Regulation of Pannexin 1 Channels by ATP

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REGULATION OF PANNEXIN 1 CHANNELS BY ATP

By

Feng Qiu

A DISSERTATION

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REGULATION OF PANNEXIN 1 CHANNELS BY ATP

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The recently discovered pannexins represent a second family of gap junction proteins in vertebrates. However, instead of forming intercellular gap junction channels like connexins, pannexins operate as unpaired pannexons, allowing the flux of molecules between the cytoplasm and the extracellular space. Pannexins appear to play a vital role in the local control loop of blood perfusion and oxygen delivery. The properties of Panx1 channels indicate that this protein is the most probable candidate for an ATP release channel and is involved in the propagation of intercellular calcium waves. It is also proposed to mediate the large pore formation of the P2X7 receptor death complex. Prolonged activation of this receptor can lead to cell death. There must be some mechanisms to stop this ATP-induced ATP release and opening of the lethal pore. Here we describe a negative feedback loop controlling pannexin 1 channel activity. ATP, permeant in pannexin 1 channels, was found to inhibit its permeation pathway when applied extracellularly. ATP analogues, including BzATP, suramine, and BBG were even more effective inhibitors of pannexin 1 currents than ATP. These compounds also attenuated the uptake of dyes by erythrocytes, which express pannexin 1. The rank order of the compounds in attenuation of pannexin 1 currents was similar to their binding
affinities to the P2X7 receptor, except that receptor agonists and antagonists both were inhibitory to the channel.

The ATP inhibitory effect is largely decreased when R75 on the first extracellular loop of Pannexin1 is mutated to alanine, strongly indicating that the ATP regulates this channel through binding. To further investigate the structural property of the ATP binding, we did alanine-scanning mutagenesis of the extracellular loops and found that mutations on W74, S237, S240, I247 and L266 on the extracellular loops severely impair the BzATP inhibitory effect indicating that they might be direct binding partners for the ligands. Mutations on R75, S82, S93, L94, D241, S249, P259 and I267 have largely decreased BzATP sensitivity. Mutations on other residues didn’t change the BzATP sensitivity compared to the wild type except for some nonfunctional mutants. All these data demonstrate that some amino acid residues on the extracellular loop of Pannexin 1 mediate ATP sensitivity. However, how these residues form the ATP-binding pocket remains to be elucidated.
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Chapter 1

Background

1.1 Gap junctions

Cell-cell communication, which is essential to coordinate cellular responses in tissues and organs, is mediated by two fundamentally different ways: a) release of secreted molecules (such as hormones, neurotransmitters) into the extracellular space, which bind to the corresponding receptors on adjacent cells; b) formation of continuous channels connecting the cytoplasm of two neighboring cells, allowing direct exchange of ions, metabolites and other messenger molecules. Direct cell-to-cell communication was first demonstrated by Furshpan and Potter in giant motor synapses of crayfish (Furshpan and Potter 1959). It is a low resistance pathway allowing direct ionic coupling. A hexagonal arrangement of apposed channels was observed in Mauthner cells of goldfish brains as potential cell-cell communication pathways (Robertson 1963). This structure was further confirmed by Revel and Karnovsky in 1967 using electron microscopy suggesting this regular sub-unit pattern at cell surface was most likely the candidate for direct communication between attached cells (Revel and Karnovsky 1967). The structure that provides direct intercellular communication was first named nexus (Dewey and Barr 1964). Later, the name of gap junction (GJ) was proposed (Revel and Sheridan 1968). Now it is generally accepted that gap junction proteins exist within most invertebrates and vertebrate tissues, with the exception in striated muscle and cells not using this model of cellular coupling including erythrocytes, platelets and sperm cells. The plaques-like expression pattern of gap junctions was established by studies using immunogold labeled freeze fracture replicas (Rash and Yasumura 1992; Rash, Yasumura et al. 1998),
immunocytochemistry (Severs, Rothery et al. 2001) and fluorescently tagged connexins (Bukauskas, Jordan et al. 2000; Falk 2000; Rutz and Hulser 2001).

Connexins were the first identified family of GJ proteins and most thoroughly studied (Dahl, Azarnia et al. 1981; Dahl, Miller et al. 1987; Willecke, Hennemann et al. 1991; Bruzzone, White et al. 1996; Willecke, Eiberger et al. 2002). It now appears that connexins are specific to chordates. To date, 20 mouse and 21 human connexins have been identified. Only in the 1990s, a second family of protein was identified to be the subunit of gap junction in invertebrate, called innexin (Phelan, Bacon et al. 1998). Surprisingly, innexins have quite different primary sequences compared to connexins. The search for innexin homologs in vertebrate yielded three new members of gap junction proteins, named pannexins (Panchin, Kelmanson et al. 2000; Baranova, Ivanov et al. 2004; Panchin 2005).

1.1.1 Structural properties of gap junctions

A complete gap junction channel is composed of two “hemichannels” residing in opposing cell membranes. In vertebrates, gap junction hemichannels are formed by six subunits of connexin proteins, called connexons. Hemichannels of six identical connexins are called homomeric, while those with different connexins are heteromeric. Two identical hemichannels form a homotypic gap junction and two differing hemichannels form a heterotypic gap junction.
Figure 1. Structure of gap junctions (a) In this model, a gap junction is a cluster of channels between two plasma membranes that are separated by a gap of about 2 – 3 nm. (b) Both membranes contain a hemichannel, called connexon, composed of six connexin subunits. (c) Each connexin subunit has four transmembrane helices and intracellular amino and carboxyl termini. Two connexons join in the gap between the cells to form a gap-junction channel, 1.5 – 2.0 nm in diameter, connecting the cytoplasms of the two cells.

The first connexin cDNA was cloned in 1986 (Paul 1986), and the invertebrate analogues were identified a decade later (Phelan, Bacon et al. 1998). A simplified
nomenclature of connexins is based on their predicted molecular weights, with a small letter indicating the species, e.g. hCx43 is the human connexin protein with molecular weight 43kDa. The amino acid sequence derived from the cloned cDNA was used to predict the structure of the proteins. The topology of the gap junction protein was predicted based on hydropathy plots, which showed four transmembrane segments (M1-M4), two extracellular loops, one intracellular loop and intracellular amino- and carboxy-terminus as shown in Figure 1c (Heynkes, Kozjek et al. 1986; Paul 1986; Beyer, Paul et al. 1987; Bauer, Loer et al. 2005). The overall structures were confirmed later by immunostaining and detailed electron crystallography analysis (Yeager and Nicholson 1996; Unger, Kumar et al. 1999; Unger, Kumar et al. 1999; Hand, Muller et al. 2002; Maeda, Nakagawa et al. 2009).

In connexins, three transmembrane regions M1, M2 and M4 contain mainly hydrophobic amino acid residues, while M3 contains a number of charged amino acid residues, being modeled as an amphipathic alpha-helix and implicated in lining of the pore (Bennett, Barrio et al. 1991). Cysteine scanning mutagenesis of connexin 46 hemichannels indicated that both M1 and M3 contribute to the lining of the pore (Zhou, Pfahnl et al. 1997; Skerrett, Aronowitz et al. 2002; Kronengold, Trexler et al. 2003; Kronengold, Trexler et al. 2003).

Recently, the electron crystallographic analysis of the connexin 26 revealed the structure of gap junction channels at 3.5Å resolution (Figure 2&3). It shows that the connexin 26 hemichannel carries a positively charged cytoplasmic entrance, named a funnel. The wall of the funnel is lined by N-terminal regions of the six subunits. The size
of the molecules which can go through the channel is determined by the narrowed pore at the funnel (Maeda, Nakagawa et al. 2009; Suga, Maeda et al. 2009).


**Figure 2.** Overall structure of the Cx26 gap junction channel in ribbon representation.
Two main methods are used to study the function of gap junction channels: electrophysiological measurement of cell-cell coupling and fluorescent dye molecule transfer (Loewenstein 1981; Dermietzel, Hwang et al. 1990; Levin 2002). The function of gap junction channels is achieved by the opening of the junctional pore. There are three major factors to affect the opening or closing states of gap junction channels, we call them gating mechanisms: transjunctional voltage \( V_j \) (the membrane potential \( V_m \) difference between two connecting cells), intracellular pH and calcium. However, due to the various isoforms of gap junction subunits and their combinatorial complexity like
homotypic, heterotypic or heteromeric, the gap junction channels display quite different gating and selectivity properties.

Like the common ion channels, gap junction channels are gated by voltage. The conductance decreases in response to the transjunctional voltage developing between the cells. Figure 4 is the panoramic view of \( V_j \)-gating properties on vertebrate gap junctions (Gonzalez, Gomez-Hernandez et al. 2007).

Figure 4. Panoramic view of \( V_j \)-gating properties on vertebrate junctions. Junctional conductance \( G_j \) is
generally maximal at $V_j=0$ and decreases symmetrically for positive and negative $V_j$’s to non-zero residual conductance.

Channels formed by connexins generally reach a residual (non-zero) steady-state conductance varying from 5% to 40% with the increase of $V_j$, which is explained by the incomplete closure of gap junction channels (Bukauskas and Weingart 1993; Weingart and Bukauskas 1993; Bukauskas and Weingart 1994). The symmetric decrease of conductance for positive and negative $V_j$ was explained by identical voltage gates in each hemichannel such that for each polarity of $V_j$, closure can be ascribed to one or the other hemichannel (Harris, Spray et al. 1981; Spray, Harris et al. 1981; Bukauskas and Verselis 2004).

The physiological role of voltage gate was proposed to restrict the passage of larger molecules, e.g. fluorescent tracers and cAMP, while having little effect on the passage of smaller electrolytes, allowing the selective exclusion of large molecules, which might have potential signaling properties, without impairing the electric coupling (Qu and Dahl 2002).

