

Chapter 12

Pannexins or Connexins?

Gerhard Dahl and Andrew L. Harris

Abstract Pannexins are a family of three vertebrate proteins that have moderate sequence homology with the innexin proteins, which compose gap junction channels in protostomes, including most invertebrates. However, it appears that in contrast to innexins, pannexins do not have the ability to form gap junction channels. They do, however, form nonjunctional plasma membrane channels (*pannexons*) that mediate regulated flux of molecules in the size range of second messengers between cytoplasm and the extracellular space. The dye permeability and pharmacological sensitivities of pannexin channels overlap those of connexin hemichannels, so it is possible that many of the phenomena that have been attributed to connexin hemichannels are in fact mediated by pannexons. For this reason, identifying which protein is involved in a particular cellular physiology requires careful evaluation of the specific conditions and requirements in each case. Several lines of experimentation have led to the suggestion that the adenosine triphosphate (ATP) release channel, a crucial element in the initiation and propagation of intercellular Ca^{2+} waves, is a connexin hemichannel. However, based on recently revealed properties of pannexin channels, which include mechanosensitivity and activation by cytoplasmic Ca^{2+} , the pannexon must be considered a prime candidate for the ATP release channel. Pannexons also appear to form the large ATP-permeable pore that is activated by the purinergic P2X7 receptor complex, which is involved in inflammation.

Keywords Pannexin · Innexin · Connexin · Gap junction · Hemichannel · Pannexon · ATP release · Calcium wave · P2Y receptor · P2X7 receptor · Cx32 · Cx38 · Cx43

G. Dahl (✉)

Department of Physiology and Biophysics, University of Miami School of Medicine,
PO Box 016430, Miami, FL 33101, United States
e-mail: gdahl@miami.edu

12.1 Introduction

Connexins form gap junction channels in deuterostomes, which include all vertebrates. A distinct family of proteins, the innexins, forms gap junction channels in protostomes, which include most invertebrates [1,2]. There is no sequence homology between these two families of gap junction proteins. A search of the human genome identified three innexin-related genes [3,4]. Because of the occurrence of homologous genes in both vertebrates and invertebrates, the corresponding proteins were termed *pannexins*. However, because subsequent studies indicate that the vertebrate homologs are functionally distinct from the *innexins*, they are treated here as a separate family, and the term refers only to the three vertebrate proteins, denoted as pannexin1 (Panx1), pannexin2 (Panx2), and pannexin3 (Panx3).

Pannexins appear to have the same transmembrane topology as connexins and innexins, with four transmembrane domains and cytoplasmic carboxyl-terminal and amino-terminal domains [5,6]. Early functional studies indicated that Panx1 could form gap junction channels in paired oocytes, as well as produce a non-junctional conductance [7]. Panx1 channels are larger than the largest connexin channels, with regard to both conductance and apparent pore width [8]. The cellular and tissue expression of pannexins overlaps with that of connexins.

This chapter summarizes current knowledge about pannexin channels, with particular attention to the criteria for resolving their cellular functions as distinct from those of connexin channels. It illustrates the major criteria for making this distinction by discussing in depth the degree of correspondence between the characteristics of the adenosine triphosphate (ATP) release channel and those of plasma membrane pannexin and connexin channels. The preponderance of data argues strongly for direct pannexin, as opposed to connexin, involvement in nonvesicular ATP release.

12.2 Do Pannexins Form Gap Junction Channels?

Despite the reports of the ability of pannexin channels to form gap junction channels in paired oocytes, they may not do so under conditions of normal expression, either in vivo or in vitro (Table 12.1). Functional assays in transfected cultured cells have failed to provide evidence of gap junctions formed by

Table 12.1 Evidence relating to gap junction function of pannexin

Evidence for gap junction function	Junctional conductance in paired oocytes [7]. Dye transfer through unspecified channels in pannexin transfected cells [9,10]
Evidence against gap junction function	No dye and electrical coupling in cell cultures expressing Panx1 [6,11] No punctate staining typical for gap junctions [11,14,15] Pannexin is glycosylated [6,17]

pannexins, with two exceptions [9,10]. No pannexin-induced dye-coupling or electrical coupling has been observed in a variety of pannexin-expressing host cells [6,11]. Thus, the only cases in which pannexins may form gap junctions are in conditions of overexpression, and in only a subset of those. Because in the overexpression studies using tissue culture cells no diagnostic test that would discriminate between connexin and pannexin channels was performed, the channel identity even in the apparent exceptions is not clear, as connexin expression could have been upregulated in the pannexin transfected cells.

