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# Pannexin membrane channels are mechanosensitive conduits for ATP

Li Bao, Silviu Locovei, Gerhard Dahl*

*Department of Physiology and Biophysics, University of Miami School of Medicine, 1600 NW 10th Ave, Miami, FL 33136, USA*

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**Abstract Intercellular calcium wave propagation initiated by mechanical stress is a phenomenon found in nearly all cell types. The waves utilize two pathways: transfer of InsP3 directly from cell to cell through gap junction channels and release of ATP onto extracellular purinergic receptors. The conduit for ATP has remained elusive and both a vesicular and a channel mediated release have been considered. Here, we describe the properties of single pannexin 1 channels. They have a wide expression spectrum, they are of large conductance and permeant for ATP, and they are mechanosensitive. Hence, pannexins are candidates for the release of ATP to the extracellular space upon mechanical stress.**


*Keywords:* Pannexin; Single channel; ATP; Mechanoasensitivity; Calcium wave

## 1. Introduction

The coordinated response of cells to mechanical stress is based on increased intracellular free calcium in the stressed cell, which spreads like a wave front from cell to cell. Propagation of the calcium wave involves the flux of InsP3 directly from cell to cell through gap junction channels and release of ATP onto extracellular purinergic receptors [1,2]. Connexins are the structural components of gap junction channels [3,4] and they have also been invoked to provide a conduit for ATP to the extracellular space through gap junction hemichannels [5,6]. However, with the exception of the lens specific connexins, cx46 and cx50, connexins form open hemichannels only under unphysiological conditions [7,8]. Furthermore, in astrocytes ablation of the main connexin, connexin 43, did not affect propagation velocity of intercellular calcium waves although coupling was reduced to 30% in spinal cord astrocytes and 5% in cortical astrocytes [9], with the residual coupling being mediated by other connexins and/or pannexins. Recently, it has been recognized that pannexins represent a second family of gap junction proteins in vertebrates [10–12]. Macroscopic currents in rat pannexin 1 expressing oocytes have indicated that this pannexin, like connexins 46 and 50, can form patent hemichannels in the non-junctional plasma membrane in physiological calcium concentrations [12]. Pannexin 1 is widely expressed in tissues with documented calcium wave propagation. We, therefore, tested whether its channel properties would be consistent with that of an ATP conduit.

## 2. Materials and methods

Preparation of oocytes and electrophysiological recording were performed as described [13]. Pannexin 1 (MRS 1) was kindly provided by Dr. Graeme Bolger, University of Alabama, in pBluescript. The plasmid was linearized with *Xho*I and in vitro transcription was performed with T3 polymerase using the mMessage mMachine kit (Ambion). 20 nl of mRNA (50 ng/μl) was injected into oocytes. The injected oocytes were then transferred into fresh OR2 (in mM: 82.5 NaCl, 2.5 KCl, 1.0 $MgCl_2$, 1.0 $CaCl_2$, 1.0 $Na_2HPO_4$, 5.0 HEPES and antibiotics (Penicillin, 10 000 U/ml amd Streptomycin, 10 mg/ml), pH 7.5) medium with elevated $Ca^{2+}$ (5 mM) and incubated at 18 °C for 48 h. For electrophysiological recordings, oocytes were transferred to regular OR2.

Single pannexin 1 hemichannels were studied by the patch-clamp technique [14] using a WPC 100 amplifier (E.S.F. Electronic, Goettingen, Germany). Unless stated otherwise, the bath and pipette contained KGlu solution (140 mM KGlu, 10 mM KCl and 5.0 mM TES, pH 7.5). Negative pressure was applied to the membrane patch pneumatically through a port on the pipette holder. The negative pressure was established first in a reservoir by applying suction with a syringe. The pressure was measured with a water manometer. Step changes of pressure were applied to the membrane patch by connecting the pipette via a valve mechanism either to the reservoir or atmospheric pressure.

ATP flux was determined by luminometry. To open pannexin channels, oocytes were depolarized by incubation in KGlu solution. The supernatant was collected and assayed with luciferase/luciferin (Promega, Madison, USA).