Gating of gap junction channels by pH has been shown decades ago (Rose and Rick 1978; Turin and Warner 1980). It is now generally accepted that intracellular acidification decreases gap junction conductance and uncouples the cells, while the degree of pH sensitivity vary among connexins. The fast response (~1ms) to low pH suggested a direct action of proton on the connexins instead of through intermediates (Pfahnl and Dahl 1998; Trexler, Bukauskas et al. 1999). The uncoupling of cells upon low pH exposure may serve an important role in limiting the spread of injury from damaged tissue to normal tissue.
The role of calcium in cell uncoupling is documented in many cell types, including cardiac cells, astrocytes, pancreatic cells, acinar cells etc. (Peracchia 1978; Peracchia 2004). The effect of Ca$^{2+}$ and acidification on gap junction channels gating was suggested to be synergistic as elevation of intracellular calcium might cause a fall in intracellular pH (Meech and Thomas 1977). The observation of channel closure upon [Ca$^{2+}$], elevation in the absence of pH decrease (Rose and Rick 1978), together with the fact that no increase in cytoplasmic Ca$^{2+}$ was observed during acidification adequate to decrease junctional conductance (Bennett and Goodenough 1978; Reber and Weingart 1982), provide the evidence that Ca$^{2+}$ and pH can work independently as cell uncouplers. The lacking of Ca$^{2+}$ high affinity binding site on the intracellular part of connexins implicate that the action of Calcium on gating might be mediated by an intermediated component, most likely calmodulin (Peracchia, Bernardini et al. 1983; Verselis, White et al. 1986; De Pina-Benabou, Srinivas et al. 2001). The sensitivity to intracellular calcium concentration is also differing among different connexin/innexin isoforms, and several connexins have been shown to be insensitive to changes in cytoplasmic calcium (Spray, White et al. 1984; Saez, Connor et al. 1989).

Beside the three typical ways of regulation we mentioned above, gap junction channels are also facing a number of other possible gating controls. One of the possibilities concerns channel activation by regulatory kinases. Recent research showed that phosphorylation plays a role in gap junction channel regulation (Lampe and Lau 2000). Indeed, most connexin proteins are phosphoproteins and the carboxyl terminus is the primary site for phosphorylation, so the carboxyl tail is an important element in gap junction channel gating. Some chemical agents, such as long-chain alkanols, anesthetics,
quinine etc. (Harris 2001) were also found capable of gap junction channel regulation, but their mechanisms are still unclear.

1.2 Cellular functions of gap junctions

The cellular function of gap junctions is coordinating the activities of cells in tissues as well as sharing metabolic materials between cells. In the heart, gap junctions allow the rapid cell-to-cell transfer of action potentials, ensuring synchronized contractions of cardiac muscles (Weidmann 1952). The presence of gap junctions is also demonstrated in the nervous system, where they are proposed to play an important role in neurogenesis and brain development in addition to their function as fast electrical synapses (Bruzzone and Dermietzel 2006). Direct electrical communication between endothelial cells and vascular smooth muscle cells via gap junctions is thought to play a relevant role in the control of vasomotor tone and arterial blood pressure (Brisset, Isakson et al. 2009; Xavier, Blanco-Rivero et al. 2009).

More information about the physiological functions of a specific isoform of connexins were obtained from studies of gap junction related inherited human diseases and connexin knock outs in mice. For example, the X-linked form of Charcot–Marie–Tooth disease (CMTX), a neuropathy resulting in progressive degeneration of peripheral nerves, is a disease linked to over 100 different Cx32 mutations (Fairweather, Bell et al. 1994; Ionasescu, Ionasescu et al. 1996; Silander, Meretoja et al. 1997). Mutations in Cx26 are responsible for non-syndromic deafness and skin disease (Kelsell, Dunlop et al. 1997; Richard, White et al. 1998; Scott, Kraft et al. 1998). Giving the high expression level of Cx43 in myocardium and other cell types, disruptions of the gene encoding Cx43
are associated with cranial and limb deformities (Britz-Cunningham, Shah et al. 1995; Paznekas, Boyadjiev et al. 2003). Homozygous Cx37 KO mice resulted in infertile females because the oocytes fail to complete growth due to the lack of gap junction-mediated signals from granulose cells (Simon, Goodenough et al. 1997). Both Cx46 KO and Cx50 KO mice develop cataracts with different timing, morphology and lens growth characteristics (White, Sellitto et al. 2001).

The existence of number of connexin-specific diseases suggests that the connexins can not be functionally replaced by each other.

1.3 Gap junction “hemichannels”

Connexons or innexons are composed of six subunits of gap junction proteins. They are called “hemichannels” because two of them can form a complete gap junction. The hemichannels remain closed until docking to each other (Dahl 1996).

Docking of hemichannels
Figure 5. Docking of two hemichannels. Generally hemichannel is closed. When two hemichannels meet each other, the interaction of extracellular loops changes the confirmation of the proteins and forms an open gap junction channel.

The existence of the hemichannels outside the gap junction region was established by surface labeling, sucrose gradient fractionation and crosslinking studies (Musil and Goodenough 1991; Levine, Werner et al. 1993; Musil and Goodenough 1993). The immunostaining against the extracellular epitopes of connexins also confirmed the existence of connexons because the extracellular space in gap junctions is too narrow for the antibody to gain access (Goodenough and Revel 1971). However, the rationality to have functional connexons is still under debate. So far, no direct effect on putative connexon non-junctional channels has been shown. The evidence for active connexons came from the observation of large channel activities in cells expressing cloned connexins. However, the conditions to obtain opening connexons were very unphysiological, either by depolarizing the cell ≥ +50mV or by elimination of extracellular calcium, with the exception of lens’ specific Cx46&Cx50 and synthesized chimera Cx32E143 (Ebihara and Steiner 1993; Li, Liu et al. 1996; Pfahnl, Zhou et al. 1997; Castro, Gomez-Hernandez et al. 1999; Hu and Dahl 1999; Beahm and Hall 2002; Contreras, Saez et al. 2003). Another complication for the proof of functional connexons is the lack of specific inhibitors for connexons. Most blockers used so far as diagnostic tool to demonstrate the existence of functional connexons are non-specific, such as CBX and mefloquine, which can block innexons and pannexons as well. The connexin mimetic peptides, which were engineered to block the connexin gap junction formation specifically (Dahl 1992; Dahl, Nonner et al. 1994; Warner, Clements et al. 1995; Chaytor, Evans et al. 1997), were used as a proof of active connexons because of their inhibition
on ATP release and/or calcium wave propagation (Braet, Vandamme et al. 2003; Leybaert, Braet et al. 2003; Gomes, Srinivas et al. 2005). But in fact, the connexin mimetic peptides have no inhibitory effect on connexon; on the contrary, they attenuated the current and dye uptake through membrane channels formed by pannexin1 (Dahl 2007; Wang, Ma et al. 2007). In addition, nearly all the active connexon data are based on the expression of exogenous connexins in cultured cells. This might be accompanied by the upregulation of other proteins, which complicates the interpretation.

The hemichannels were initially considered as precursors and components of gap junction channels, without physiological or pathological roles of their own. The reason was that due to their properties, unopposed hemichannels’ opening would be deleterious to the cells. However, growing evidence suggest that pathways similar to hemichannels may exist. For example, cells can take up large extracellular dyes that are only permeable for gap junction channels. Recent findings indicate that large membrane channels can also be modulated under several pathophysiological conditions, such as ischemia. Ischemia opens neuronal large membrane channels, which dramatically alter the permeability properties of membranes and lead to cell death through ionic dysregulation, loss of metabolites, and changes in intracellular ATP (Thompson, Zhou et al. 2006). It seems inappropriate to call this fully functional structure “hemichannel” any more. Instead, they are functional membrane channels with unusually large conductance.

The activity of innexons under physiological conditions was demonstrated by our group recently (Bao, Samuels et al. 2007). In vertebrate, if connexon is unlikely to be the physiological candidate for non-junctional channel, there must be another family of proteins to accomplish these unique cellular functions.
1.4 A second gap junction family in vertebrates--pannexins

Mammalian analogs of innexins, termed pannexins, were recently discovered and classified to be a second family of gap junction proteins in vertebrates (Panchin, Kelmanson et al. 2000; Bruzzone, Hormuzdi et al. 2003). They have some sequence similarity to invertebrate innexin proteins, but share no homology with vertebrate connexins. The human pannexin family consists of three members: pannexin1 (Panx1, 426aa, 47.6kDa), pannexin 2 (Panx2, 664aa, 73.3kDa), and pannexin 3 (Panx3, 392aa, 44.7kDa). Pannexins are widely expressed in different tissues, and at higher level in vertebrate central nervous system (Vogt, Hormuzdi et al. 2005). Pannexin1 is widely expressed in different tissues in rodents and human. The expression of Pannexin 2 is mainly found in the nervous system, and Pannexin 3 is only detected in the skin (Bruzzone, Hormuzdi et al. 2003; Baranova, Ivanov et al. 2004; Vogt, Hormuzdi et al. 2005; Litvin, Tiunova et al. 2006). Electrophysiology studies in oocytes expressing human pannexin1 indicate that this pannexin, like connexin 46 and 50, can form functional channels in the non-junctional plasma membrane, i.e., a membrane channel allowing flux of ions and larger molecules, such as ATP, across the cell membrane (Bao, Locovei et al. 2004; Locovei, Bao et al. 2006). The undocked membrane channels of pannexin 2 or pannexin 3 didn’t show any channel activity, at least in oocytes (Bruzzone, Hormuzdi et al. 2003).

The pharmacological properties of pannexin non-junctional channels were first tested in the heterologus expression system (Bruzzone, Barbe et al. 2005). Several compounds that have widely used to block connexin channels were also inhibitory for pannexin
channels, such as CBX, flufenamic acid, mefloquine, but the magnitude is quite different. Pannexin1 channels showed remarkably high sensitivity to CBX while relative insensitive to flufenamic acid, indicating these chemicals affect connexins and pannexins in different ways.

By hydrophobicity analysis, it was predicted that the pannexins, like connexins and innexins, have four transmembrane domains, two extracellular loops, one intracellular loop and intracellular amino and carboxyl termini (Figure 6a). This prediction was later confirmed by immunostaining, with antibodies directed against the putative first extracellular loop and the carboxyl terminal (Locovei, Bao et al. 2006) (Figure 6b).

Figure 6a. Putative topology of human pannexin1. Antibody 4512 and 4515 were directed against the putative first extracellular loop and carboxyl terminal respectively.
Figure 6b. Antibodies staining of oocytes expressing human pannexin1. Extracellular application of antibody 4512 (a) but not 4515 (c) to intact oocytes expressing human pannexin1 yielded immunostaining of the oocyte surface. Application of antibody 4512 to uninjected intact oocytes did not yield staining (b). Application of antibody 4515 to sectioned oocytes resulted in both membrane and intracellular staining.