Gap junctions are characterized by punctate immunohistochemical staining at regions of intercellular contact [12,13]. The puncta correspond to gap junction plaques. Immunostaining for Panx1 does not show punctate staining [11,14], but rather the diffuse staining of the cell surface expected of a nonclustered protein distribution. In addition, in the epithelia tested to date, pannexin expression in the plasma membrane is limited to luminal rather than the basolateral surfaces where gap junctions are typically located [15]. At neuronal synapses, Panx1 is found only in the postsynaptic, and not the presynaptic, membrane, excluding a junctional role [16].

Unlike connexins, which are not glycosylated, Panx1 is glycosylated in the second extracellular loop [6,17]. This modification adds substantial bulk to the extracellular-facing aspect of the protein. Insertion of glycosylation sites into the extracellular loop domains of connexins blocks formation of junctional channels when these sites are glycosylated [18]. Therefore, it appears that bulky carbohydrates on the extracellular loops prevent the tight docking interactions between connexin hemichannels required for formation of a patent cell-cell channel. On this basis, it is difficult to imagine that glycosylated pannexins can form intercellular channels.

How, then, can the formation of gap junctions by pannexin be explained, in the few cases where it occurs when (over)expressed? *Xenopus* oocytes are prone to faulty glycosylation [19,20,21]. Furthermore, in any transfected cell overexpression of exogenous protein may overwhelm the glycosylation machinery in the Golgi apparatus and allow an appreciable amount of aberrantly unglycosylated protein to proceed through the synthesis and insertion pathways. Thus, the reports of gap junction channel formation by Panx1 in oocytes and perhaps some other expression systems may be an expression system artifact and not reflect the ability of the protein to form an intercellular channel in vivo or under conditions of endogenous expression levels.

The ability of Panx2 or Panx3 to form gap junctions, or any type of functional channel, has not been demonstrated. Bruzzone et al. [7] found that expression of Panx2 or Panx3 does not produce channel activity under normal test conditions. Panx2, however, did modulate Panx1 channel activity when the two pannexins were coexpressed. The gene structures predict that both Panx2 and Panx3 can be expressed as different isoforms due to alternative RNA splicing [22]. It is therefore possible that the channel-forming isoform(s) have not yet been studied, or that the experimental conditions favorable for channel activity of these pannexins have not yet been found.

12.3 Properties of Pannexin Plasma Membrane Channels

Cells that never form gap junctions, such as mature erythrocytes, express Panx1 [5]. Panx1 expression in oocytes leads to a nonjunctional membrane conductance [7]. The channels have a large unitary conductance (475 picoSiemens [pS]) and exhibit numerous subconductance states (Fig. 12.1) [8]. Panx1 channels are highly permeable to ATP, to the extent that current carried through the channel

