit zinc binding site in rat P2X2 receptors." J Biol Chem **280**(28): 25982-93.
Nakazawa, K. and P. Hess (1993). "Block by calcium of ATP-activated channels in pheochromocytoma cells." J Gen Physiol **101**(3): 377-92.
Neelands, T. R., E. C. Burgard, et al. (2003). "2', 3'-O-(2,4,6,trinitrophenyl)-ATP and A-317491 are competitive antagonists at a slowly desensitizing chimeric human P2X3 receptor." Br J Pharmacol **140**(1): 202-10.
Newbolt, A., R. Stoop, et al. (1998). "Membrane topology of an ATP-gated ion channel (P2X receptor)." J Biol Chem **273**(24): 15177-82.
Nicke, A., H. G. Baumert, et al. (1998). "P2X1 and P2X3 receptors form stable trimers: a novel structural motif of ligand-gated ion channels." Embo J **17**(11): 3016-28.
Nicke, A., D. Kerschensteiner, et al. (2005). "Biochemical and functional evidence for heteromeric assembly of P2X1 and P2X4 subunits." J Neurochem **92**(4): 925-33.
North, R. A. (2002). "Molecular physiology of P2X receptors." Physiol Rev **82**(4): 1013-67.
North, R. A. (2002). "Molecular physiology of P2X receptors." Physiol Rev **82**(4): 1013-67.
North, R. A. (2003). "The P2X3 subunit: a molecular target in pain therapeutics." Curr Opin Investig Drugs **4**(7): 833-40.
North, R. A. (2004). "P2X3 receptors and peripheral pain mechanisms." J Physiol **554**(Pt 2): 301-8.
North, R. A. and A. Surprenant (2000). "Pharmacology of cloned P2X receptors." Annu Rev Pharmacol Toxicol **40**: 563-80.
North, R. A. and A. Verkhratsky (2006). "Purinergic transmission in the central nervous system." Pflugers Arch **452**(5): 479-85.
Ormond, S. J., N. P. Barrera, et al. (2006). "An uncharged region within the N terminus of the P2X6 receptor inhibits its assembly and exit from the endoplasmic reticulum." Mol Pharmacol **69**(5): 1692-700.
Patel, M. K., B. S. Khakh, et al. (2001). "Properties of native P2X receptors in rat trigeminal mesencephalic nucleus neurones: lack of correlation with known, heterologously expressed P2X receptors." Neuropharmacology **40**(1): 96-105.
Popova, M., L. Asatryan, et al. (2010). "A point mutation in the ectodomain-transmembrane 2 interface eliminates the inhibitory effects of ethanol in P2X4 receptors." J Neurochem **112**: 307-17.
Priel, A. and S. D. Silberberg (2004). "Mechanism of ivermectin facilitation of human P2X4 receptor channels." J Gen Physiol **123**(3): 281-93.
Prive, G. G. (2007). "Detergents for the stabilization and crystallization of membrane proteins." Methods **41**(4): 388-97.
Ralevic, V. and G. Burnstock (1998). "Receptors for purines and pyrimidines." Pharmacol Rev **50**(3): 413-92.

Ralevic, V. and G. Burnstock (1998). "Receptors for purines and pyrimidines." Pharmacol Rev **50**(3): 413-92.
Rassendren, F., G. Buell, et al. (1997). "Identification of amino acid residues contributing to the pore of a P2X receptor." Embo J **16**(12): 3446-54.
Rettinger, J., A. Aschrafi, et al. (2000). "Roles of individual N-glycans for ATP potency and expression of the rat P2X1 receptor." J Biol Chem **275**(43): 33542-7.
Rettinger, J., K. Braun, et al. (2005). "Profiling at recombinant homomeric and heteromeric rat P2X receptors identifies the suramin analogue NF449 as a highly potent P2X1 receptor antagonist." Neuropharmacology **48**(3): 461-8.
Roberts, J. A., H. R. Digby, et al. (2008). "Cysteine substitution mutagenesis and the effects of methanethiosulfonate reagents at P2X2 and P2X4 receptors support a core common mode of ATP action at P2X receptors." J Biol Chem.
Roberts, J. A. and R. J. Evans (2004). "ATP binding at human P2X1 receptors. Contribution of aromatic and basic amino acids revealed using mutagenesis and partial agonists." J Biol Chem **279**(10): 9043-55.
Roberts, J. A. and R. J. Evans (2006). "Contribution of conserved polar glutamine, asparagine and threonine residues and glycosylation to agonist action at human P2X1 receptors for ATP." J Neurochem **96**(3): 843-52.
Roberts, J. A. and R. J. Evans (2007). "Cysteine substitution mutants give structural insight and identify ATP binding and activation sites at P2X receptors." J Neurosci **27**(15): 4072-82.