1.5 Function of pannexin1 as ATP release channel

1.5.1 ATP signaling

The first evidence of extracellular actions of purine nucleotides and nucleosides in the cardiovascular system was described by Drury and Szent-Gyorgyi in 1929 (Drury and Szent-Gyorgyi 1929). However, it took more than 40 years for people to realize the
importance of adenosine as an autocrine and paracrine mediator in extracellular signaling. In 1972, Burnstock postulated that ATP could act as the transmitter in nerve-mediated responses of the smooth muscles in the gut and bladder (Burnstock, Dumsday et al. 1972). This concept was strengthened by the identification of purinergic receptors several years later (Burnstock 1976).

Today, there is no longer any question that extracellular ATP signaling through purinergic receptors plays crucial biological roles not only in excitable cells but also in non-excitable cells, ranging from neurotransmission, smooth muscle contraction, chemosensory signaling, secretion and vasodilatation, to more complex phenomena such as immune responses, pain, male reproduction, fertilization and embryonic development (Schwiebert and Zsembery 2003; Burnstock 2006; Burnstock 2006).

Calcium wave propagation is a phenomenon observed during several physiological activities, including ciliary beat in airway epithelial cells, synaptic transmission between neurons, metabolic coordination in glia cells and vascular perfusion modulation (Sanderson, Charles et al. 1990; Sigurdson, Sachs et al. 1993; Charles 1998; Newman 2001). The involvement of ATP in the calcium wave propagation has been recognized: Mechanical stimulation or increase of intracellular calcium can induce the ATP release. After release, ATP can act on the purinergic receptors on the nearby cell membrane, which will elicit the increase of cytoplasmic calcium concentration either through direct calcium entry through P2X receptors or through opening of intracellular calcium store by intermediate IP3 involving P2Y receptors (Osipchuk and Cahalan 1992; Frame and De Feijter 1997).
1.5.2 ATP release

The steady-state intracellular ATP concentration is 1–10 mM (Beis and Newsholme 1975), the energy source for metabolic and enzymatic reactions inside the cell. Because ATP is essential for metabolism, release of this precious energy substrate seems counter-intuitive. Moreover, if one assumes that the normal extracellular ATP is approximately 10 nM under basal conditions and intracellular ATP is 10 mM, the gradient for ATP secretion or efflux is approximately $10^6$-fold. If a pathway is activated or opened for ATP release, ATP would exit the cell down a very favorable chemical concentration gradient. However, only 1% or less of the intracellular ATP pool needs to be released to activate maximally any and all receptors. Thus, accomplishment of extracellular ATP signaling can occur without compromising cellular metabolism or essential enzymatic reactions. Furthermore, any alternative signaling mechanism would also consume ATP for synthesis, packaging etc. of the alternate compound.

Two general release modes for ATP release have been proposed: (i) vesicular release, the exocytotic release of transmitters and (ii) channel-mediated release. Although vesicular ATP release is well documented and marked by its sensitivity to Brefeldin A and nocodazole which destroy the Golgi complex and vesicle trafficking to the cell surface (Maroto and Hamill 2001; Bal-Price, Moneer et al. 2002), it cannot account for all of the ATP release phenomena. In particular, ATP is released from erythrocytes, which, under physiological conditions, are vesicle-free (Bergfeld and Forrester 1992). Various channels have been implicated in this process, including CFTR (cystic fibrosis transmembrane conductance regulator) (Reisin, Prat et al. 1994), connexin 43 (Cx43)
hemichannels (Cotrina, Lin et al. 1998), a volume regulated channel (VRAC) (Hisadome, Koyama et al. 2002), maxi-anion channel (Sabirov and Okada 2004) and the purinergic receptor P2X7 (Suadicani, Brosnan et al. 2006). However, the precise molecular nature of these interactions is still not clear, and the lacking of specific pharmacological blockers for any of these candidates hampered documentation of their involvement.

Once ATP is released from the cell, it will be broken down rapidly into its basic components ADP or adenosine; thus, it is thought to act locally in an autocrine or paracrine manner within tissues and tissue microenvironments.

1.5.3 Pannexin1 and ATP

Mechanical stress is a prime stimulus for ATP release in many cell types, including erythrocytes (Sprague, Ellsworth et al. 1998). A channel which is both mechano-sensitive and ATP-permeable would be the most probable candidate for the efficient release. Pannexin1 fulfills these specifications: First of all, pannexin1 is permeable for ATP (Figure 7). Pannexin1 is found in endothelium cells and erythrocytes where calcium waves are documented (Locovei, Bao et al. 2006). It forms a mechano-sensitive, ATP-permeable channel when expressed in *Xenopus* oocytes (Bao, Locovei et al. 2004). ATP release reduced significantly with application of pannexin1 channel blockers on the red blood cells (Locovei, Bao et al. 2006; Silverman, Locovei et al. 2008). In addition, increased intracellular calcium level opened pannexin1 channels. Thus, it is reasonable to propose that pannexin1 membrane channel may function as the ATP release channel and mediate the calcium wave propagation.
Figure 7. ATP flux through pannexin1 channels. (a) ATP release from oocytes was determined by luminometry. When depolarized by elevated potassium in the extracellular fluid, uninjected oocytes and oocytes expressing connexin 43 released ATP to the extracellular space, probably by a Brefeldin-sensitive, vesicular mechanism [Maroto, 2001]. Expression of pannexin1 resulted in significantly increased ATP release (p < 0.01, versus control KGlu). (b) A voltage ramp was applied to an excised (inside-out) patch containing a single pannexin1 channel. To assess permeability properties of the channel, an ion gradient was applied. The pipette solution contained 50 mM and the bath solution 5 mM K2ATP. The average reversal potential from five independent measurements was +25 mV, indicating substantial permeability of the channel for ATP3-

Giving the fact that pannexin1 channels can be activated by extracellular ATP when coexpressed with P2Y receptors (Locovei, Wang et al. 2006), this channel is proposed to mediate ATP-induced ATP release, which is observed in several cell types, including erythrocytes (Di Virgilio, Chiozzi et al. 2001). Recent studies on pannexin1 have shown that this protein is also part of the pore forming unit of the P2X7 receptor death complex,
which means it is the molecular substrate for the permeabilization pore (or death receptor channel) recruited into P2X7 receptor signaling complex (Pelegrin and Surprenant 2006; Locovei, Scemes et al. 2007). The proposed activation scheme is: ATP binds to P2X7 receptors which somehow induce the opening of the pannexin channels. More ATP can be released from this channel to the extracellular space and stimulate the receptors on the neighboring cells and itself. Indeed, if these channels are to be kept open, cell constituents would rapidly leak out of the cell, causing cell death (Figure 8).

![Figure 8](image)

**Figure 8.** Membrane currents and morphology of oocytes co-expressing P2X7 receptor and Panx1 with prolonged exposure to ATP. (a) Brief exposure to 200 µM ATP resulted in a reversible inward current. Longer exposure to 500 µM ATP resulted in a current that initially reversed partially but subsequently increased to levels that could not be clamped anymore, indicating membrane breakdown. (b) Non-injected control oocytes (upper left quadrant) and oocytes co-expressing P2X7 receptor and Panx1 (lower right quadrant) were exposed to 300 µM ATP. (c) Within 3 min a dramatic change of pigmentation was observed in the co-expressing but not in the control oocytes. (d) After 5 min all co-expressing oocytes sprang leaks with yolk oozing out of the cells.

### 1.6 Regulation of pannexin1

Current hypotheses of pannexin1 channel function in the mammalian brain are: 1) intercellular pannexin1 channels might represent a novel class of electrical synapses with possible roles in the generation of oscillatory and synchronous activity. 2) Pannexin1 in
cell membranes forms functional membrane channels, connecting the cytoplasm to the extracellular space (Dahl and Locovei 2006). Conforming to the first hypothesis, earlier studies by Bruzzone et al. (Bruzzone, Hormuzdi et al. 2003) in paired *Xenopus* oocytes found that expression of pannexin1, alone and in combination with pannexin2, forms intercellular channels. However, this gap junction formation is less than 1% effective as that by connexins (Boassa, Ambrosi et al. 2007). Studies in erythrocytes, cultured neurons and glia did not detect the canonical gap junctions of pannexins; they only observed plasma membrane staining (Locovei, Bao et al. 2006; Huang, Grinspan et al. 2007). The glycosylation site on the extracellular loop of pannexin1 determines its trafficking to the cell surface but likely impedes formation of intercellular channels (Boassa, Gaietta et al. 2006; Penuela, Bhalla et al. 2007), pointing towards the second hypothesis that the main physiological role of pannexin1 on cell membrane may be forming nonjunctional membrane channels.

Pannexin1 membrane channels as proposed by hypothesis 2 could release small molecules to the extracellular space. If ATP were released, the channel may promote the propagation of calcium waves. Pannexins are expressed in many tissues, and at particularly high level in vertebrate central nervous system (Vogt, Hormuzdi et al. 2005). Electrophysiological studies in *Xenopus* oocytes expressing human pannexin1 have observed that pannexin1, like connexin 46 and 50, forms a membrane channel conducting ions and larger molecules, such as ATP, between the intra- and extracellular spaces (Bruzzone, Hormuzdi et al. 2003; Bao, Locovei et al. 2004). The pannexin1 channel can be activated by positive membrane potentials to produce a unitary conductance up to 475pS in 150mM potassium gluconate or 550pS in 150 potassium chloride solutions. The
individual channel conducts in at least five levels. Besides the maximal level of conductance, at least four levels of subconductance occur with 5%, 25%, 30% and 90% of the full conductance. Channel activity is increased by mechanical stretch, indicating pannexin1 is mechanosensitive. When depolarized by potassium solution, oocytes expressing pannexin1 exhibit significantly large ATP efflux than noninjected oocytes. These properties of pannexin1 are consistent with a role in ATP release.