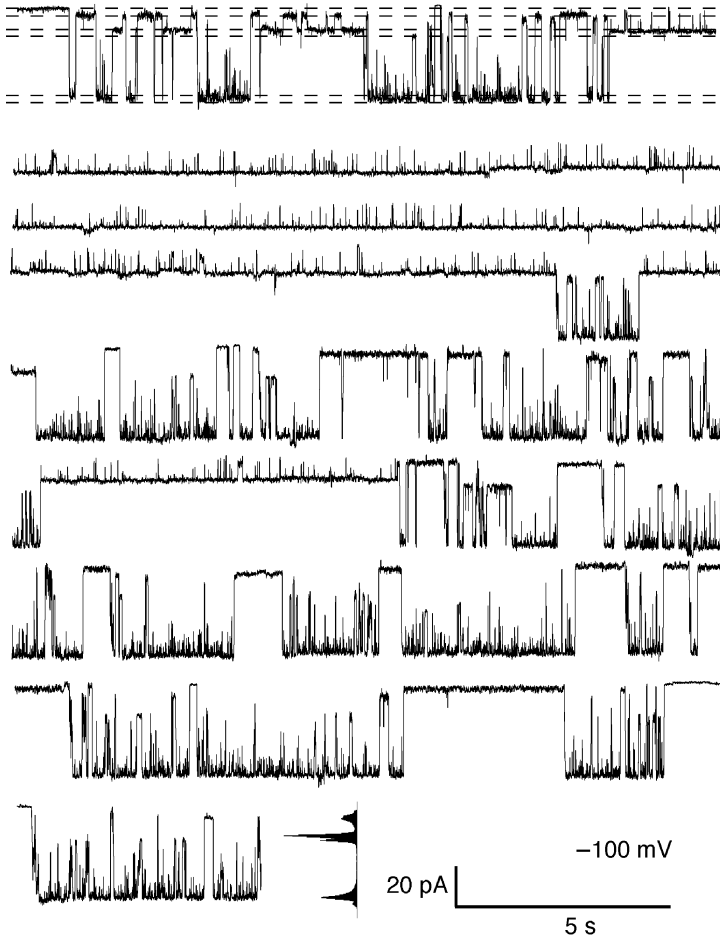


Fig. 12.1 Single-channel currents in an inside-out membrane patch excised from an oocyte expressing Panx1. An uninterrupted recording segment of 140 seconds is shown together with an all point histogram of the entire segment. Characteristic of pannexin channels, several subconductance levels can be discerned (indicated by *dotted lines*). When actively gating, fully closed and fully open states are rare events. The unitary conductance of the full open state is 475 pS. (A high-resolution version of this figure is available on the accompanying CD and online at www.springerlink.com) (From Bao et al. [8] with permission.)

by ATP itself can be discerned. The channel is permeable to dyes that permeate connexin hemichannels and is accessible to polyethylene glycols up to ~ 1.5 kDa [23]. Panx1 channels are closed at negative potentials, open at positive potentials, and inactivate over time. Panx1 channels can be activated at normal resting membrane potentials by mechanical stress [8] or by increases of cytoplasmic Ca^{2+} concentration to the micromolar range [24].

These properties suggest that pannexin plasma membrane channels have a physiological role: to regulate the flux of molecules in the size range of second messengers between cytoplasm and extracellular space [14]. The nonplaque, diffuse plasma membrane localization of pannexin protein seen immunohistochemically (mentioned above) is consistent with such a role.

12.4 The Adenosine Triphosphate Release Channel

Intercellular Ca^{2+} waves are widespread. They serve diverse functions, including control of ciliary beat in airway epithelia, control of peripheral vascular perfusion, modulation of synaptic transmission by glia, and ossification [25,26,27,28]. Ca^{2+} waves are initiated by various physiological stimuli, including extracellular ATP and mechanical stress. Ca^{2+} wave propagation can involve two pathways: direct intercellular flux of inositol triphosphate (IP_3) through gap junction channels and an extracellular pathway involving ATP release and purinergic receptors [25,29,30]. In the latter, ATP is released from the initiating cell (in response to mechanical stress, for example) and from cells in the wavefront in response to activation of purinergic receptors. The most compelling evidence for channel-mediated as opposed to vesicular release is that ATP can be released from cells that do not contain vesicles, such as erythrocytes [31]. On this basis, a channel for ATP release would be expected to be mechanosensitive and to open upon activation of purinergic receptors.

On the basis of several experimental findings, connexin channels have been proposed to mediate ATP release. Gap junction inhibitors interfere with ATP release [32,33], and, under conditions of ATP release, cells take up extracellular dyes known to permeate connexin channels [34,35]. Conventional gap junction channel blockers also inhibit the dye uptake. In addition, there is a notable correlation between induced expression of connexin and ATP release [36]. Thus, it was reasonable to infer that connexins, in addition to forming gap junctions, provided the ATP release pathway in the form of connexin hemichannels in the nonjunctional membrane.

