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Activation of pannexin 1 channels by ATP through P2Y receptors and by cytoplasmic calcium

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Abstract The ability for long-range communication through intercellular calcium waves is inherent to cells of many tissues. A dual propagation mode for these waves includes passage of IP3 through gap junctions as well as an extracellular pathway involving ATP. The wave can be regenerative and include ATP-induced ATP release via an unknown mechanism. Here, we show that pannexin 1 channels can be activated by extracellular ATP acting through purinergic receptors of the P2Y group as well as by cytoplasmic calcium. Based on its properties, including ATP permeability, pannexin 1 may be involved in both initiation and propagation of calcium waves.

Keywords: Pannexin; Purinergic receptor; Activation by calcium; ATP-induced ATP release; Calcium wave

1. Introduction

In many tissues stimulation of a single cell can initiate a propagated calcium wave traveling from cell to cell. Functional roles of intercellular calcium waves include regulation of ciliary beat in airway epithelia [1], modulation of synaptic transmission between neurons by glia cells [2], coordination of metabolism by glial cells [3], control of vascular perfusion by endothelial cells [4], and ossification by osteoblasts [5].

A variety of stimuli, including mechanical stress or depolarization, can elicit an increase of cytoplasmic calcium concentration. Somehow intracellular IP3 is generated, which then diffuses to neighboring cells through gap junction channels resulting in calcium increases in neighboring cells [1]. However, wave propagation is not restricted to contiguous cells. In addition to the gap junction mediated expansion, an extracellular propagation mode involving ATP as messenger has been identified [6,7]. Binding of extracellular ATP to P2Y receptors results in the production of intracellular inositol triphosphate (IP3) and consequently to an increase in the cytoplasmic calcium concentration of contiguous and non-contiguous cells. Wave propagation appears to be regenerative, although with a low safety margin [8–14], suggesting that ATP is also released from cells not subjected to the initial stimulus. While gap junction mediated wave propagation is well documented [1], the ATP mediated propagation mode is sufficient. Calcium waves in astrocytes of Cx43 null mice propagate at the same speed as in wild type cells, where Cx43 is the major gap junction protein [15,16].

A series of mysteries shroud the extracellular wave propagation scheme. What molecules sense a stimulus such as mechanical stress? What links the stimulus to ATP release from the cell? What is the ATP release mechanism in the stimulated cell? Does the same ATP release mechanism operate in the non-stimulated cells? If so, how is ATP release in the non-stimulated cell activated?

In a simple scenario calcium wave initiation and propagation involves a channel that is mechanosensitive and permeable to ATP for initiation of the wave and is activated by ATP through purinergic receptors for wave propagation. Concerning the molecular mechanism of ATP release, both a vesicular mechanism and channel-mediated release have been proposed. Although vesicular release can be documented by, for example, its sensitivity to Brefeldin [17], it cannot be the only release mechanism, as ATP release can be observed in vesicle free cells, like erythrocytes [18]. Many channels, including a voltage dependent anion channel (VDAC), a volume regulated anion channel (VRAC), the cystic fibrosis transmembrane regulator (CFTR), a maxi-anion channel, the purinergic receptor P2X7 and connexin 43 [19–24] have been proposed to represent release channels. None of them fulfills all the criteria stated above.

We have shown recently that pannexin1 forms a membrane channel that is mechanosensitive and permeable to ATP [25]. This channel protein is widely expressed and thus is probably found in the right places, i.e., where calcium waves occur. Thus, pannexin 1 should be considered as a candidate ATP release channel involved in the initiation of calcium waves. Here, we test whether pannexin 1 can be activated through purinergic receptors and thus also fulfills the third requirement for an ATP release channel involved in calcium wave propagation.

2. Materials and methods

Preparation of oocytes and electrophysiological recording were performed as described [26]. Human pannexin 1 (MRS1) was kindly provided by Dr. Graeme Bolger, University of Alabama, and human P2Y1 by Dr. T. Kendall Harden, University of North Carolina. Human P2Y2 was obtained from the American Type Culture Collection (Manassas, VA, USA). Pannexin 1, in Bluescript, was linearized with *Kpn*I, P2Y1, in pcDNA3.1, with *Sma*I and P2Y2, in pCMVV. Sport 6, with *Nhe*I. In vitro transcription was performed with the polymerases T3 (pannexin 1), T7 (P2Y1) and SP6 (P2Y2) using the mMessage mMachine kit (Ambion, Austin, TX, USA). mRNAs were quantified by absorbance (260 nm), and the proportion of full-length transcripts

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was checked by agarose gel electrophoresis. In vitro transcribed mRNAs (~20 nL) were injected into *Xenopus* oocytes. The oocytes were incubated at 18 °C for 18–24 h in Oocyte Ringer's Solution (OR2) (in mM: 82.5 NaCl, 2.5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 1 Na_2HPO_4, 5 HEPES, pH 7.5).

Whole cell membrane current of single oocytes was measured using a two-microelectrode voltage clamp and recorded with a chart recorder. Both voltage-measuring and current-passing microelectrodes were pulled with a vertical Puller (Kopf) and filled with 3 M KCl. The recording chamber was perfused continuously with solution. Membrane conductance was determined using voltage pulses, typically of 5 s duration and of 5 mV amplitude.