The role of ATP as an extracellular signaling molecule is well recognized (Burnstock and Knight 2004). This signaling is mediated by two types of purinergic receptors: the metabotropic P2Y receptors and the ionotropic P2X receptors. When coexpressed with P2Y and P2X7 receptors pannexin1 channels can be activated by extracellular ATP (Locovei, Wang et al. 2006; Locovei, Scemes et al. 2007). Application of ATP to oocytes co-expressing pannexin1 and P2Y2 results in large inward currents (Figure 10). Cells expressing P2Y2 or pannexin1 alone did not exhibit this response. Ionotropic P2X7 receptors respond to ATP or analogue agonists by opening small non-selective cation channels (Burnstock 1995; North 2002). In oocytes, ATP induced an inward current in cells expressing P2X7 receptor, but not in non-transfected oocytes; co-expression of P2X7 and pannexin1 increased the inward current about 6 fold (from 0.5µA in P2X7 to 3µA in P2X7+pannexin1 (Locovei, Scemes et al. 2007)). We know that pannexin1 is a large channel; prolonged opening of this channel would allow leakage of vital cell constituents and cause cell death. Therefore, there must be some deactivation mechanisms to keep this potentially harmful channel in check. Two mechanisms have been proposed to regulate pannexin1 activity. One involves the conserved intracellular serine/threonine phosphorylation sites of pannexin1. These sites are potential targets for
regulatory kinases, such as PKC, PKA, PKG and CKII, which modify pannexin1 channel activity (Barbe, Monyer et al. 2006). Another mechanism concerns the involvement of beta-subunit of voltage-dependent potassium channels (Kvβ3) (Bunse, Haghika et al. 2005). However, neither mechanism provides straight negative-feedback. In fact, Kvβ3 even attenuates pannexin1 sensitivity to inhibitors (Bunse, Locovei et al. 2009). When co-expressed with purinergic receptors, Pannexin1 channel is opened by extracellular ATP. Due to the large ATP permeability, this should result in a positive feedback loop keeping the channels permanently open. However, the opposite is observed. Exposure of cells co-expressing pannexin1 with either P2Y2 or P2X7 receptors to ATP results only in a transient activation of pannexin1 channels. They inactivate in the prolonged presence of ATP (Figure 9). Thus, we hypothesize that ATP itself exerts negative feedback by acting directly on the pannexin1 channel to prevent the prolonged lethal leak from the cell. This mechanism could contribute to the deactivation of long-range calcium wave propagation.

**Figure 9.** Dual ATP effect on membrane currents of oocytes expressing P2Y2+Panx1 or P2X7+Panx1. (a) 100 µM ATP induced a large inward current in P2Y2 and pannexin1 coexpressed oocyte, which inactivated
in the presence of ATP. (b) Brief exposure to 200 µM ATP resulted in a reversible inward current. Longer exposure to 500 µM ATP resulted in a current that inactivated at the presence of ATP. The subsequent increase to levels that could not be clamped anymore is the indication of membrane breakdown.
Chapter 2

Materials and Methods

2.1 Plasmid and synthesis of mRNA

Mouse pannexin1 was kindly provided by Dr. Rolf Dermietzel (University of Bochum), and Cx46 was obtained from Dr. D. L. Paul (Harvard University). The gene encoding mouse pannexin1 was inserted in vector pCS2 and Cx46 was contained in plasmid rSP64T.

Mouse pannexin1 and Cx46 were linearized with \textit{Not I} and \textit{EcoR1} respectively. All restriction enzymes were purchased from New England BioLabs, Ipswich, MA, USA. In vitro transcription was performed with SP6 polymerase, using the Message Machine kit (Ambion, Austin, TX). mRNAs were quantified by absorbance (260nm), and the proportion of full-length transcripts was checked by agarose gel electrophoresis.

2.2 Preparation of oocytes

All procedures were conducted in accordance with the “Guiding Principles for Research Involving Animals and Human Beings” of the American Physiology Society. \textit{Xenopus laevis} oocytes are prepared by collagenase treatment. First, we cut the ovary into small pieces and incubate them by stirring in Ca$^{2+}$ free Ringer solution with 2.5mg/ml collagenase (Worhington, Lakewood, NJ, USA) at room temperature. The regular Ringer solution contains 82.5mM NaCl, 2.5mM KCl, 1mM Na$_2$HPO$_4$, 1mM MgCl$_2$, 1mM CaCl$_2$ and 5mM HEPES and is called OR2 solution. After 3~4 hours incubation, the follicle cells were digested and the oocytes were isolated. The oocytes were washed thoroughly with regular OR2 and mature cells (>1000 micron) with even
pigmentation were selected (Figure 10) and kept in regular OR2 with 10mg/ml streptomycin and 10,000 U/ml penicillin at 18°C.

![Figure 10. Selected oocytes under microscope](image)

2.3 Preparation of erythrocytes

Xenopus Laevis blood was collected in Ca^{2+} free OR2 with 5mM EGTA and spin at low speed. The upper layer of the suspension was removed and the packed erythrocytes were washed three times in OR2 solution with 5mM Glucose by low-speed centrifugation. Erythrocytes were suspended in OR2 with antibiotics and Glucose at 20% hematocrit and kept at 4°C. Cells were diluted to 0.1% by regular OR2 for dye-uptake experiment.

2.4 Electrophysiology-two electrodes voltage clamp

Whole cell membrane current of single oocytes was measured using a two-electrode voltage clamp and recorded with a chart recorder. The oocyte was impaled with two electrodes, one called voltage electrode which is for clamping the membrane potential at a certain level and the other called current electrode for current injection to maintain the desired voltage. Both voltage-measuring and current-passing microelectrodes were pulled with a vertical puller (Kopf) and filled with 3M KCl. The resistance of these pipettes was
1 to 5 M\(\Omega\) in regular OR2. An Ag/Cl reference electrode was inserted in a chamber filled with 3M KCl, which was electronically connected to the bath via an agar bridge. The recording chamber was perfused continuously with solution powered by a low-flow pump (VWR). Membrane conductance was determined using voltage pulse. Oocytes expressing Cx46 were held at -10mV, and depolarizing pulses of 5s duration and of 10mV amplitude were applied. Oocytes expressing pannexin1 were held at -50mV, and pulses to +50mV were applied to transiently open the channels.

![Illustration of two-electrodes voltage clamp setup.](image)

**2.5 Single-channel patch clamp**

Single pannexin1 channels were studied by the patch-clamp technique (Hamill, Marty et al. 1981) using a WPC 100 amplifier (E.S.F. Electronic, Goettingen, Germany). The vitelline membrane of the oocyte was manually removed and the oocyte was washed once before transfer into a new dish containing sodium chloride solution (140mM NaCl, 10mM KCl and 5mM TES, pH 7.5). Electrode pipettes made from glass capillary tubing (1.5-0.86mm, #GC150F-15, Warner Instrument Corporation) were pulled using a Flaming-Brown Micropipette Puller (Model P-97, Sutter Instrument Company) and
polished with a microforge (Narishige Scientific Instruments) to 0.5-1 mm with resistances of 10-20 MΩ in NaCl solution. Both the standard pipette and bath solution were NaCl solution. After an outside-out patch was excised from the membrane and the pannexin1 channel was identified, the patch was transferred into a microperfusion chamber, which was continuously perfused with solution. The perfusion system was driven by gravity at a flow rate of 100µl/s.

2.6 Dye uptake

*Xenopus* erythrocytes were washed three times in Ringer solution by low-speed centrifugation. Erythrocytes were suspended at 0.1% hematocrit and aliquots of 75µl were plated onto poly-D-lysine coated 96-well plates (BioCoat, Becton Dickinson). 25µl OR2 alone or with 4 times concentration of drugs were added and pre-incubated for 10 min (final volume 100 µl). 85µl of solution was removed from the well and dye uptake was initiated by adding 100µl KGlu (140mM KGluconate, 10mM KCl, 5mM TES, pH 7.5) solution with 5 µM YoPro-1 with or without drugs. Addition of 100 µl OR2 with YoPro-1 instead of KGlu served as negative control. Images were acquired with a Canon Powershot S3 IS digital camera with an exposure time of 6 sec and an aperture setting of 3.2 attached to the phototube of an inverted fluorescence microscope (Leica, model DMIL).

2.7 ATP release assay

ATP flux was determined by luminometry. Oocytes after two days injection of pannexin1 messenger RNA were pretreated in OR2 solution with and without BBG for 10 minutes and stimulated by incubation in OR2 solution (negative control), KGlu
solutions (positive control), KGluc solution with BBG, respectively, for 10 minutes. The supernatant was collected and assayed with luciferase/luciferin (Promega, Madison, USA).

2.8 Site-directed mutagenesis

The alanine mutants were engineered with QuikChange II site-directed mutagenesis kit (Stratagene) according to the manufacturer’s specifications (see Appendix).
Chapter 3

RESULT

3.1 Inhibition of pannexin1 channels by ATP and its analogues

To test whether pannexin1 is blocked by ATP, two different approaches were designed: (1) Test for an inhibitory effect of ATP on electrical currents carried by pannexin1 in whole cells and in membrane patches; (2) Monitor the ATP effect on dye-uptake and ATP release of pannexin1.

3.1.1 Inhibition of pannexin1 currents by ATP and ATP analogues

At negative potentials, pannexin1 channels are closed. Application of ATP does not activate pannexin1 unless purinergic receptors are also present. Pannexin1 channels open at positive membrane potentials. To test ATP effects on open pannexin1 channels I used *Xenopus* oocytes expressing mouse pannexin1 exogenously. From a holding potential of -50 mV I applied voltage steps to +50 mV with 5s duration to induce pannexin1 mediated currents (Figure 12). Application of ATP attenuated the pannexin1 currents reversibly. The effect of ATP on pannexin1 channels appeared to be specific as channels of similar pore size formed by Cx 46 were not affected by ATP. As shown in Figure 12a, 200µM ATP inhibits pannexin1 current by more than 20% and this inhibition is reversible and repeatable. BzATP, a more potent P2X receptor agonist, is more efficient than ATP in pannexin1 channel inhibition; 20µM BzATP inhibits the current about 20% (Fig. 12b). The depolarization-induced Cx46 current is not attenuated by ATP or BzATP. Rather a small augmentation is observed, suggesting that the inhibitory effect of ATP on the pannexin1 channel is not general for channels formed by gap junction proteins (Fig. 12c).
Figure 12. ATP and BzATP inhibit pannexin1 membrane currents but not currents carried by Cx46. (a) -50mv to +50mv voltage pulses induce large membrane currents in oocytes expressing mouse pannexin1 channels, which are reversibly attenuated by 200 µM ATP. (b) The pannexin1 currents are similarly inhibited by 20 µM BzATP. (c) 200 mM ATP and 50 mM BzATP had no inhibitory effect on currents carried by Cx46, which were induced by -10mV to 0mV voltage steps.