The mechanosensitivity of the ATP release channel combined with its ATP permeability raise the possibility that pannexons play a role in the initiation of Ca^{2+} waves. Wave propagation may involve pannexons as well, since when coexpressed with either P2Y or P2X7 receptors [24,37] they open in the presence of extracellular ATP. Activation of the metabotropic P2Y receptors leads to the release of Ca^{2+} from intracellular stores, which in turn can activate pannexin

channels. In the case of P2X7 receptors, activation of pannexin channels does not require influx of extracellular Ca^{2+} through the inotropic receptor [37]. Instead, pannexin activation may occur by protein–protein interaction. A P2X7-Panx1 interaction is indicated by co-immunoprecipitation of the two proteins [38].

12.4.1 Which of the Candidate Channels Are Active Under the Required Conditions?

Under physiological conditions most connexins form hemichannels in the plasma membrane [39,40] that are closed until they dock during gap junction formation to form cell–cell channels. With some exceptions, connexin hemichannel currents tend to be activated by strong depolarization or reduction of extracellular Ca^{2+} below 0.5 mM [41,42]. Thus, the activity of endogenous connexin hemichannels is unlikely to be significant under normal physiological conditions, such as those under which Ca^{2+} waves occur.

The situation for pannexin channels is quite different. The physiological stimuli for Ca^{2+} wave initiation and propagation — mechanical stress and activation of purinergic receptors — can induce pannexin channel activity at the resting membrane potential in a normal ionic environment (Fig. 12.2) [8,24,37].

By definition, during Ca^{2+} waves there is a propagated increase in cytoplasmic Ca^{2+} . Gap junction channels are either closed by increased cytoplasmic Ca^{2+} or unaffected by it, depending on the particular connexin [43,44]. Opening of connexin hemichannels by cytoplasmic Ca^{2+} would require a reversal of Ca^{2+} sensitivity between single connexin hemichannels and hemichannels that are in gap junction channels. Such a reversal of gating sensitivity is not only conceptually unappealing but also has not been observed in connexins that form functional single hemichannels [45,46,47,48]. Pannexons, on the other

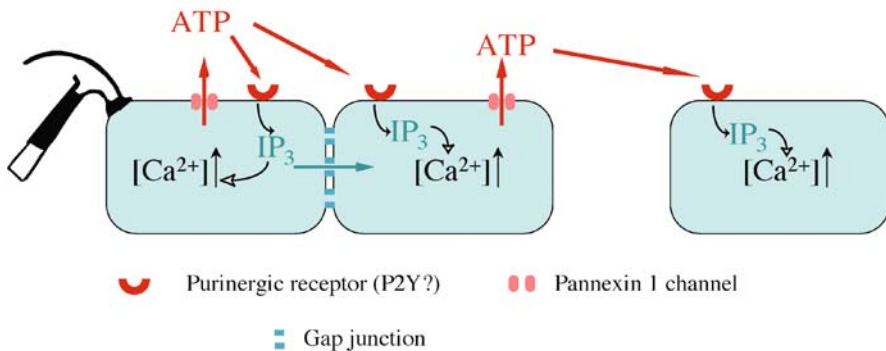


Fig. 12.2 Scheme depicting possible involvement of Panx1 channels in calcium wave initiation and propagation. (A high-resolution version of this figure is available on the accompanying CD and online at www.springerlink.com) (From Locovei et al. [24] with permission.)

hand, can be activated at the resting membrane potential by increases in Ca^{2+} concentration comparable to those in Ca^{2+} waves [24].

ATP release and Ca^{2+} wave propagation are facilitated by reduction of extracellular Ca^{2+} to the micromolar range [32,49,50]. This treatment also can open connexin hemichannels [48,51]. However, reduction of extracellular Ca^{2+} typically leads to a rise in cytoplasmic Ca^{2+} [52], which could activate pannexin channels. Therefore, the sensitivity of ATP release to low extracellular Ca^{2+} cannot be considered as unambiguous evidence for connexin as opposed to pannexin involvement.

