Pannexin-1 Couples to Maitotoxin- and Nigericin-induced Interleukin-1β Release through a Dye Uptake-independent Pathway*

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Pannexin-1 is a recently identified membrane protein that can act as a nonselective pore permeable to dyes such as ethidium when ectopically expressed. Blockade of pannexin-1 in macrophage endogenously expressing the ATP-gated P2X<sub>7</sub> receptor (P2X<sub>7</sub>R) blocks the initial dye uptake, but not the ionic current, and also blocks processing and release of interleukin-1β (IL-1β) in response to P2X<sub>7</sub>R activation. These results suggest that pannexin-1 may be a hemichannel activated by the P2X<sub>7</sub>-R to provide the conduit for dye uptake and downstream signaling to processing and release of IL-1β. We have pursued this hypothesis by measuring dye uptake and IL-1β processing and release in mouse J774 macrophage in response to P2X<sub>7</sub>R activation and to maitotoxin and nigericin, two agents considered to evoke IL-1β release via the same mechanism. The experiments were carried out over time periods during which no lactate dehydrogenase was released from cells to examine only noncytolytic pathways. P2X<sub>7</sub>-R activation evoked dye uptake that could be separated into two components by pannexin-1 inhibition: an initial rapid phase and a slower pannexin-1-independent phase. Maitotoxin-evoked dye uptake was unaltered by pannexin-1 inhibition. Nigericin did not induce dye uptake. Inhibition of pannexin-1 blocked caspase-1 and IL-1β processing and release in response to all three stimuli. Thus, although pannexin-1 is required for IL-1β release in response to maitotoxin, nigericin, and ATP, a mechanism distinct from pannexin-1 hemichannel activation must underlie the former two processes.

Interleukin-1β (IL-1β)<sup>2</sup> is the crucial pro-inflammatory cytokine whose early release from activated macrophage leads to the generation of the host defense response to bacterial toxins, injuries, and other inflammatory challenges (1–3). IL-1β is synthesized as a 34-kDa pro-peptide that has no biological activity until it is cleaved by activated caspase-1 resulting in its mature, bioactive 17-kDa form (2–4). IL-1β synthesis is initiated by inflammatory stimuli such as lipopolysaccharide (LPS), but little or none is released in the absence of a secondary stimulus (2, 3). Although much is known about the formation of the cytosolic multiprotein complex that is responsible for caspase-1 activation (the inflammasome) (2–4), how secondary stimuli act to trigger inflammasome activation and subsequent release of bioactive IL-1β remains poorly understood. One of the most potent physiological triggers for the processing and release of mature IL-1β is activation of the ATP-gated P2X<sub>7</sub> receptor channel, which itself is up-regulated in response to LPS and other inflammatory stimuli (2).

The P2X<sub>7</sub>-R receptor is an unusual cation-selective ion channel whose activation by relatively high levels of extracellular ATP evokes not only ionic current but also results in the passage of large molecules up to about 900 Da (2, 5). Passage of these larger molecules, usually assayed by measuring the uptake of fluorescent dyes such as ethidium and YoPro1, has been considered to represent the opening of a “large pore” (2, 5). The P2X<sub>7</sub>-R ion channel opens with kinetics generally similar to other ligand-gated ion channels (i.e. within milliseconds), whereas the large pore is activated some seconds later (2–10 s depending on receptor density and agonist concentration) (2, 5, 6). Until recently, the general consensus was that the P2X<sub>7</sub>-R channel protein itself dilated over several seconds to a size sufficient to allow dye uptake (5). However, several indirect lines of evidence obtained over the past 3–4 years led to the more likely idea that a distinct, accessory protein linked to the activation of the P2X<sub>7</sub>-R is directly responsible for the dye uptake (5, 6–8). We have recently identified this accessory protein as pannexin-1 (9).

Panxins are a three-membered family of mammalian membrane proteins (Panx1, 2, and 3) about which little is currently known. They were identified in 2003–2004 by low stringency homology to invertebrate gap junction proteins, innexins (10, 11), and Panx1 can form gap junction-like connections that allow intercellular passage of dyes when overexpressed in two adjacent oocytes or mammalian epithelial cell lines (10, 12). However, there is no evidence to date that endogenous Panx1 forms gap junctions. Overexpression of Panx1 in single oocytes or mammalian cells also results in the formation of “hemichannel-like” nonselective pores (9, 10, 13, 14), and we have obtained evidence that endogenous Panx1 forms the dye-permeable uptake path upon activation of P2X<sub>7</sub>-Rs (9). We found that Panx1 and P2X<sub>7</sub>-R co-immunoprecipitate in P2X<sub>7</sub>-R-expressing cells and that selective inhibition of Panx1 by siRNA silencing or by a Panx1-mimetic inhibitory peptide blocked ATP-evoked dye uptake but not receptor activation or its...
resulting ionic current. Of general physiological significance, Panx1 protein was up-regulated in macrophage in response to inflammatory challenge with LPS, and its selective inhibition in these activated macrophage blocked the P2X,R-mediated processing of caspase-1 and subsequent processing and release of IL-1β (9). These results suggest a direct functional link between P2X,R-mediated dye uptake and activation of caspase-1/IL-1β processing and release.