Purinergic receptors interact with a variety of ATP analogues and ATP metabolites, some activating, others inhibiting the receptors (Burnstock 1995). The affinity of these agents to different purinergic receptors varies and has given rise to a classification of
purinergic receptors which was subsequently confirmed by cloning of the various P2Y and P2X subtypes (Burnstock and Kennedy 1985). The higher affinity of BzATP over ATP is a characteristic property of the P2X7 receptor. To test the specificity of ATP on pannexin1 currents we tested different purinergic receptor agonists and antagonists. Figure 12 shows that the ATP metabolites ADP and AMP were ineffective in inhibiting currents through pannexin1 channels, which is consistent with their relatively weak interaction with P2X7 receptors. Suramin is a broad antagonist of purinergic receptors and most potent for P2Y2 receptor, blocking with an IC$_{50}$ of ~10µM (Wilkinson, McKechnie et al. 1994; Wilkinson, Purkiss et al. 1994; Wildman, Unwin et al. 2003). It inhibits pannexin1 channel as effective as BzATP (IC$_{50}$~100µM) (Figure 14). The strongest inhibition is observed with BBG, which attenuates channel activity almost completely at a concentration ~10µM (Figure 13). Effects of UTP, cAMP, and A438079 (‘specific antagonist’ for P2X7 receptor (King 2007)) are also assessed and shown in figure 13.
Figure 13. Pannexin1 current inhibition by various purinergic receptor agonists and antagonists.

3.1.2 Concentration dependence of pannexin1 current inhibition

The dose-response curves are usually used in toxicology and pharmacology to determine “safe” or “hazardous” levels and the dosages for drugs, potential pollutants, and other substances exposed to our life. The dose-response relationship in our
experiment is actually the concentration response relationship, which tells us the degree of inhibition with different concentrations of effective blockers. In contrast to a traditional receptor binding curve, the full active response of our channel is elicited by voltage pulses instead of a full agonist. From the dose-response curves, we can get some information about the apparent affinity of a drug to the pannexin1 channel as well as comparison the pharmacologic profiles of different drugs.

![Dose-response curves for inhibition of pannexin1 currents by ATP and various analogues.](image)

**Figure 14.** Dose-response curves for inhibition of pannexin1 currents by ATP and various analogues. Means ± SEM are plotted (n = 3-5).

We tested various concentrations of BBG, BzATP, Suramin, and ATP on pannexin1 currents (Figure 14). The highest ATP concentration we could test on oocytes expressing pannexin1 was 1mM. Higher concentrations of ATP induced an unknown inward current in both injected and uninjected cells (Figure 15). The ATP inhibitory effect on pannexin1 will therefore be underestimated at ATP concentration higher than 1 mM. The activity
spectrum with which the various substances affected pannexin1 currents closely resembles that on the P2X7 receptor (North and Barnard 1997), except that both P2X7 agonists and antagonists inhibited pannexin1 currents.

**Figure 15.** An inward current of unknown origin was induced in uninjected oocyte by 5mM ATP but not 1mM ATP.

Figure 16 shows the different concentration requirement for activation and inhibition of pannexin1. The BzATP concentration required for inhibition of pannexin1 is higher than required for activation of pannexin1 by ATP through P2X7. This makes the ATP-induced ATP release mechanism at least transiently possible despite the inhibitory effect of ATP on the release channel. Low concentration of ATP activates the purinergic receptors and initiates the signaling pathway, so that more ATP is released through pannexin1 channels. As the released ATP builds up to a higher concentration, it terminates further ATP release by closing the ATP release channel-pannexin1 before causing any damage.
3.1.3 Possible inhibitory mechanism

Typically, a larger ligand yielded stronger channel inhibition as if the molecules plugged up the pore and thereby inhibited the channel. However, not all compounds obeyed this relationship (table 1). Compounds like BBG are known to have high affinity for purinergic receptors. It thus appears that the inhibition of pannexin channels is on the
basis of an affinity dominated steric block of the channel. The effect of carbenoxolone is also listed in the table as a reference.

Table 1 Inhibitory effect of various chemicals on currents carried by pannexin1

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Molecular Weight</th>
<th>Concentration tested</th>
<th>Inhibitory effect on Panx1</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>347.2</td>
<td>500µM</td>
<td>8%</td>
</tr>
<tr>
<td>cyclic AMP</td>
<td>351.2</td>
<td>100µM</td>
<td>No effect</td>
</tr>
<tr>
<td>ADP</td>
<td>427.2</td>
<td>500µM</td>
<td>8%</td>
</tr>
<tr>
<td>Pyrophosphate</td>
<td>446.06</td>
<td>1mM</td>
<td>7%</td>
</tr>
<tr>
<td>Oxidized ATP</td>
<td>505.2</td>
<td>100µM</td>
<td>No effect</td>
</tr>
<tr>
<td>UTP</td>
<td>550.1</td>
<td>250µM</td>
<td>No effect</td>
</tr>
<tr>
<td>ATP</td>
<td>551.1</td>
<td>100µM</td>
<td>20%</td>
</tr>
<tr>
<td>PPADS</td>
<td>599.31</td>
<td>10µM</td>
<td>10%</td>
</tr>
<tr>
<td>BzATP</td>
<td>715.4</td>
<td>100µM</td>
<td>53%</td>
</tr>
<tr>
<td>KN-62</td>
<td>721.84</td>
<td>50µM</td>
<td>40%</td>
</tr>
<tr>
<td>Brilliant blue G</td>
<td>854</td>
<td>5.58µM</td>
<td>66%</td>
</tr>
<tr>
<td>Suramin</td>
<td>1429.2</td>
<td>100µM</td>
<td>52.5%</td>
</tr>
<tr>
<td>Polyethylene glycol</td>
<td>1500</td>
<td>50mM</td>
<td>19%</td>
</tr>
<tr>
<td>Carbenoxolone</td>
<td>614.7</td>
<td>20µM</td>
<td>90%</td>
</tr>
</tbody>
</table>

Discrimination of gating and steric effects on channels typically can easily be achieved by single channel analysis. However, pannexin1 channels are not conducive for
this type of analysis because of their unusual properties. Already in the absence of interfering agents pannexin1 channels exhibit multiple subconductance levels. Figure 17 shows a record from an excised outside-out membrane patch containing more than one pannexin1 channel. The channel is activated when clamped at +50mV and inactivated when the clamping potential jumps to -50mV. Channel activity can be ascribed to pannexin1 because of the attenuation by carbenoxolone (CBX), a known pannexin1 inhibitor (Bruzzone, Barbe et al. 2005), which has no endogenous target channel in Xenopus oocytes. Application of BzATP or BBG had similar effects on channel activity as carbenoxolone. The complex channel currents were reduced to discernible unitary events (Figure 17b) with conductances that are only a fraction of the full conductance of ~500 pS of pannexin1 channels. The drugs, therefore, either attenuated the channel conductance by steric block or they forced the channel into any one of the lower subconductance states.

3.1.4 ATP release from pannexin1 expressing oocytes is inhibited by BBG

As mentioned before, cumulative evidence show that pannexin1 is the most probable candidate to be the ATP release channel. To verify this hypothesis, I measured ATP release from pannexin1 channel expressing oocytes. Non-transfected oocytes, when depolarized by extracellular potassium, release ATP via an endogenous mechanism sensitive to Brefeldin and thus thought to be vesicular (Maroto and Hamill 2001).
Figure 17. Channel activities in an outside-out membrane patches excised from oocytes expressing pannexin1 are decreased by BzATP and BBG. (a) BzATP and BBG have an acute inhibitory effect on pannexin1 channel which is identified by the inhibition of Carbenoxolone. (b) the current trace in the box was amplified to give a clear view of channel activities.

Oocytes expressing pannexin1 release ATP at a significantly higher level than uninjected oocytes when exposed to high potassium solution (Bao, Locovei et al. 2004;
The released ATP was measured by luciferin/luciferase assay. The effect of ATP analogues on pannexin1 channels was not limited to inhibition of ionic currents. BBG, for example, also inhibits ATP release from oocytes expressing pannexin1 exogenously in a concentration dependent fashion (Figure 18). Among those drugs effective for pannexin1 current inhibition, BBG is the only one that does not interfere with the luciferin/luciferase assay.

**Figure 18.** ATP release from oocytes expressing pannexin1 was inhibited by BBG. The ATP release was induced by high potassium solution and the ATP level was measured by luminometry using a luciferase
assay. This ATP release is attenuated by BBG in a dose-dependent way (n=5). The control was done in solutions containing 10nM ATP.

3.1.5 ATP inhibition of dye uptake of erythrocytes

To test whether the inhibition of pannexin1 channels by ATP and its analogues may also apply in vivo, we examined the effect of these chemicals on erythrocytes. These cells release ATP under shear stress and in response to a low oxygen environment, and the release appears to be mediated by pannexin1 channels (Bergfeld and Forrester 1992; Sprague, Ellsworth et al. 1998; Locovei, Bao et al. 2006). Under conditions of ATP release, a concomitant uptake of extracellular dyes like carboxyfluorescein can be observed (Locovei, Bao et al. 2006), which is a convenient surrogate measure for ATP release and has been used as such in several cell types.

Here we used the nucleated frog erythrocytes in combination with YoPro-1, a dye that fluoresces highly only when bound to nucleic acid, to test the drug inhibition on dye-uptake. Figure 19 shows that the uptake of YoPro-1 by erythrocytes was attenuated by BBG. Curiously, when applied at their respective IC_{50} concentrations for current inhibition the rank order for inhibition was altered, BzATP was most effective in inhibiting dye uptake, followed by ATP and BBG was least effective (Figure 19&20).
Figure 19. Uptake of the fluorescent molecule YoPro by erythrocytes from *Xenopus*. (a) Fluorescent micrographs were taken 15 minutes after application of YoPro (5µM) in OR2 (left), KGlù (middle) and KGlù with 5µM BBG (right). (b) YoPro will be fluorescent when it binds to the nucleotides. So the double exposure to the fluorescent light and transmitted light pictures are taken to differentiate the living cells from the naked nuclei. (c) Quantitative analysis of dye uptake in (a) using the NIH image analysis program. n>50, p<0.0001. (d) Quantitative result from the plate reader. n=9, p=0.0034.
Figure 20. Uptake of dye molecule YoPro in *Xenopus* erythrocytes is attenuated by ATP and BzATP. n>40, p=0.0019.