12.4.2 Which of the Candidate Proteins Are Expressed in the Right Places?

A candidate ATP release channel must exhibit an expression pattern consistent with its function. Many tissues express both connexins and pannexins and therefore cannot help to discriminate between the contributions of the two candidates. However, there are cases where ATP is released from cells that express pannexin but not connexin. A prime example is the erythrocyte [5], which releases ATP in a low oxygen environment or in response to shear stress [31,53]. ATP release in the presence of pannexin and absence of connexin also occurs in the receptor cells of the taste bud [54]. Although the gustatory epithelium expresses both Panx1 and several connexins [55], analysis of the expression patterns of individual cell types within taste buds revealed that the cells that release ATP express Panx1 but no connexins [54]. Conversely, there are no known cases where there is nonvesicular ATP release in the absence of pannexin, whether or not connexin is present.

A candidate ATP release channel also must be expressed at the appropriate cellular sites. In the airway epithelium, ATP is released from the apical aspect of the cell. Panx1 is expressed at high concentration in these cells at the apical membrane, and not at all at the basolateral membrane [15]. Though not established for these particular cells, connexin expression in polarized cells is typically in the basolateral membrane and not in the apical membrane [12].

12.4.3 The Dilemma of Genetic Manipulation

The strongest evidence for connexins serving as ATP release channels comes from studies where forced expression of connexins is correlated with increased ATP release [32,36]. However, this inference is directly contradicted by findings from cells derived from Cx43 knockout (KO) mice in which Ca^{2+} wave propagation proceeds at approximately the same speed in astrocytes from Cx43KO mice as in those from wild-type mice [56]. Because Cx43 is the major connexin expressed in these cells, both pathways for wave propagation, gap junctional

and extracellular, ought to be eliminated in the Cx43KO cells. Electrical coupling was greatly attenuated, indicating that the persisting Ca^{2+} waves propagated via the extracellular pathway. Clearly, the ATP release required for this could not occur through Cx43 channels in these Cx43KO cells.

What, then, can explain the experiments in which presence of ATP release channels seems to correlate with connexin expression? Compensatory mechanisms have been recognized to be common phenomena in both knockout and forced expression studies. Specifically, gene knockout or overexpression can cause widespread changes in expression of other genes, particularly in the case of connexin genes, which affect a key mechanism of intercellular signaling. For example, Jacobas et al. [57,58,59] have shown that up to 10% of the proteome is altered in Cx43KO mice; some proteins are upregulated and others downregulated. The consequence is a reversal of conventional scientific wisdom; in this case, only a negative result is interpretable: persistence of propagated Ca^{2+} in the absence of Cx43 indicates lack of involvement of Cx43 [56]. Although not established, abolition of Ca^{2+} waves could be due to downstream effects of the genetic manipulation. In this case, to draw a conclusion about the role of Cx43, it would be necessary to document whether the expression of pannexin, for example, was also downregulated. Similarly, ATP release studies in which connexin is upregulated consequently must also assess changes in pannexin expression, when the connexin manipulation has an effect.

Acute expression in oocytes by injection of specific messenger RNA is less likely to suffer from compensatory complications. Panx1 expressed in oocytes results in ATP release in response to depolarization [8]. Under the same conditions, Cx43 expression did not lead to ATP release by these cells [8]. In 0 mM extracellular Ca^{2+} , Cx38 may do so [60], although ATP release in these cells has been shown to be sensitive to short exposure to brefeldin, indicating a vesicular release mechanism [61]. Consistent with an ATP release function of Panx1, its knockdown by interfering antisense oligodeoxyribonucleotides (RNAi) in an astrocytoma cell line attenuates ATP-induced dye uptake [37].

12.4.4 The Dilemma of Pharmacology

Reagents commonly used to block connexin channels are not specific for those channels; they also affect other membrane channels [14,62,63,64] (see Chapter 8). Importantly, carbenoxolone and niflumic acid block both connexin and pannexin channels. More relevant to ATP release, some compounds, such as octanol and 18 α -glycyrrhetic acid, block gap junction channels at concentrations that do not affect Ca^{2+} wave propagation [36]. The lipid oleamide has been suggested to discriminate between gap junction communication and Ca^{2+} wave propagation in glial cells [65], an apparent contradiction if connexin channels are involved in the latter.

