Intrinsic properties and regulation of Pannexin 1 channel

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Abbreviations: ATP, adenosine triphosphate; CALHM1, calcium homeostasis modulator 1; CBX, Carbenoxolone; COP II, coat protein II; Erev, reversal potential; HEK, human embryonic kidney; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HIV, Human Immunodeficiency Virus; NMDG+, N-methyl-d-glucamine; Panx1, Pannexin 1; TEV, Tobacco Etch Virus; pS, picosiemens; SNP, single nucleotide polymorphism; TRP, transient receptor potential; UTP, uridine triphosphate

Pannexin 1 (Panx1) channels are generally represented as non-selective, large-pore channels that release ATP. Emerging roles have been described for Panx1 in mediating purinergic signaling in the normal nervous, cardiovascular, and immune systems, where they may be activated by mechanical stress, ionotropic and metabotropic receptor signaling, and via proteolytic cleavage of the Panx1 C-terminus. Panx1 channels are widely expressed in various cell types, and it is now thought that targeting these channels therapeutically may be beneficial in a number of pathophysiological contexts, such as asthma, atherosclerosis, hypertension, and ischemic-induced seizures. Even as interest in Panx1 channels is burgeoning, despite early descriptions of electrical coupling in paired, Panx1-expressing oocytes, some of their basic properties, mechanisms of modulation, and proposed functions remain controversial, with recent reports challenging some long-held views regarding Panx1 channels. In this brief review, we summarize some well-established features of Panx1 channels; we then address some current confounding issues surrounding Panx1 channels, especially with respect to intrinsic channel properties, in order to raise awareness of these unsettled issues for future research.

Introduction

The first member of the Pannexin family of membrane channels was discovered with the cloning of Pannexin 1 in 1998 (Panx1, then called MRS1, GenBank accession number AF093239). It was soon recognized that Panx1 shares weak sequence homology with the "innexin" family of invertebrate gap junction channels.1 Much effort has since been dedicated toward defining the cellular and physiological roles of Panx1. This has led to the view that Panx1 is a surface membrane channel that permeates ions and various vital dyes, and serves as a conduit for regulated ATP release in support of purinergic signaling in numerous biological contexts. Indeed, Panx1 channels have already been implicated in ischemia-induced seizure, tumor formation or metastasis, hypertension, inflammation, HIV infection, migraine, and neuropathic pain.2,6 Despite this widespread interest, however, some basic properties of Panx1 channels still remain uncertain. In this brief review, we first provide some background information on the characteristics and functions of Panx1 that have been well-established; then, we turn to some areas of recent controversy, where existing data cannot yet unequivocally resolve key properties of the channel. We consider potential explanations for these inconsistencies and propose future directions for exploring properties and regulation of Panx1 channels in diverse physiological contexts.

Background

Three Pannexin family proteins have been identified (Panx1, Panx2, and Panx3) that belong to innexin/pannexin/connexin superfamily of channels. Within this family, the presumed subunit topology includes 4 transmembrane domains with both the N- and C-termini located intracellularly.1,3,5 Among the 3 Pannexin family proteins, Panx1 is the most widely expressed, while Panx2 and Panx3 show more restricted localization (to central nervous system and to skin and cartilage, respectively).8,10 Commensurate with its broader distribution, Panx1 has also been the best studied member of the family, and is the primary focus of this review.