Channel activity was analyzed only for patches containing single channels. For assessment of the effects of mechanical stress on open probability of single channels, Student's paired *t* tests were performed and the *p*-values are indicated in the figures.

## 3. Results and discussion

To study the properties of single pannexin channels, human pannexin 1 [10] (originally cloned as MRS1, GenBank Accession No. AF093239) was expressed in *Xenopus oocytes* and analyzed by patch clamp. Consistent with the observed macroscopic membrane currents [12], expression of human pannexin 1 in single *Xenopus oocytes* resulted in the appearance of a novel type of membrane channel with a large unitary conductance of 475 pS in 150 mM potassium gluconate (Fig. 1) and 550 pS in 150 mM KCl (not shown). The channel activity has to be attributed to pannexin 1 because these large conductance channels were not observed in control oocytes and because of the distinctive features of the channel, not described for other channels expressed in oocytes in the literature, in-

* Corresponding author. Fax: +1-305-243-5931.
*E-mail address:* gdahl@miami.edu (G. Dahl).

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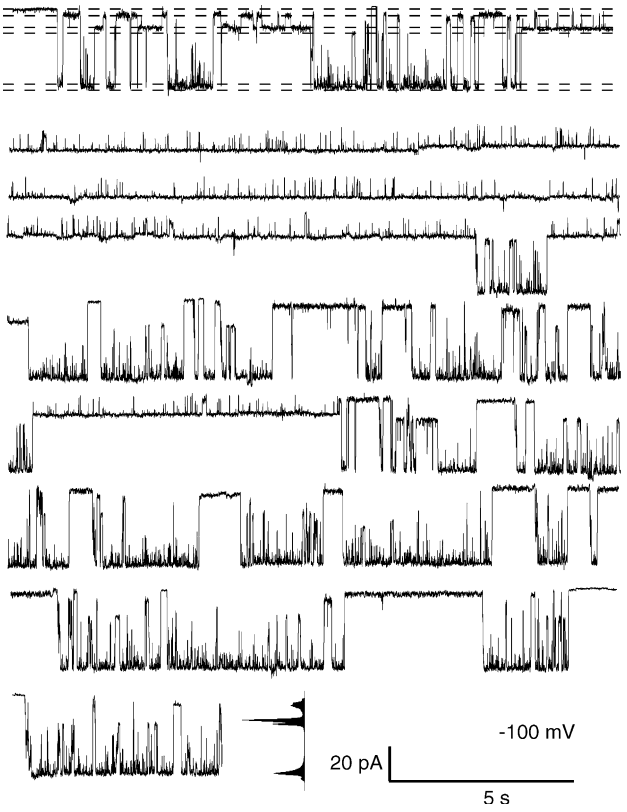


Fig. 1. Single channel currents in an inside-out membrane patch excised from an oocyte expressing human pannexin 1. The patch was held at a potential favorable for channel opening (−20 mV) before switching to −100 mV to facilitate identification of various current levels. An uninterrupted recording segment of 140 s is shown together with an all point histogram of the entire segment. In addition to the closed (c) and the full open state (o) at least one, possibly two, low level subconductance states in addition to two intermediate and one high subconductance state can be discerned.