It was therefore seen as a major step toward connexin-specific agents when connexin mimetic peptides were found to attenuate ATP release and Ca^{2+} wave

propagation [66]. Over two dozen publications have appeared in which connexin mimetic peptides were used to assess the contribution of connexin channels to Ca^{2+} waves. However, the specificity of these peptides for connexin channels has never been assessed. The currents through hemichannels formed by the connexin chimera Cx32*43E1 [47,67] are not acutely altered by the connexin mimetic peptides [23]. On the other hand, these peptides, termed *GAP24* and *GAP27*, rapidly inhibit pannexin channel currents [23] (see Chapter 8).

Pannexin mimetic peptides, based on the extracellular loop domains of Panx1, acutely inhibit both pannexin and connexin channels [23]. These data suggest that the effects of these peptides are not sequence-specific but arise from steric block of these large channels. No matter what the mechanism, the above findings argue that acute effects of the connexin peptides on a process, such as dye uptake, ATP release, and Ca^{2+} waves, indicate the involvement of pannexin rather than connexin channels. This point has been experimentally validated by two recent publications that together show that connexin mimetic peptides acutely inhibit ATP release (interpreted to indicate connexin involvement [55]) and that the cells so affected express pannexin but not connexin [54].

In summary, prior to knowledge about pannexin channel properties, connexin channel activity best explained some of the phenomena associated with ATP release in some tissues. However, as indicated above and summarized in Table 12.2, pannexons are now the stronger candidates for ATP release channels.

Table 12.2 Comparison of properties of connexin and pannexin channels

	Connexin	Pannexin
Channel opens under physiological conditions	No	Yes [5,8,24]
Channel is mechanosensitive	Demonstrated only for Cx46 [72]	Yes [8]
Channel can be opened by ATP through P2 receptors	Not demonstrated	Yes [24,37]
Channel is activated by cytoplasmic calcium	No	Yes [24]
Channel activity is rapidly attenuated by connexin mimetic peptides	No [23]	Yes [23]
Channel expression pattern matches ATP release	Poorly ¹	Yes [5,15,54]
Localization is consistent with sites of ATP release	No	Yes [15].
Protein knockout or knock-down decreases ATP release or surrogate measurements ²	No [56]	Yes [37,38]
Exogenous expression increases ATP release or surrogate measurements ²	Yes [36,73]	Yes [8]
Channel is inhibited by gap junction blockers	Yes	Yes [70]
Channel mediates dye uptake	Yes [74]	Yes [37,38]

¹ATP release and Ca^{2+} waves are found in cells in the absence of connexin including invertebrates and vertebrate erythrocytes and taste receptor cells.

²Typical surrogate measures for ATP release are dye uptake and Ca^{2+} wave propagation measurements.

12.5 Pannexins, Purinergic Receptors, and Cell Death

A second large, dye-permeable pore that has sometimes been confused with connexin channels is that activated by stimulation of the purinergic receptor P2X7R (also known as the P2Z receptor; [68]). This receptor is a member of the ionotropic P2X family of proteins. This receptor behaves like the other P2X receptors when exposed briefly to the agonists ATP or benoylbenzoyl-ATP by opening a relatively small 20 pS cation pore. With prolonged or repetitive exposure, it induces opening of a large, lytic, dye-permeable, and ATP-permeable pore. The properties of the P2X7R large pore are similar to those of Panx1 and connexin channels. Common features include unitary conductance, permeability to dyes typically used to study gap junction channels, sensitivity to cytoplasmic acidification, and sensitivity to gap junction channel blockers [8,24,69,70,71].