One possible explanation for the discrepancy between current inhibition and dye-uptake inhibition rank order is: BzATP and ATP may inhibit the current through
decreasing the conductance, either by steric block or by forcing the channel into subconductance state, leading to the exclusion of the large molecules like fluorescent dyes; Whereas BBG may decrease the macro current through lowering the open probability, which will still allow the large molecules to go through if given enough time.

### 3.2 Inhibitory mechanism: ATP-binding site on pannexin1

The inhibition of pannexin1 mediated currents by ATP and analogues is probably not by simple partitioning of these molecules in the channel and thereby interfering with the flow of smaller ions. The concentrations of the reagents required for observing current inhibition are too low for such an effect. Furthermore, the deviation from a strict size-dependence is not consistent with such a mechanism. Instead, binding of ATP to the pannexin1 protein appears to be a plausible mechanism. Now the question is where is the putative binding site on pannexin1 for ATP.

The effective concentrations for channel inhibition by ATP applied from the extracellular site are considerably lower than the ATP concentration prevailing in the cytoplasm. Thus, putative binding sites can be expected to be on the external portion of the pannexin1 protein. Further support for an extracellular site comes from the observation that extracellular application of drugs to closed pannexin1 channel affected the channel before the channel was opening (Figure 21).
Figure 21. BBG has an inhibitory effect on pannexin1 channel before the channel was open.

Site-directed mutagenesis is the most common method to probe the functional structure of a protein. In ion channel structure-function studies, mutagenesis analysis is applied to identify amino acids important for channel gating, such as voltage sensors for a voltage-gated ion channel and ligand-binding sites for a ligand-gated ion channel.

3.2.1 Alanine mutation on R/K residues on the extracellular loops

Giving the observation that the inhibition of pannexin1 channels exhibited a similar pharmacology as the P2X7 receptor, we hypothesized that ATP might act on pannexin1 in a similar way as P2X7 receptor by ligand binding. None of the canonical ATP binding sites was found in the pannexin1 sequence. The same applies to P2X7 receptors, for which, however, the contribution of positively charged amino acids to ATP binding has been established (Ennion, Hagan et al. 2000; Jiang, Rassendren et al. 2000). There are also several positively charged residues in the putative extracellular loops of pannexin1.
We started with mutations of these residues to see whether they are involved in the effect of ATP on pannexin1 channels.

**Figure 22.** Positively charged residues in the putative extracellular loops of pannexin1

Alanine mutations of K248 and K265 yielded no channel activity, either due to disturbed membrane expression or malfunction of the channel. Figure 23 shows that conversion of arginine in position 75 to alanine attenuated the inhibitory effect of ATP and BzATP on channel currents. Mutation of any other of the remaining 4 arginines or lysines was inconsequential for both channel activity and its inhibition by ATP (Figure 24).
Figure 23. ATP and BzATP effects on R75A mutant. (a) The large membrane currents through R75A mutant expressed oocytes were not affected by 500µM ATP and decreased a little by 50µM BzATP. (b) BzATP dose response curve of wild type pannexin1 and R75A mutant.
We, therefore, focused on position 75 and made additional mutations. We did detailed mutational analysis on BzATP effect because the following reasons: 1) high concentration of ATP would induce an unknown current from even non-transfected cells; 2) BzATP is much more stable than ATP, which would benefit the massive and laborious mutation screening; 3) ATP and BzATP have almost the same effect on pannexin1 channel inhibition except that BzATP is a stronger blocker, indicating they might share the same binding site. As figure 25 shows, replacement of the arginine with lysine at R75 had no consequence for the inhibitory effect of BzATP on pannexin1 currents. Replacement of R75 with glutamate completely abolished the effect of BzATP on pannexin1 currents. Cysteine replacement decreased the sensitivity for BzATP to a similar extent as the alanine replacement. The channel properties of these mutants are almost the
same to wt including expression time course in oocytes, membrane conductance, voltage activation and sensitivity to carbenoxolone. Therefore, the effects of drugs on these mutants and wt are comparable. Figure 25 shows the BzATP inhibitory effect on different mutations and the difference between these mutants are more clear. The mutagenesis data suggest that the charge carried by arginine at 75 position is important for BzATP effect, while the side chain effect can not be excluded so far. The slower action of BzATP on R75K (figure 25d) may be caused by the laminar flow of the solution in the chamber, which has also been found in the control condition sometimes.
**Figure 25.** BzATP effects on different R75 mutants. (a-e) effect of 100µM BzATP on wt and R75A, R75C, R75k, R75E mutants. (f) dose-response curve of BzATP on wt and different R75 mutants.

Furthermore, we tested the BzATP and BBG, two representative P2X7 receptor agonist and antagonist, to see how different mutations affect different drugs (Figure 26). Comparing to the wt, R75K mutation did not change the BzATP sensitivity but showed no response to BBG, and the glutamate replacement of R75 eliminated both BzATP and BBG inhibition. The quantitative inhibitory result of 100µM BzATP and 1µM BBG on mutants 75K and 75E indicates that these two drugs have distinct but overlapping binding site on pannexin1.

![Figure 25](image)

**Figure 26.** Effect of BzATP and BBG on R75K and R75E mutants. (a) The 1 µM BBG inhibitory effect on the pannexin1 current was disappeared in R75K and R75E mutants. (b) Comparing the BzATP and BBG effect on the R75 mutants with wt.
3.2.2 Alanine scanning mutagenesis on the extracellular loops

The previous mutagenesis data implies that ATP works on pannexin1 through ligand-binding and R75 plays an important role in the binding. To further characterize the ATP binding site, we did alanine scanning mutagenesis on the extracellular loops of pannexin1.

Alanine scanning mutagenesis is a simple and widely used technique to probe protein structure and function. Alanine is considered to be a relatively conservative substitution since it ‘eliminates the side chain effect without altering the main-chain conformation or imposing any extreme electrostatic or steric effects’ (Cunningham and Wells 1989; Lefevre, Remy et al. 1997). The traditional alanine scanning mutagenesis is to replace a stretch of residues by alanines in order to avoid laborious single amino acid replacements. Here we substitute the residues on the extracellular loops with alanine one at a time to get more detailed information about functional residues for ATP inhibition.

Several online softwares, i.e. TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) and TMHMM (http://www.cbs.dtu.dk/services/TMHMM) were used to predict the transmembrane segments so as the extracellular loops of mouse pannexin1. The first extracellular loop is from I58 to K107 and the second extracellular loop is from S237 to G273.

All the residues on the putative extracellular loops were systematically substituted by alanine one at a time and the endogenous alanine were replaced by cysteine. Of the 87 mutants we engineered, 30 mutants did not exhibit any channel activity when expressed on oocytes. Again, we don’t know whether it’s due to impaired channel function or interrupted transportation to the cell membrane as a result of the mutations.
The common feature for all the gap junction proteins is they bear several conservative cysteines on the extracellular loops, 6 for connexins and 4/6 for innexins, which form 2(3) disulfide bonds and are proposed to be critical for the structure of the proteins and gap junction formation (Dahl, Levine et al. 1991). There are also 4 characteristic cysteines on the extracellular loops of pannexin1. Mutations on any of the four cysteines led to loss of channel function. This result confirms the importance of these extracellular cysteines for protein structure.

Except for those null mutants, mutations on other residues resulted in functional membrane channels similar to wt. The effect of 100µM BzATP on these mutants was tested. Reversible inhibition are still displayed in most of the mutants with various levels, but disappeared completely in mutants W74A, S237A, S240A and L266A, indicating these four amino acids are critical for ligand binding (Figure 27).

Figure 28 shows the quantitative result from the first extracellular loop. We tested 5 mutants in a row for each batch of oocytes and used the wt as the control. The 100µM BzATP inhibition on wt has some variation between different batches of cells, so we normalized the data to the wt in each batch. The W74 seemed so essential for BzATP interaction that mutations on these two residues did not respond to 100µM BzATP at all. Mutations on other three amino acids R75, S82, S93 and L94 have obviously decreased BzATP sensitivity, suggesting their supporting roles in the binding.
Figure 27. 500 μM ATP and 100 μM BzATP inhibited the pannexin1 channels but were ineffective in some of the alanine mutations on the extracellular loops.
Figure 28. Normalized 100 µM BzATP effect on alanine mutations of the residues on the first extracellular loop.

Mutations on S59, Q63, I64, S65, C66, F67, F72, F79, D81, Y83, C84, A86, A87, Q89, Q90, K91, Q95, G99, P102, L103 are not shown in the figure because these mutants did not give normal channel activity.

On the second extracellular loops, mutation on S237, S240, I247 and L266 decrease the BzATP effect dramatically and mutation on D241, S249, P259 and I267 decreased the BzATP inhibitory effect partially (Figure 29).
Figure 29. Normalized 100 μM BzATP effect on alanine mutations of the residues on the second extracellular loop.

Figure 30 shows the relative localization of those effective amino acids on the extracellular loops. All the previous mutagenesis data demonstrate that these amino acids are critical for ligand binding in mediating ATP sensitivity. They are either direct binding partner for ATP or essential for holding of the ATP binding pocket. But to answer the question how they work together to form the ATP binding site needs detailed crystal structure information about the pannexin1.
Figure 30. Relative localization of amino acids that are effective in changing the ATP effect in pannexin1 channel inhibition.
Chapter 4

Discussion

4.1 Does pannexin1 form gap junctions?

Pannexins are a recently identified second gap junction family in vertebrates. They are expressed in many organs of mammals and thus are expected to perform a basic function. The coexistence of two unrelated gap junction families in vertebrate raises the questions whether they are functionally redundant to each other and whether they are diverged from a common ancestor to form similar channels. The existence of a series of connexin diseases suggests that specific connexins are not functionally replaced by other connexins or by pannexins despite their overlapping expressions.