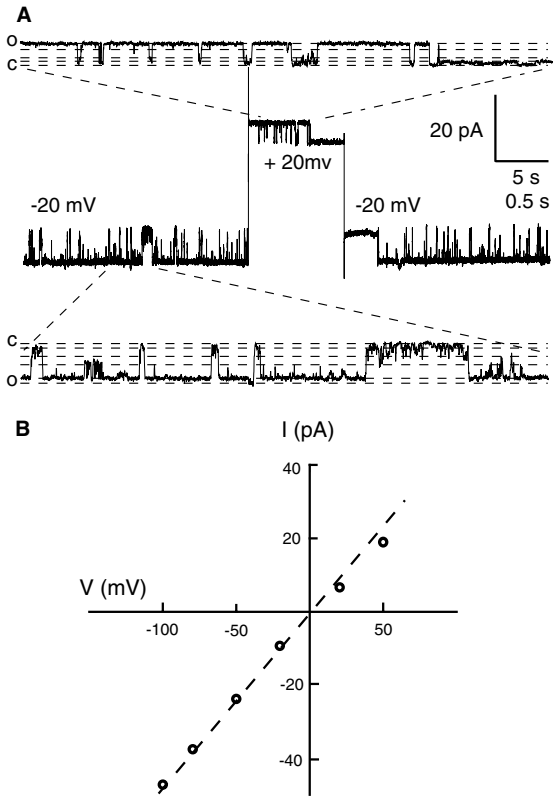


Fig. 2. (A) Single channel currents at −20 and at +20 mV. Multiple conductance levels can be observed over a wide range of voltages. At positive potential, the current excursions are smaller than at negative potentials because of the channel's preference for subconductance states. (B) The current–voltage relationship for pannexin 1 single-channel currents reveals a slope conductance of 475 pS at negative potential with potassium gluconate as charge carrier.

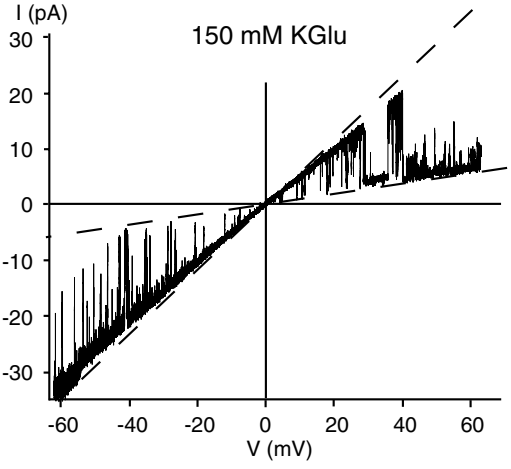


Fig. 3. Responses of pannexin 1 channels to voltage ramps. A 70-s voltage ramp from −60 to +60 mV was applied to an on-cell membrane patch containing a single pannexin 1 channel. The bath and pipette solutions contained 150 mM potassium gluconate.

cluding connexin channels [15–17]. The channel has unusual properties: it exhibited at least five open states. Besides the full open state, no less than four subconductance states with 5%, 25%, 30% and 90% of the maximal conductance can be discerned. These subconductance states were observed with KGlu (Fig. 1) as well as KCl solutions (not shown) in pipette and bath. Transitions between states were fast and highly variable. They occurred between substates but also between closed and full open states. The subconductance states were observed over the wide range of voltages examined (−100 to +50 mV). The channel, when active, mainly dwelled in the subconductance states, while sojourns to the full open and closed states were scarce (Fig. 2). Like cx46 hemichannels [15], pannexin 1 channels are gated by voltage in a complex manner. A slow gating mechanism, probably equivalent to the loop gate of cx46, closed the channel at potentials more negative than −20 mV. Held at positive potentials, the channels mainly dwelled in the low subconductance states. These single channel properties are consistent with macroscopic currents. When held more negative than −20 mV the channels were closed, and voltage steps to +20 mV and more positive values resulted in rapid inactivation of the currents (data not shown, see also [12]).

Fig. 3 illustrates the response of a single channel to voltage ramps applied after holding the patch at a potential favorable for channel opening (−20 to +20 mV). The currents at negative potentials are larger than at positive potentials, indicating rectification. In addition, the preference for subconductance states at positive potentials is noticeable.

The permeability properties of pannexin channels are not known. To test whether pannexin channels allow the passage of molecules larger than the standard charge carriers, sodium, potassium and chloride, the flux of ATP was examined. The efflux of ATP from oocytes expressing pannexin 1 was determined by luminometry. Oocytes expressing pannexin 1 exhibited an elevated ATP efflux only under conditions favoring channel opening, i.e., when depolarized by high potassium solution (Fig. 4). The depolarization-induced ATP release in control oocytes and in oocytes expressing cx43 probably represents the vesicular release of ATP reported previously [18].