Robertson, S. J., M. G. Rae, et al. (1996). "Characterization of a P2X-purinoceptor in cultured neurones of the rat dorsal root ganglia." Br J Pharmacol **118**(4): 951-6.
Ruppelt, A., W. Ma, et al. (2001). "Genomic structure, developmental distribution and functional properties of the chicken P2X(5) receptor." J Neurochem **77**(5): 1256-65.
Samways, D. S. and T. M. Egan (2007). "Acidic amino acids impart enhanced Ca2+ permeability and flux in two members of the ATP-gated P2X receptor family." J Gen Physiol **129**(3): 245-56.
Samways, D. S., B. S. Khakh, et al. (2011). "Preferential use of unobstructed lateral portals as the access route to the pore of human ATP-gated ion channels (P2X receptors)." Proc Natl Acad Sci U S A **108**(33): 13800-5.
Samways, D. S., B. S. Khakh, et al. (2012). "Allosteric Modulation of Ca2+ flux in Ligand-gated Cation Channel (P2X4) by Actions on Lateral Portals." J Biol Chem **287**(10): 7594-602.
Seguela, P., A. Haghighi, et al. (1996). "A novel neuronal P2x ATP receptor ion channel with widespread distribution in the brain." J Neurosci **16**(2): 448-55.
Shinozaki, Y., K. Sumitomo, et al. (2009). "Direct Observation of ATP-Induced Conformational Changes in Single P2X(4) Receptors." PLoS Biol **7**(5): e1000103.
Silberberg, S. D., T. H. Chang, et al. (2005). "Secondary Structure and Gating Rearrangements of Transmembrane Segments in Rat P2X4 Receptor Channels." J Gen Physiol **125**(4): 347-59.
Silberberg, S. D., M. Li, et al. (2007). "Ivermectin Interaction with Transmembrane Helices Reveals Widespread Rearrangements during Opening of P2X Receptor Channels." Neuron **54**(2): 263-274.

Sokolova, E., A. Skorinkin, et al. (2004). "Agonist-dependence of recovery from desensitization of P2X(3) receptors provides a novel and sensitive approach for their rapid up or downregulation." Br J Pharmacol **141**(6): 1048-58.
Sokolova, E., A. Skorinkin, et al. (2006). "Experimental and modeling studies of desensitization of P2X3 receptors." Mol Pharmacol **70**(1): 373-82.
Soto, F., M. Garcia-Guzman, et al. (1996). "P2X4: an ATP-activated ionotropic receptor cloned from rat brain." Proc Natl Acad Sci U S A **93**(8): 3684-8.
Soto, F., M. Garcia-Guzman, et al. (1996). "Cloning and tissue distribution of a novel P2X receptor from rat brain." Biochem Biophys Res Commun **223**(2): 456-60.
Soto, F., G. Lambrecht, et al. (1999). "Antagonistic properties of the suramin analogue NF023 at heterologously expressed P2X receptors." Neuropharmacology **38**(1): 141-9.
Steinberg, T. H., A. S. Newman, et al. (1987). "ATP4- permeabilizes the plasma membrane of mouse macrophages to fluorescent dyes." J Biol Chem **262**(18): 8884-8.
Stoner, H. B. and H. N. Green (1950). "Studies on the role of the adrenal cortex in adenosine triphosphate shock." Br J Exp Pathol **31**(5): 603-14.
Stoop, R., S. Thomas, et al. (1999). "Contribution of individual subunits to the multimeric P2X(2) receptor: estimates based on methanethiosulfonate block at T336C." Mol Pharmacol **56**(5): 973-81.
Surprenant, A. and R. A. North (2009). "Signaling at purinergic P2X receptors." Annu Rev Physiol **71**: 333-59.
Surprenant, A., D. A. Schneider, et al. (2000). "Functional properties of heteromeric P2X(1/5) receptors expressed in HEK cells and excitatory junction potentials in guinea-pig submucosal arterioles." J Auton Nerv Syst **81**(1-3): 249-63.
Tatham, P. E. and M. Lindau (1990). "ATP-induced pore formation in the plasma membrane of rat peritoneal mast cells." J Gen Physiol **95**(3): 459-76.
Tenneti, L., S. J. Gibbons, et al. (1998). "Expression and trans-synaptic regulation of P2x4 and P2z receptors for extracellular ATP in parotid acinar cells. Effects of parasympathetic denervation." J Biol Chem **273**(41): 26799-808.
Thomas, S. A. and R. I. Hume (1990). "Permeation of both cations and anions through a single class of ATP-activated ion channels in developing chick skeletal muscle." J Gen Physiol **95**(4): 569-90.
Torres, G. E., T. M. Egan, et al. (1998). "N-Linked glycosylation is essential for the functional expression of the recombinant P2X2 receptor." Biochemistry **37**(42): 14845-51.