Connexins are the first identified gap junction family and their ability to mediate direct cell-cell coupling is well established. A membrane channel activity of connexons has been inferred from the effect on membrane current of drugs known to block intercellular coupling. Opening of connexin membrane channels is seen under unphysiological conditions, such as at membrane potentials $\geq 50\text{mV}$, or at extremely low extracellular calcium concentration (Li, Liu et al. 1996; Contreras, Saez et al. 2003). Most of the evidence for the connexons to be the ATP release channel comes from pharmacological intervention. Nevertheless, most connexon blockers are also inhibitory for pannexons, even the connexin mimetic peptides, which was proposed to be specific inhibitors for connexins.

Human pannexins are orthologs of innexins that form gap junctions in invertebrate. Thus, one might expect that pannexins exert the same function in vertebrates, i.e. forming
gap junctions. So far, no experimental evidence for pannexin gap junction in vivo has been presented. Earlier studies by Bruzzone et al. in paired Xenopus oocytes found that pannexin1 alone and in combination with pannexin 2 induced the formation of intercellular channels. However, in the paired oocytes assay, two cells were forced into a dimension of contact area never feasible in vivo, and the paring time is long enough (24-48 hours) for the possible formation of low affinity Cx38 gap junction channels endogenously expressed in oocytes. Moreover, there is a large difference between the gap junction formation by pannexins and connexins (Figure 31).

Figure 31. Junctional conductance of oocyte pairs expressing mouse or rat pannexin1 (mPanx1, rPanx1) or rat connexin 46 (rCx46). Uninjected oocytes served as control. Conductance was determined 6 (open bars) and 24 h (black bars) after pairing. cRNA was injected 2–3 days before pairing. The junctional

conductance of rCx46 expressing oocytes paired for 24 h were too large to be determined accurately.

The study of expression and localization of pannexin1 showed only diffuse staining of this protein on the membrane surface (Dahl and Locovei 2006; Locovei, Bao et al. 2006) instead of the punctuate staining typical for gap junctions made of either connexins (Paul 1986) or innexins (Bauer, Lehmann et al. 2004). Moreover, the finding of pannexin1 at the postsynaptic sites but none at the presynaptic sites in the hippocampal and cortical neurons makes the gap junction formation of this protein very unlikely in this special synaptic structure. Pannexin1 may be part of the postsynaptic channel complex and involved in regulation of postsynaptic activity in its undocked membrane channel configuration (Zoidl, Petrasch-Parwez et al. 2007).

Another argument in favor of pannexin1 functioning as an unpaired pannexons comes from the recent discovery of large channel activities, including large inward currents and permeation for fluorescent dye molecules, in the hippocampal neurons with physiological characteristics similar to pannexin1 occurring during OGD (oxygen/glucose deprivation) (Thompson, Zhou et al. 2006).

Further support for the unlikelihood of pannexin1 forming gap junctions comes from the investigation of post-translational modification of the membrane proteins, specifically glycosylations. Glycosylation is addition of saccharides to a protein or lipid, which serves a variety of structural and functional roles in membrane and secreted proteins (Varki, Freeze et al. 2009). The two main glycosylations is N-glycosylation and O-glycosylation, named by the atoms the saccharides are attached to. There is an N-glycosylation site on the putative second extracellular loop of pannexin1. Glycosylation of this site represents a steric hindrance for docking of two hemichannels to form gap junctions. This
assumption was confirmed by the largely increased coupling of pannexin1 expressing cells after removal of the steric obstacles by treatment with N-glycosidase F (Figure 32).

![Bar chart showing effect of deglycosylation treatments on the junctional conductance of oocyte pairs expressing mPanx1. Panx1-WT cRNA was injected 2-3 days before pairing. The oocytes were treated with tunicamycin (1 µg/ml), or PNGase F (10 units/ml) for 30 minutes before pairing. Junctional conductance was determined 6 hours after pairing. Treatments that promoted the removal of carbohydrate groups from the extracellular surface significantly increased junctional conductance.](Figure 32)

**Figure 32.** Effect of deglycosylation treatments on the junctional conductance of oocyte pairs expressing mPanx1. Panx1-WT cRNA was injected 2-3 days before pairing. The oocytes were treated with tunicamycin (1 µg/ml), or PNGase F (10 units/ml) for 30 minutes before pairing. Junctional conductance was determined 6 hours after pairing. Treatments that promoted the removal of carbohydrate groups from the extracellular surface significantly increased junctional conductance.

Tunicamycin is a mixture of antibiotics, which blocks the glycoprotein synthesis so as to inhibit the pannexin1 glycosylation. This drug did not induce the increase of coupling as PNGase F because glycosylation is important for pannexin1 transportation to the cell membrane (Boassa, Ambrosi et al. 2007).
One current hypothesis is that pannexins do not duplicate the function of connexins in connecting directly the cytoplasms of adjacent cells, but rather allow the communication between cytoplasm and the extracellular space. However, in the absence of more specific evidence, it is probably premature to rule out a gap junction function of pannexins. From the evolutionary point view, in invertebrate, innexins form either gap junction channels for cell-cell coupling or non-junctional membrane channels for intra-extracellular spaces communication. These abilities are inherited to vertebrates but accomplished by two non-related gap junction protein families, with connexins forming the gap junctions and pannexins retaining the function as membrane channels (Figure 33).

Figure 33. Evolution of gap junctional proteins. Innexins forms not only membrane channels but also gap junctions in invertebrate, and these two functions were carried out by two different families of proteins pannexins and connexins respectively in vertebrate.
4.2 Pannexin1 as an ATP release channel

An increasing number of observations indicate that pannexin1 forms ATP release channels. Biophysical properties of pannexin1 channels are consistent with such a role, as well as the expression of pannexin1 at the cellular and subcellular levels. 1) Pannexin1 is expressed in cells that release ATP, including erythrocytes, endothelial cells, astrocytes, and bronchial epithelial cells. Especially in the bronchial epithelial cells, pannexin1 is localized to the luminal membrane, the identified site of ATP release (Ransford, Dahl et al. 2007; Ransford, Fregien et al. 2009). 2) The pannexin1 membrane channel is highly permeable to ATP. The unitary conductance of pannexin1 is around 500pS. It is not only permeable for regular charge carriers, like sodium, potassium etc., but also allow the passage of large molecules as ATP and fluorescent dyes. 3) Pannexin1 is mechanosensitive. It is activated at the resting membrane potential when subjected to mechanical stress. Mechanical stress is the physiological stimulus for initiation of calcium waves. The roles of ATP in calcium wave propagation are schematically summarized in figure 34.

![Diagram of ATP release and calcium wave propagation](image)
Figure 34. Scheme depicting the involvement of ATP and pannexin1 channels in the calcium wave propagation.

Pannexin1 channels can be activated by extracellular ATP acting through the purinergic receptors P2Y or P2X7, as well as by increase of cytoplasmic calcium, indicating this channel is involved in ATP-induced ATP release as shown in figure 34.

Erythrocytes are good preparation for study of channel-mediated ATP release because they do not implement vesicular release. Erythrocytes release ATP in response to shear stress or -physiologically more important- in low-oxygen environment. The released ATP binds to purinergic receptors on the endothelium cells, which induces further ATP release from these cells and triggers a calcium wave (Figure 35). The calcium wave results in the release of nitric oxide initiating relaxation of the vascular smooth muscle and allowing more oxygen to be delivered to the tissue. This peripheral control mechanism for oxygen delivery operates in many species including humans. The expression of pannexin 1 in erythrocytes and endothelial cells, the activation of pannexin 1 by increased intracellular calcium and/or by purinergic receptors all are consistent with pannexin 1 underlying ATP release in this control system.
Figure 35. Scheme depicting the involvement of pannexin 1 channels in local blood-flow regulation.

Besides Pannexin 1, other proteins have been implicated to serve as ATP release channel including the purinergic receptor P2X7 (Suadicani, Brosnan et al. 2006). The P2X7 receptor is a unique ionotropic purinergic receptor. When activated by ATP, it opens a small ‘typical’ cation channel permeable for calcium. Repeated or prolonged application of ATP leads to the gradual formation over seconds to minutes of a larger pore allowing passage of molecules up to 900 Da (Coutinho-Silva and Persechini 1997; Ugur, Drummond et al. 1997; North 2002; Ferrari, Pizzirani et al. 2006). The ‘large pore’ is usually studied by measuring uptake of fluorescent dyes, which is also a traditional assay to monitor gap junction permeability. Two hypotheses have been proposed to explain the formation of the pore: 1) the ‘small pore’ or ion channel itself dilates to the extent that dye can pass as if by accretion of more subunits into an increasing multimer; 2) an additional component, a large channel, is activated through opening of P2X7 receptor. The failure to reconstitute the P2X7 ‘large pore’ in several exogeneous expression systems favors the involvement of another channel (Nuttle, el-Moatassim et al. 1993; Petrou, Ugur et al. 1997). On the other hand, recent studies show that the ‘large pore’
could be the pannexin 1 membrane channel (Pelegrin and Surprenant 2006; Locovei, Scemes et al. 2007). The finding of similar pharmacological spectra of pannexin 1 and P2X7 in our study also confirms this hypothesis. Previous publications describing the P2X7 receptor as the ATP release channel based on the antagonist inhibition need to be reevaluated. Regardless of the mechanism, the present findings indicate that caution is warranted when data involving these drugs are interpreted. The effects may be attributable to either P2X7 receptors, or pannexin 1 channels, or both. The issue is more complicated by the association of pannexin 1 with the P2X7 receptor (Pelegrin and Surprenant 2006; Locovei, Scemes et al. 2007; Iglesias, Locovei et al. 2008). For example, ATP release and its surrogate measure, dye uptake, are inhibited by BBG, as is the propagation of calcium waves (Suadicani, Brosnan et al. 2006). In the past this has been claimed as a clear indication that the P2X7 receptor itself mediates ATP release and calcium wave propagation. Although that conclusion is not tenable anymore, an involvement of the P2X7 receptor in calcium wave propagation as activator of pannexin 1 channels is conceivable.

4.3 Is it counterintuitive that the permeant ion inhibiting its own permeable channels?

If pannexin 1 forms ATP release channels it seems counterintuitive that ATP would inhibit channel activity. However, negative regulation of a channel by its permeant has previously been reported. For example, as part of desensitization at synapses calcium ion entry inactivates the permeation pore Ca_{2.1} (Mochida, Few et al. 2008). However, the action of calcium is not direct but involves a Ca^{2+} sensor protein. A direct action of the
permeant ion on its conduction pathway has been demonstrated for Cl⁻ on ClC channels, but its effect is facilitation of ion permeation (Chen 2003).