As an independent measure of ATP permeability, 10:1 gradients of potassium ATP were applied to excised patches containing single pannexin 1 channels. In the absence of ATP permeability, i.e., with exclusive potassium permeability, the reversal potential would be expected to be close to +60 mV on the side of the lower salt concentration (cytoplasmic side). Membrane currents instead reversed at ~+25 mV, indicating that ATP carries current in the pannexin channel (Fig. 4B).

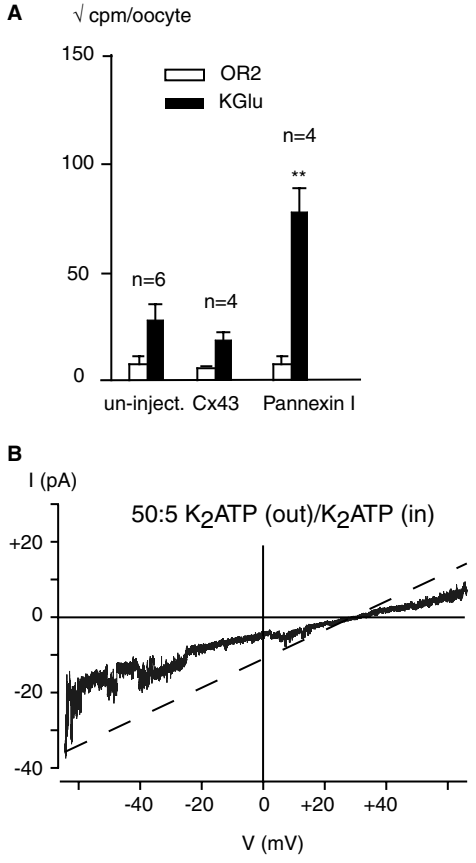


Fig. 4. ATP flux through pannexin 1 channels. (A) ATP release from oocytes was determined by luminometry. When depolarized by elevated potassium in the extracellular fluid, uninjected oocytes and oocytes expressing connexin 43 released ATP to the extracellular space, probably by a Brefeldin-sensitive, vesicular mechanism [18]. Expression of pannexin 1 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 pannexin 1 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 $K_2ATP$. The average reversal potential from five independent measurements was +25 mV, indicating substantial permeability of the channel for $ATP^{3-}$.

The permeability for ATP is, however, less than the potassium permeability because the reversal potential is positive.

We have observed that cx46 hemichannels are sensitive to mechanical stress (Bao, Sachs and Dahl, unpublished). To test whether pannexin channels are also mechanosensitive, we used a single channel patch clamp. Cell-attached or excised membrane patches with single pannexin 1 hemichannels were mechanically stressed by suction applied to the patch pipette [19]. Fig. 5 shows typical responses of pannexin 1 channels to stretch. Over a wide range of potentials (−50 to +50 mV), the channels exhibited increased activity when stressed mechanically. Increased channel activity occured by a switch from a low subconductance to a higher conductance level (Fig. 5A) or started from a totally closed channel (Fig. 5B). For quantitative analysis (Fig. 5C), we used a detection threshold for openings above the low subconductance level to capture both types of increased activity. During prolonged stretch, channel activity often subsided spontaneously.

These properties of pannexin 1 suggest that a non-junctional membrane channel (gap junction hemichannel) formed by this protein could be involved in the widespread phenomenon of calcium wave propagation between cells. Typically elicited by mechanical stimulation, the response of many cell types to the stimulus is to increase cytoplasmic free calcium concentration that propagates to neighboring and distant cells like a wave front [1]. Propagation occurs by two pathways. One involves