Due to its similar topology to connexins, which form vertebrate gap junction channels, and to its moderate sequence homology to innexins, the invertebrate analog of connexin, Panx1 was initially considered an alternative gap junction in vertebrates.1,9,11 However, despite early descriptions of electrical coupling in paired, Panx1-expressing oocytes5 and later reports of Panx1-dependent, dye-coupling in glioma cells,12 it is now clear that formation of those intercellular gap junction channels by Panx1 is likely a rare event that occurs only under special circumstances (see refs. 13 and 14 for detailed discussion). Instead, Panx1 primarily forms uncoupled...
channels at the plasma membrane surface (i.e., equivalent to connexon “hemichannels”). Cell surface expression of Panx1 requires an intact C-terminus, and is balanced by COPII (coat protein II)-dependent ER-to-Golgi forward trafficking and channel internalization that is independent of clathrin/caveolin/dynamin II mechanisms.\textsuperscript{15,16} In addition, Panx1 channels on the cell surface are multiply-glycosylated, and it has been suggested that the complex glycosylation on the second extracellular loop (at Asn254) may interfere with gap junction formation and thereby favor generation of membrane channels.\textsuperscript{10,17,18}

Figure 1. Channel properties of C-terminally truncated hPanx1. (A) Examples of carbenoxolone-sensitive current obtained from HEK293T cell expressing full-length or C-terminal truncated (ΔC; same as Δ371 in ref. 22) hPanx1 using whole-cell voltage ramp. Note that the full-length hPanx1 does not generate appreciable current. (B) Representative whole-cell recording of hPanx1ΔC with different concentration of HEPES \textsuperscript{−} in the bath. Voltage ramps started with symmetrical Cl \textsuperscript{−} (151 mM), followed by replacing Cl \textsuperscript{−} with HEPES \textsuperscript{−} in the bath while maintaining total anion concentration (Cl \textsuperscript{−} + HEPES \textsuperscript{−} = 161 mM). Note the shift in reversal potential (E\textsubscript{rev}). Similar results were obtained by measuring tail currents at different repolarization steps following a constant depolarization step (data not shown). (C) Plot of reversal potential obtained in bath solutions containing different HEPES concentrations. Red dots indicated averaged E\textsubscript{rev} from recordings shown in (B). The dashed lines represent fits to an extended constant field (GHK) equation\textsuperscript{69} using relative permeability ratios (P\textsubscript{HEPES}:P\textsubscript{Cl} of either 0.3 or 0). The data indicate a substantial HEPES permeability in the C-terminally truncated hPanx1 channel. (D) Example of cell-attached recordings of hPanx1ΔC in HEK293T cells at the indicated patch potentials. The patch contained at least 2 active Panx1 channels, as noted by the transitions from the closed state (C) to 1 (O1) and 2 (O2) channel openings of equal amplitude. Carbenoxolone (CBX) inhibited open probability without changing unitary current amplitude, as reported previously.\textsuperscript{34} We did not observe multiple subconductance states,\textsuperscript{23} even in long duration recordings. (E) Unitary conductance (γ) obtained by analyzing single-channel current amplitude at various patch potentials (from data in [D]).
The structural details of Panx1 membrane channels have not been resolved at high resolution. However, based on protein crosslinking and initial electron micrographs, it appears that Panx1 channels likely form as hexamers, similar to non-junctional connexin “hemichannels.” By using a cysteine scanning approach, Wang and Dahl proposed a pore structure for Panx1 in which the first transmembrane domain and first extracellular loop formed the outer mouth of the channel pore. Interestingly, their data also suggested that the distal end of the putatively intracellular C-terminus contributed to the channel pore. Consistent with this, our group showed that the distal Panx1 C-terminus serves as an autoinhibitory region that must dissociate from the pore in order to allow for a cleavage-based form of Panx1 activation (see below).

Multiple physicochemical factors and cell-signaling processes can modulate the activity of membrane-associated Panx1 channels. For example, Panx1 is activated by membrane depolarization, by elevated extracellular potassium concentrations, and by mechanical deformation resulting from changes in osmolarity or application of negative pressure. The mechanisms mediating these various forms of channel activation remain to be determined. It appears likely that both the voltage gating and the mechanosensitivity, which are retained in isolated membrane patches, are intrinsic properties of Panx1 channel. However, the regions of Panx1 responsible for sensing change in membrane potential or stretch have not yet been identified. In terms of modulatory cell signaling events, Panx1 can be inhibited by direct S-nitrosylation at multiple sites on the channel. In addition, Panx1 is activated by Gq-coupled receptors. This receptor-mediated form of modulation appears to involve signaling pathways that include Rho and various downstream kinase activities, but how those activities are translated into effects on the channel remains to be established.