Single pannexin 1 channels were studied by the patch-clamp technique [27] using an Axopatch-1B amplifier (Axon Instruments). Currents were filtered at 5 kHz, digitized using a VR-10B digital data recorder, and stored on video tape. The recordings were transferred to a Power Macintosh (Apple) computer using an ITC-18 Computer Interface (Instrutech Corporation) and analyzed. Acquisition and analysis were done with the Acquire and TAC programs (both from Bruxton Corporation).

The vitelline membrane of the oocyte was removed and the oocyte was washed once before transfer into a new dish containing potassium chloride solution (150 mM KCl, 5.0 mM TES, 1 mM EGTA, pH 7.5). The amount of Ca^{2+} added to the intracellular solution to obtain approximate free Ca^{2+} concentrations of 0.1–100 μM was calculated using MaxChelator (http://www.stanford.edu/~cpatton/maxc.html).

Electrode pipettes made from glass capillary tubing (1.5–0.86 mm, #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 μm with a resistance of 10–20 MΩ in KCl solution. Both the standard pipette and bath solution were KCl solution. After an inside-out patch was excised from the membrane and the pannexin 1 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 100 μl/s.

3. Results

3.1. Activation of pannexin 1 channels through P2Y receptors

As reported previously [25,28], pannexin 1 expressed in *Xenopus* oocytes forms channels in the non-junctional membrane in addition to forming gap junction channels. The pannexin 1 channels connecting the cytoplasm with the extracellular space are closed at the resting membrane potential and can be opened by depolarization. Like gap junction channels formed by connexins, pannexin 1 channels are inhibited by cytoplasmic acidification (Fig. 1A).

We have shown previously that pannexin 1 channels are mechanosensitive and highly permeable to ATP and thus qualify as a channel to release ATP to the extracellular space for the initiation of intercellular calcium waves [25]. If the channel were also involved in ATP-induced ATP release, i.e., in wave propagation, it should be activated by extracellular ATP through purinergic receptors.

To test whether pannexin 1 can be activated by extracellular ATP, we co-expressed it with the purinergic receptors P2Y1 or P2Y2, known to be expressed in tissues exhibiting intercellular calcium waves [16,29–31]. As a control, oocytes expressing exclusively pannexin 1 channels were exposed to extracellular ATP and no response was observed (Fig. 1B). ATP applied to oocytes expressing exclusively P2Y receptors exhibited small currents (Fig. 1C and E), probably carried by calcium-activated chloride channels and a cation permeability [32,33].

Application of ATP to oocytes co-expressing pannexin 1 together with either P2Y1 or P2Y2 resulted in large currents (Figs. 1D and F and 2). The ATP-induced currents were inhibited by cytoplasmic acidification, consistent with their origin in pannexin 1 channels. The concentration requirements for ATP to induce the currents differed; P2Y1 required higher concentrations than P2Y2. This is consistent with the different sensitivities of the receptors to the ligand [34].

Although both P2Y receptors were able to activate pannexin 1 channels, the mode of activation was distinct. P2Y1 activated the channels slowly and the response was sustained for the period of ATP application. In contrast, activation by P2Y2 was fast and transient even with prolonged application of ATP. Interestingly, a similar difference has been observed for the kinetics of intercellular calcium wave propagation mediated by these two purinergic receptors. Calcium waves mediated by P2Y2 receptors propagate at higher velocity than those mediated via P2Y1 [35].

3.2. Activation of pannexin 1 channels by cytoplasmic calcium

Activation of pannexin 1 channels via P2Y receptors could occur through a membrane-delimited mechanism or through a second messenger pathway. P2Y1 and P2Y2 receptors are G-protein linked and activate phospholipase C to produce IP3 [36]. As a result of receptor activation, cytoplasmic calcium concentration increases. To test whether this signaling pathway is involved, we tested the effect of calcium on pannexin 1 channels. First, we increased cytoplasmic calcium with the calcium ionophore A23186 in oocytes expressing pannexin 1. The ionophore application resulted in a current that was suppressed by cytoplasmic acidification, consistent with a pannexin 1 mediated current (Fig. 3A). In uninjected oocytes, the ionophore induced a small membrane conductance (not shown) that was not affected by acidification and is probably attributable to endogenous calcium-activated chloride channels.

To test whether calcium affects pannexin 1 channels directly, we used the patch clamp technique. Pannexin 1 channels have a unique profile [25]: their unitary conductance is 550 pS in potassium chloride solution, they exhibit long-lived multiple subconductance states and they have a characteristic voltage dependence. The channels are closed at negative transmembrane potential and open at positive potential. Once open, returning the potential to negative leaves the channels active for tens of seconds. Among the channels endogenous to oocytes is a calcium activated chloride channel with a unitary conductance of 3 pS [37]. Thus, the channels can easily be discriminated.

After identification of pannexin channels at potentials permissive for channel opening, the potential was held at −50 mV, which closed the channels. Application of calcium to the cytoplasmic face of the channels contained in excised inside-out membrane patches resulted in channel activity that ceased upon washout of the calcium containing solution (Fig. 3). Free calcium concentrations in the micromolar range were sufficient to elicit this effect (Fig. 4).

4. Discussion

The properties of pannexin 1 channels are suggestive of a prime role of these channels in both initiation and propagation of calcium waves. The channels formed by pannexin 1 on the surface membrane are closed at the resting membrane potential [28] but can be opened at this potential by mechanical