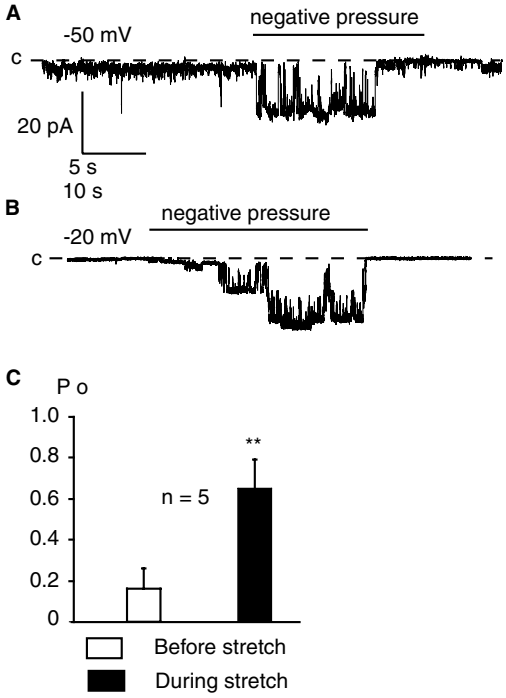


Fig. 5. Stretch-activation of pannexin 1 hemichannels. Single channel currents were recorded from a membrane patch of an oocyte exogenously expressing pannexin 1. The membrane potential was held at −50 mV (A) and −20 mV (B). Negative pressure (~40 mbar) was applied by suction to the patch pipette during the time indicated by the line. The patch in (B) contained two channels. Bath and pipette solutions contained potassium gluconate. Open probability (C) was determined for five membrane patches from different oocytes. Means ± S.E. are plotted; statistical significance of differences between the activity before and during application of stretch uses a paired $t$ test as indicated: **, $P < 0.01$.

gap junction channels that permit the transit of a messenger, probably Insp3, directly from cell to cell, bypassing the extracellular space. The other is an extracellular pathway that involves release of ATP from one cell and binding of ATP to purinergic receptors on neighboring cells.

While the involvement of connexins is obvious for the first pathway, several studies have implicated cx43 in the extracellular pathway, too [5,6,20]. It has been proposed that hemi gap junction channels composed of connexin 43 subunits provide the conduit for ATP release. The proposal is based on cx43 being the major connexin in the investigated cell types and the use of drugs that are known to interfere with gap junction channels. While some studies support the notion that cx43 hemichannels indeed can be functional, typically such channel activity is only observed in unphysiological low calcium concentrations [7] or at potentials only achieved in experimental settings, i.e., >+60 mV [21]. Furthermore, ablation of cx43 in mice did not change the velocity of intercellular calcium wave propagation in spinal cord astrocytes, suggesting that efficient ATP release can occur in the total absence of cx43 [22]. Calcium waves propagate in cx43 deficient cells mainly through an extracellular pathway. The latter pathway is augmented by a switch in subtypes of purinergic receptors, which compensates for the loss of the gap junction mediated propagation mode [23].

Pannexin 1 hemichannels, on the other hand, fulfill criteria required for an involvement in calcium wave propagation. Pannexin 1 is widely distributed among tissues with cell communication via calcium waves. The channel formed by this protein can be opened by mechanical perturbation at the resting membrane potential. The channel is permeable for ATP and, finally, pannexin 1 channels can be opened at physiological calcium concentration [12]. Hence, a contribution of pannexin 1 channels to the initiation of calcium waves is plausible. Whether pannexin 1 channels are also involved in wave propagation remains to be determined. Such an involvement is theoretically feasible in many ways.

As shown by Bruzzone et al. [12] and the present study, pannexin 1 forms channels in the non-junctional membrane that connects the cytoplasm with the extracellular space. Open gap junction hemichannels, because of their large size and permeability to ATP and second messenger molecules [24,25], are generally deleterious to cell health. For example, the lens connexins (cx46 and cx50) form open hemichannels in oocytes expressing these connexins exogenously, and the cells die unless the hemichannels are kept shut by an elevated extracellular calcium concentration [26]. The voltage dependence of pannexin 1 channels ensures that the channels do not threaten cell viability. They will be closed at the resting membrane potential with a large safety margin towards depolarization unless challenged by mechanical stress, whereupon they can directly signal to other cells via ATP.

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