Our group has extensively characterized a novel form of cleavage-based Panx1 modulation that is responsible for nucleotide release from apoptotic T lymphocytes; the released nucleotide acts as a “find-me” signal to facilitate phagocyte-mediated cell clearance. The contribution we described for Panx1 was later verified by another group using macrophages from Panx1 knockout mice. We identified a caspase recognition consensus site within the C-terminus of Panx1 and showed that channel activation (and ATP release) during apoptosis requires a caspase-mediated proteolytic cleavage of the C-terminal tail. Consistent with this, we showed that C-terminal truncation of Panx1 renders the channel constitutively active (Fig. 1A).

In inside-out patches, we showed that purified, activated caspase 3 could activate wild-type Panx1, and that Tobacco Etch Virus (TEV) protease could activate a form of Panx1 engineered to contain a TEV recognition sequence at the caspase site, further demonstrating that this cleavage-based Panx1 activation could occur at the plasma membrane in the absence of other apoptotic signals. Furthermore, we showed that purified Panx1 C-termini can inhibit cleavage-activated channels, and that cysteine-mediated cross-linking of the C-terminus to presumptive pore-associated residues interfered with cleavage-mediated activation. This suggested that the C-terminus blocks Panx1 via a “ball-and-chain” mechanism. According to this current model, cleavage of the C-terminus allows dissociation of the autoinhibitory region from the pore to allow for channel activation. Notably, although it is clear that Panx1 activation can occur in non-apoptotic cells in the absence of C-terminal cleavage, our findings nevertheless suggest that altered interactions between the C-terminus and the pore region may represent a common site for regulation of Panx1 by various channel modulators.

The aforementioned characteristics of Panx1 channels, although not yet fully elaborated, are relatively well accepted. We now turn our attention to some more controversial points regarding channel intrinsic properties and regulatory mechanisms.

**Ionic Selectivity and Unitary Conductance**

It is often stated that Panx1 forms a large-conductance, non-selective channel that allows passage of several high molecular weight fluorescent dyes and, importantly, that also permits release of nucleotides such as ATP and UTP. Here, we examine some recent work that has challenged these widely held views, especially with respect to the ionic selectivity and unitary conductance of the channels.

Several lines of evidence support Panx1 being a non-selective channel. First, by using inside-out patch recordings with asymmetrical concentration of K+ATP across the membrane, the Dahl laboratory reported that the reversal potential (Erev) of Panx1 single-channel currents was at a point between the predicted Erev for potassium and ATP, suggesting both cation and anion contributions to Panx1 current. It is noteworthy that these measures of an ATP current in single channels remain the only direct demonstration to date of ATP permeation through Panx1. Second, various fluorescent dyes can flux across the plasma membrane when Panx1 channels are activated, and this includes both cationic dyes (To-Pro, Yo-Pro) and anionic dyes (fluorescein, Lucifer Yellow). This dye transfer requires Panx1 activity since it can be abrogated by Panx1 channel blockers or gene knockdown/knockout and enhanced by Panx1 overexpression. However, these studies do not directly demonstrate that the dyes traverse the membrane via the channel itself, which would require measurement of a dye-dependent Panx1 channel current (i.e., similar to the Erev experiments with K+ATP, described above). Nevertheless, the simplest interpretation from these sets of studies is that both anions and cations can permeate through Panx1 channels.