Regulation of channel activities by ATP has been described for several channels, such as the regulation of CFTR chloride channel by ATP (Anderson and Welsh 1992) and the ATP sensitive potassium (K\textsubscript{ATP}) channel (Ashcroft and Kakei 1989).

Pannexin 1 forms a potentially deadly channel. Large pore size and low selectivity will quickly run down electrochemical gradients and dissipate vital cellular constituents. Indeed, prolonged or repeated activation of pannexin 1 channels can kill Xenopus oocytes (Locovei, Scemes et al. 2007). Pannexin 1 channels can be opened by extracellular ATP via P2Y or P2X7 receptors at resting membrane potentials. This forms a loop of positive feedback that probably underlies ATP-induced ATP release in many tissues. Nevertheless, cell death is not the typical outcome when pannexin 1 channels are activated through purinergic receptors, with the exception of prolonged activation of P2X7 receptors. Rather, activation through P2Y2 results in a transient response. The ionic current decays spontaneously in the presence of ATP (Locovei, Wang et al. 2006), as if the receptors desensitize or channels become inactivated. My results indicate that channel inhibition occurs and thus can contribute negative feedback to pannexin 1 channel activity.
Figure 36. Scheme depicting the involvement of ATP in P2X7 receptor and Pannexin 1 complex: external ATP activates the P2X7 receptor which consequently opens pannexin 1 channel and allows more ATP release, forming an ATP-induced ATP release positive feedback loop; The released ATP accumulates to a higher concentration and inhibits its own permeation pore, preventing excessive ATP leakage and protecting cell integrity.

4.4 Where is the putative ATP binding site?

The effect of ATP on pannexin 1 channels appears to be direct; no intermediates probably are involved. Aside from the effect of ATP on channels in excised membrane patches, this is further indicated by the attenuation of the effect of ATP and its analogues by mutations in pannexin 1. Although no overt canonical binding site for ATP is in the pannexin 1 sequence, replacement of an arginine in position 75 with alanine drastically
attenuated the inhibitory action of ATP and analogues on pannexin 1 currents. Of the other 5 basic amino acids in the extracellular portions and conserved in pannexin 1 of different species, alanine substitutions resulted in loss of function in two of them (K248A and R265A), while alanine substitutions of the remaining basic amino acids had no consequence for ATP effects on channel currents. In most ATP-binding proteins, positively charged amino acids are thought to interact with the phosphate groups of ATP directly. For example, in the well-known ATP-binding motif Walker loop (Walker, Saraste et al. 1982), the key component is a lysine residue, which is proposed to be directly involved in coordinating ATP binding (Muller, Bakos et al. 1996). Site-directed mutagenesis of P2U (P2Y2) receptors, which do not have the consensus ATP-binding motifs, showed that positively charged amino acids in transmembrane helices alter the ATP potency dramatically (Erb, Garrad et al. 1995). Typically, ATP binding sites involve more than one basic amino acid. It is, therefore, important to evaluate the basic amino acids in positions 248 and 265 in more detail. Even if these two amino acids do not affect the ATP potency on pannexin 1, the arginine 75 contributed by part/all of the six subunits of the pannexin 1 channel can still form a positively charged binding pocket for ATP. The ligand-binding site formed from the interaction of residues between neighboring subunits has been demonstrated for ion channel families as γ-aminobutyric acid A (Smith and Olsen 1995) and nicotinic acetylcholine receptors (Czajkowski, Kaufmann et al. 1993; Czajkowski and Karlin 1995). The crystal structure of the ATP-gated P2X4 receptor has recently been described and the putative ATP-binding pocket is suggested to be localized in the groove between two adjacent subunits of the trimer (Kawate, Michel et al. 2009).
Table 2 Compare the effect of various chemicals on P2X7 receptor and Pannexin 1

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Type</th>
<th>EC$<em>{50}$/IC$</em>{50}$ on P2X7 (µM)</th>
<th>IC$_{50}$ on Panx1 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>Agonist for Purinergic Receptors</td>
<td>No effect $^5$</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>cyclic AMP</td>
<td>Product of Purinergic signaling</td>
<td>No effect $^5$</td>
<td>No effect</td>
</tr>
<tr>
<td>ADP</td>
<td>Agonist for P2Y receptors</td>
<td>No effect $^5$</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Pyrophosphate</td>
<td>ATP metabolite</td>
<td>No effect</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>UTP</td>
<td>Agonist for P2Y receptors</td>
<td>No effect $^4$</td>
<td>No effect</td>
</tr>
<tr>
<td>ATP</td>
<td>Agonist for Purinergic Receptors</td>
<td>100 $^2$</td>
<td>~600</td>
</tr>
<tr>
<td>BzATP</td>
<td>Agonist for P2X7</td>
<td>4.7 $^1$</td>
<td>100</td>
</tr>
<tr>
<td>PPADS</td>
<td>Antagonist for Purinergic Receptors</td>
<td>5.4 $^6$</td>
<td>~200</td>
</tr>
<tr>
<td>KN-62</td>
<td>Antagonist for P2X7 receptor</td>
<td>4.88 $^6$</td>
<td>~100</td>
</tr>
<tr>
<td>Brilliant blue G</td>
<td>Antagonist for P2X7 receptors</td>
<td>0.01 $^7$</td>
<td>1</td>
</tr>
<tr>
<td>Suramin</td>
<td>Antagonist for Purinergic Receptors</td>
<td>40 $^8$ ~500 $^2$</td>
<td>100</td>
</tr>
<tr>
<td>A438079</td>
<td>Antagonist for P2X7 receptors</td>
<td>6.9 $^6$</td>
<td>100</td>
</tr>
</tbody>
</table>

$^1$ (Bianchi, Lynch et al. 1999)

$^2$ (Khakh, Burnstock et al. 2001)
The pharmacology of pannexin 1 inhibition is very similar to that of the P2X7 receptor (Table 2), except that receptor agonists and antagonists are both inhibitory for pannexin 1 channels and higher concentrations are required. The similarity refers to the rank order of compounds to affect the receptor and the channel. However, for all compounds the IC₅₀ for Pannexin 1 is considerably higher than the EC₅₀/IC₅₀ for P2X7. Thus, it can be expected that the binding site in pannexin 1 is probably similar to that of the P2X7 receptor. The binding sites for agonists and antagonists on P2X receptors are different but in the vicinity (Kawate, Michel et al. 2009), consisting with our finding of different but overlapping BzATP and BBG effect on different mutants. Although speculative, we suggest that pannexin 1 shares a similar ligand-binding pocket with P2X7 receptor due to their similar pharmacological property found in our study and their close relationship in the P2X7 death complex (Locovei, Scemes et al. 2007). The ATP-binding site is still unclear for P2X7 receptor, although it is speculated to be similar to other P2X receptors. The unique higher affinity to BzATP than ATP of P2X7 receptor is similar to pannexin 1. So the finding of possible ATP binding partner in pannexin 1 may give some reference to look for the putative ATP binding site on P2X7 receptor. Figure 37 shows the alignment of the second extracellular loop of pannexin 1 and the part of the
extracellular loop of P2X7 receptor. The red amino acids are the ones having impact on the ATP effect when substituted with alanine. These amino acids are also found in the extracellular loop of P2X7 receptor, but are widely distributed so the alignment requires gaps.

Figure 37. Alignment of amino acid residues in the extracellular region of pannexin 1 and P2X7 receptor. The amino acid sequence of the second extracellular loop of pannexin 1 is shown and the red ones are effective for ATP binding. The equivalent residues are pointed out in the extracellular region of P2X7 receptor with gaps in between.

The mechanism of action of ATP in inhibiting pannexin 1 channel activity appears to have a steric component. This is indicated by the considerably higher inhibitory effect of ATP or analogues on ATP release and dye uptake than on ion flux. The potency of the various drugs to inhibit pannexin currents, however, is only poorly correlated with molecular size of the drugs. For example suramin, the largest molecule used in this study, was less effective in inhibiting pannexin 1 currents than the smaller BBG. However, if the affinity of the drugs to the binding site in pannexin 1 is similar to that in the P2X7 receptor, suramin would have the lowest affinity to pannexin 1 (table 2). Thus, it is plausible that a combination of affinity and molecular size determine the potency of the drugs to inhibit pannexin 1 activity.
Appendix

Site Directed Mutagenesis

1. The first step for the site-directed mutation is to design and synthesize two complimentary oligonucleotides containing the desired mutation, called primers. The mutation primers were designed following the primer design guidelines.

2. Prepare the PCR reaction as indicated below:

   5µl  10× reaction buffer
   2µl  (15 ng) of dsDNA template
   1.25µl (125 ng) of oligonucleotide primer #1
   1.25µl (125 ng) of oligonucleotide primer #2
   1µl   dNTP mix
   1µl   *PfuUltra* HF DNA polymerase (2.5 U/µl)
   38.5µl nuclease free H₂O
   50µl   Total
Incubate reaction mix in PCR machine. The temperature cycling parameters are listed in table below. The whole PCR procedure takes about 2.5 hours.

<table>
<thead>
<tr>
<th>Segment</th>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>95°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>95°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>55°C</td>
<td>1 minute</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68°C</td>
<td>7 minutes</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>68°C</td>
<td>7 minutes</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>4°C</td>
<td>2 minutes – 2 hours</td>
</tr>
</tbody>
</table>

3. After the thermal cycle, add 1µl of the Dpn I restriction enzyme (10 U/µl) directly to each amplification reaction, mix well and incubate the reaction at 37°C for 1 hour to digest the template (parental) DNA.

4. Transfer 1µl of Dpn I-treated DNA from each reaction to 50µl supercompetent cells, gently mix well and incubate on ice for 30min.

5. Heat shock the transformation reaction for 45 seconds at 42°C and place the reaction back on ice for 2min.

6. Add 200µl S.O.C. medium to the reaction and shake at 225rpm in 37°C incubator for 1 hour.
7. Spread 10-50μl of the reaction on agar plates containing the appropriate antibiotic for the plasmid vector and incubate the transformation plates at 37°C for about 16 hours.

(Adapted from protocol of QuikChange II site-directed mutagenesis kit, Stratagene)
References