Several lines of experimentation indicate that P2X7R activation of the large pore requires Panx1 channels. It has been recently shown that P2X7R-mediated cell death involves Panx1 (Fig. 12.3) [37]. ATP-induced dye uptake in macrophages and in astrocytes is attenuated by RNAi targeting Panx1 [37,38]. Coexpression of P2X7R with Panx1 results in ATP-induced dye uptake in *Xenopus* oocytes, while neither of the two proteins alone mediates this phenomenon [37]. The activation of pannexon currents by ATP through P2X7R does not require influx of Ca^{2+} [37] but may occur through protein-protein interaction [38].

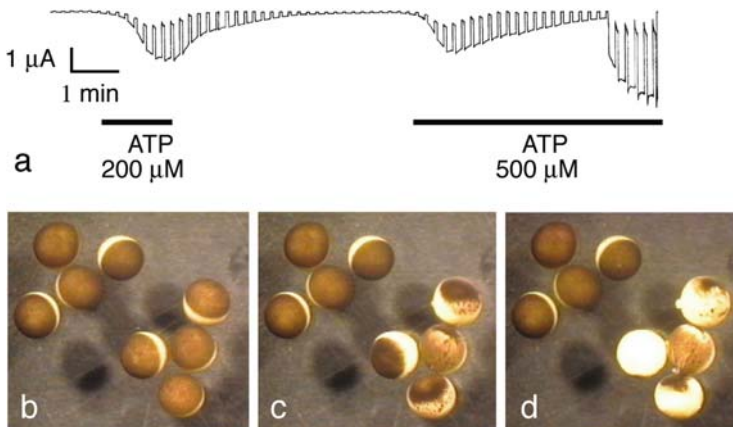


Fig. 12.3 Membrane currents and morphology of oocytes coexpressing P2X7R and Panx1 with exposure to ATP. (a) Brief exposure to 200 μM ATP resulted in a reversible inward current. Longer exposure to 500 μM ATP resulted in a current that initially reversed partially but subsequently increased to levels that escaped voltage clamp, indicating membrane breakdown. (b) Noninjected control oocytes (*upper left group*) and oocytes coexpressing P2X7R and Panx1 (*lower right group*) were exposed to 300 μM ATP. (c) Within three minutes a dramatic change of pigmentation was observed in the coexpressing but not in the control oocytes. (d) After five-minutes exposure to ATP, all coexpressing oocytes lost cellular integrity, indicated by yolk oozing out of the cells. (A high-resolution version of this figure is available on the accompanying CD and online at www.springerlink.com) (From Locovei et al. [37] with permission.)

There is also evidence that activation of metabotropic P2Y receptors also results in Panx1 currents [24]. In this case, the activation is most likely via the increase in cytoplasmic Ca^{2+} that follows ligand binding to the receptor.

12.6 Conclusion

Pannexins were discovered only recently and knowledge about their functional roles is just emerging. Although they were discovered based on their limited sequence homology with the invertebrate gap junction innexin proteins, pannexins do not form cell–cell channels. Instead their physiological role may be exclusively to provide a highly regulated pathway for molecules in the size range of second messengers to cross the plasma membrane.

Considering the amino acid sequence relationship between innexins and pannexins, the following evolutionary scenario becomes plausible: An invertebrate *ur*-channel acquired the ability to dock to its counterpart in another cell to form a cell–cell channel. Gene duplications occurred in the innexin family. Some or all of the innexins formed dual function channels; in addition to gap junction function they retained the ability to act as nonjunctional channels, allowing the exchange of molecules between cytoplasm and extracellular space. With the advent of connexins the gap junction function was usurped by these proteins, while the modern innexins, the pannexins, were retained for nonjunctional purposes.

Acknowledgments The authors thank Drs. Nirupa Chaudhari, Silviu Locovei, Ken Muller, and William Silverman for helpful discussions and reading the manuscript. Work in the lab of G.D. on pannexin channels is supported by National Institutes of Health (NIH) grant GM48610.

Note added in proof: A reagent known to attenuate nucleotide release has been recently reported to inhibit pannexin but not connexin channels; Silverman W, Locovei S, Dahl GP. Proenecid, a gout remedy, inhibits pannexin 1 channels. *Am J Physiol.* 2008;295:C761-7.

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