Two independent reports recently challenged this idea by measuring the Erev of whole-cell Panx1 currents during substitution of either cations or anions in the bath. In both studies, it was suggested that Panx1 was strictly anion-selective since Erev was unchanged when extracellular Na+ was replaced by N-methyl-d-glucamine (NMDG+), but shifted noticeably when Cl- was replaced with other anions. Although this provides strong evidence for anion permeation through Panx1, these data cannot exclude the possibility that Panx1 allows cations to pass, albeit with equal selectivity for Na+ and NMDG+; indeed,
that alternative interpretation was previously advanced based on similar results with Na’/NMDG’ substitution in an earlier study. At this point, studies of cation permeation via Panx1 channels have been limited to Na’/NMDG’ substitution and it may be informative to examine permeability of other monovalent and divalent cations. In addition, these studies examined voltage-activated Panx1 channels under basal, unstimulated conditions, and it is possible that selectivity is modified under conditions when channels are stimulated, and when Panx1-dependent cationic dye-uptake is observed. In this respect, it is thought that TRP channels become permeable to large cations, including fluorescent dyes, during sustained stimulation, perhaps via a pore dilation mechanism. Thus, it seems premature to rule out any cationic permeability of Panx1 at this point.

In terms of anion permeation via Panx1, a relative permeability profile was established as NO$_3^−$ > Cl$^−$ > glutamate-aspartate > > HEPES. In addition, because Romanov and colleagues were unable to detect ATP release from exogenously expressed Panx1, they suggested that ATP might not be able to pass through Panx1 channel, perhaps due to its larger molecular weight than HEPES. However, that work did not assess ATP permeation via Panx1 directly, relying instead on an indirect cell-based, calcium mobilization assay system. In addition, molecular size is not the sole determinant of permeability, which can also be influenced by the 3-dimensional structure of the permeants and their interactions with the channel pore. Finally, as mentioned above, these permeation and ATP release studies were performed during membrane depolarization of Panx1 channels recorded under basal, unstimulated conditions, and large molecules like ATP or fluorescent dyes may transit Panx1 more effectively when the channels are activated (e.g., by mechanical stretch, receptor activation, or caspase cleavage). Consistent with the idea that permeability characteristics could change with channel activation, we found a permeability ratio for P$_{\text{HEPES}}$/P$_{\text{Cl}}$ $\approx$ 0.3 in human Panx1 (hPanx1) channels activated by C-terminal truncation (Fig. 1B and C), a value that was measurably higher than that reported for full-length mouse Panx1 (mPanx1, P$_{\text{HEPES}}$/P$_{\text{Cl}}$ < 0.01). In addition to the provocative new results regarding ion selectivity reported in these recent studies, the work also presents an alternative to the view that Panx1 is a very large conductance channel. Early recordings of ATP-permeant hPanx1 channels expressed in Xenopus oocytes revealed a channel with a high unitary conductance (~500 pS) and multiple sub-conductance states. In addition, native Panx1-like channels with a similarly large conductance were identified in mouse hippocampal neurons and human erythrocytes, and were associated with either release of fluorescent dye or ATP. By contrast, the recent work reported a much smaller conductance (~75 pS) for mPanx1 when measured in mammalian cell lines. The reasons for these discrepancies in single-channel conductance are not obvious. Although one might implicate different expression systems or species variants employed in the studies of cloned channels, these considerations cannot explain the reports of native, large-conductance Panx1-like channels from both mouse and human cells. Alternatively, it might be suggested that the large Panx1 conductance becomes apparent under conditions favoring ATP release or dye permeation. However, we find that C-terminally truncated hPanx1 expressed in mammalian cells displays the smaller unitary conductance (~75 pS, Fig. 1D and E), even though it is clear that those cleavage-activated Panx1 channels are associated with both ATP release and dye uptake in apoptotic cells. Therefore, our data do not support a correlation between a large single-channel Panx1 conductance and either ATP release or dye uptake.