Torres, G. E., T. M. Egan, et al. (1999). "Hetero-oligomeric assembly of P2X receptor subunits. Specificities exist with regard to possible partners." J Biol Chem **274**(10): 6653-9.
Toulme, E., F. Soto, et al. (2006). "Functional properties of internalization-deficient P2X4 receptors reveal a novel mechanism of ligand-gated channel facilitation by ivermectin." Mol Pharmacol **69**(2): 576-87.
Valera, S., N. Hussy, et al. (1994). "A new class of ligand-gated ion channel defined by P2x receptor for extracellular ATP." Nature **371**(6497): 516-9.
Vassort, G. (2001). "Adenosine 5'-triphosphate: a P2-purinergic agonist in the myocardium." Physiol Rev **81**(2): 767-806.

Vávra, V., A. Balik, et al. (2008). "Differential expression of P2X receptors at suprachiasmatic and supraoptic nuclei." Purinergic Signalling **4**(Suppl.5): S192.
Vavra, V., A. Bhattacharya, et al. (2011). "Facilitation of glutamate and GABA release by P2X receptor activation in supraoptic neurons from freshly isolated rat brain slices." Neuroscience **188**: 1-12.
Virginio, C., A. MacKenzie, et al. (1999). "Pore dilation of neuronal P2X receptor channels." Nat Neurosci **2**(4): 315-21.
Virginio, C., A. MacKenzie, et al. (1999). "Pore dilation of neuronal P2X receptor channels." Nat Neurosci **2**(4): 315-21.
Virginio, C., R. A. North, et al. (1998). "Calcium permeability and block at homomeric and heteromeric P2X2 and P2X3 receptors, and P2X receptors in rat nodose neurones." J Physiol **510 (Pt 1)**: 27-35.
Whitlock, A., G. Burnstock, et al. (2001). "The single-channel properties of purinergic P2X ATP receptors in outside-out patches from rat hypothalamic paraventricular parvocells." Pflugers Arch **443**(1): 115-22.
Wong, A. Y., G. Burnstock, et al. (2000). "Single channel properties of P2X ATP receptors in outside-out patches from rat hippocampal granule cells." J Physiol **527 Pt 3**: 529-47.
Xiong, K., X. Q. Hu, et al. (2005). "The mechanism by which ethanol inhibits rat P2X4 receptors is altered by mutation of histidine 241." Br J Pharmacol **145**(5): 576-86.
Yamamoto, K., R. Korenaga, et al. (2000). "Fluid shear stress activates Ca(2+) influx into human endothelial cells via P2X4 purinoceptors." Circ Res **87**(5): 385-91.
Yan, Z., S. Li, et al. (2008). "The P2X7 receptor channel pore dilates under physiological ion conditions." J Gen Physiol **132**(5): 563-73.
Yan, Z., Z. Liang, et al. (2006). "Participation of the Lys313-Ile333 sequence of the purinergic P2X4 receptor in agonist binding and transduction of signals to the channel gate." J Biol Chem **281**(43): 32649-59.
Yi, C. L., Y. W. Liu, et al. (2009). "Conserved extracellular cysteines differentially regulate the inhibitory effect of ethanol in rat P2X4 receptors." Biochem Biophys Res Commun **381**(1): 102-6.
Zemkova, H., A. Balik, et al. (2008). "Molecular structure of purinergic P2X receptors and their expression in the hypothalamus and pituitary." Physiol Res.
Zemkova, H., M. L. He, et al. (2004). "Identification of ectodomain regions contributing to gating, deactivation, and resensitization of purinergic P2X receptors." J Neurosci **24**(31): 6968-78.
Zemkova, H., M. Kucka, et al. (2010). "Characterization of purinergic P2X4 receptor channels expressed in anterior pituitary cells." Am J Physiol Endocrinol Metab **298**(3): E644-51.
Zemkova, H., Z. Yan, et al. (2007). "Role of Aromatic and Charged Ectodomain Residues in the P2X4 Receptor Functions." J Neurochem **102**(4): 1139-50.
Zhao, J. and S. J. Ennion (2006). "Sp1/3 and NF-1 mediate basal transcription of the human P2X1 gene in megakaryoblastic MEG-01 cells." BMC Mol Biol **7**: 10.
Zhou, L., L. Luo, et al. (2009). "Regulation of P2X(7) gene transcription." Purinergic Signal **5**(3): 409-26.

Zhou, Z. and R. I. Hume (1998). "Two mechanisms for inward rectification of current flow through the purinoceptor P2X2 class of ATP-gated channels." J Physiol **507**(Pt 2): 353-64.
Zimmermann, H. (2000). "Extracellular metabolism of ATP and other nucleotides." Naunyn Schmiedebergs Arch Pharmacol **362**(4-5): 299-309.