In summary, these new observations raise a number of questions regarding long-held views on the basic properties of Panx1 channels. For example, what are the permeability/selectivity properties of the channel in its basal state? Is channel activation associated with alterations in those properties that allow both ATP release and dye uptake? What is the relationship between channel-mediated ionic currents and channel-mediated ATP release and dye uptake? How many C-termini must be cleaved to relieve the block of multimeric Panx1 channels? Are the requirements the same for ionic current, ATP release and dye uptake? What channel properties are affected with different forms of Panx1 activation (e.g., receptor-mediated, stretch-activated)? When designing experiments to address these questions, it will be important to consider the species orthologs of the channel. That is, despite ~90% similarity in protein sequences, the unstimulated full-length hPanx1 does not generate basal current even at depolarized potentials (see Figure 1A), whereas mPanx1 shows some basal activity across a wide range of membrane potentials without additional stimulation.

Other Areas of Controversy

Another point of controversy regarding Panx1 function has centered around its proposed role as the “large-pore conductance” associated with P2X7 purinergic receptor activation. Two models have been advanced to explain the time-dependent change in membrane permeability that follows P2X receptor activation. In one model, the phenomenon is viewed as an intrinsic property of P2X ionotropic receptors, which themselves undergo pore dilation during continued stimulation; in the other, it is envisaged that P2X receptor activation leads to recruitment of additional, large-pore channels that account for the enhanced permeation state. It has been suggested that Panx1 could fulfill this latter role, since Panx1 was co-immunoprecipitated with P2X receptors and inhibition of Panx1 channel using genetic or pharmacological approaches reduced ATP-induced dye-uptake. However, a number of recent studies have demonstrated that dye uptake following P2X receptor activation was unaffected by genetic deletion or pharmacological block of Panx1. There is no obvious explanation for the opposite conclusions reached by different groups. It is possible that Panx1 is able to couple with P2X receptors only in specific conditions, but might be dispensable in others due to genetic compensation by other large-pore channels (e.g., Panx2). Indeed, there is evidence that more than one pathway may be involved in dye uptake resulting from sustained activation of P2X receptors. In addition, it has also been demonstrated that coupling between channels...
P2X7 receptors and Panx1 channels is sensitive to a single nucleotide polymorphism (SNP) of P2X7 receptors, and contrasting results might thus reflect the specific P2X7 isoforms that are present in different systems. Finally, discrepant results could be due to different levels of endogenous P2X receptors or Panx1 channels in different expression systems, examination of Panx1 orthologs from various species, or even the types of fluorescent dye used in different studies. In any case, since P2X-activated large pore activity and dye uptake can occur in the absence of Panx1, it seems clear that other pathways must contribute in some circumstances. Therefore, the phenomenon of P2X-activated dye uptake and ATP release should not be associated reflexively with Panx1.

We have not touched on additional inconsistencies in literature regarding the regulation and function of Panx1 channels. For example, it remains to be determined whether Panx1 can be activated by intracellular calcium; whether there is a role for Panx1 in inflammasome activation and release of IL-1β; (see comment in ref. 14); whether Panx1 serves as an pro- or anti-ontocortic factor; and whether Panx1 is involved in astrocytic ATP release. The early idea that Panx1 is required for ATP release from type 2 taste cells was recently questioned, and a contribution from the related calcium homeostasis modulator 1 (CALHM1) channel, rather than Panx1, in taste-stimuli-induced ATP release was demonstrated. Since CALHM1 expression also supports dye uptake from cells, this new channel represents another candidate that should be considered for other functions previously ascribed to Panx1, but one that may be distinguished based on its differential pharmacology.

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Conclusion Remarks
Since its initial identification some 15 y ago, Panx1 has been associated with diverse physiological mechanisms and pathological conditions. However, a number of basic channel properties and regulatory mechanisms are still largely unclear, even when studied in heterologous systems. There is also an urgent need to identify selective, small-molecule modulators of Panx1 in order to better characterize its actions in situ, and to determine its potential as a therapeutic target. With respect to defining the actions of native Panx1 channels, new studies using Panx1 knockout mice are likely to continue to establish functional effects in a wide variety of contexts where ATP release and purinergic signaling are important.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Concluding Remarks
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