To further elucidate Panx1 signaling processes underlying IL-1β processing/release, we have examined whether and how Panx1 may be involved in IL-1β release evoked by two well known nonphysiological secondary stimuli that have been thought to act via the same mechanism(s) as P2X,R, the marine toxin, maitotoxin, which activates (or forms) calcium-permeable, cationic currents and also leads to ethidium and YoPro1 uptake, and the K+;/H+ antiport ionophore, nigericin (7, 15–19). We compared dye uptake induced by ATP, maitotoxin, and nigericin in HEK cells stably expressing P2X,R and both dye uptake and caspase-1/IL-1β processing/release, but Panx1 is involved only with an initial phase of P2X,R-mediated dye uptake, whereas dye-uptake pathways are not involved in IL-1β processing/release induced by maitotoxin or nigericin.

**EXPERIMENTAL PROCEDURES**

**Materials**—Carbenoxolone (CBX), ethidium bromide, ATP, nigericin, and *Escherichia coli* LPS were from Sigma, and maitotoxin was from Alexis. The Panx1 mimetic blocking peptide 10panx1 (WRQAAFVDSY), the connexin 32 blocking peptide 32gap27 (SRPTEKTVF), and the other Panx1 peptides (GTQISCSFS, CFSPSSFWRQAA, QKNSLQSESGNL, YCWAAVQQKNSLQSESGNL, LRDNSTVPDQFQ, and SGILRNDS-TVPDQF) were synthesized by Sigma-Genosys and Alta Biosciences.

**Cell Culture**—*J774* murine macrophages cell line was cultured in RPMI and human embryonic kidney 293 cells in F-12 medium, all supplemented with 10% fetal calf serum (Invitrogen).

**RNA Interference**—Small interference RNAs against human Panx1 was carried out as described previously (9). Briefly, HEK293 cells stably expressing P2X7 receptors were transfected using Lipofectamine2000 (Invitrogen) and 200 pmol of Panx1-siRNA (5'-GCUCAGAGGUAUGAACAU-3') or Silencer® negative control 1 siRNA (Ambion) as the negative scrambled control. The cells were cultured for 48 h at 37 °C before experiments, and mRNA expression levels for Panx1, P2X7, and β-actin were determined by reverse transcriptase-PCR.

**Reverse Transcriptase-PCR Analysis**—Total RNA was isolated using the RNaseasy mini kit (Qiagen), followed by reverse transcription using SUPERSCRIPT™ III (Invitrogen) RNase H-reverse transcriptase with oligo(dT). Specific primers for Panx1, rat P2X7, or β-actin were previously reported (9) and used in PCR. The obtained product sizes for Panx1 (610 bp), rat P2X7 (585 bp), and β-actin (1.1 kb) were as expected from their mRNA sequences.

**Fluorescence Assays**—Dye uptake experiments were carried out using a Nikon confocal microscope under 20× objective, and ethidium fluorescence was measured at 543/590 nm excitation/emission. The cells were preincubated for 15 min at 37 °C with 500 μM of 10panx1 or 1 mM of other Panx1 peptides or 32gap27 peptide, following the addition of 20 μM ethidium. The images were recorded at 5–10-s intervals for 2 min before and during superfusion at 37 °C with ATP, nigericin, or maitotoxin for 5–20 min in standard physiological extracellular solution (except otherwise stated) consisting of 147 mM NaCl, 10 mM HEPES, 13 mM glucose, 2 mM CaCl2, 1 mM MgCl2, and 2 mM KCl. For each experiment, the time course of ethidium fluorescence was measured for 30 isolated (not touching) cells and then averaged to obtain the mean fluorescent signal, and the slope of the fluorescent signal versus the time was used as the most accurate and consistent measurement for comparisons. Digitonin (100 μM) was used at the end of the experiments to induce maximum dye uptake.

**Western Blotting**—*J774* macrophages were seeded at 2 × 10⁶ cells/well in a 6-well dish on the night before treatment. The cells were stimulated with LPS (1 μg/ml) for 4 h, washed twice with the same solution used for dye uptake experiments, and preincubated for 30 min with 500 μM 10panx1 or 1 mM of other Panx1 peptides or 32gap27 peptide and 5 mM ATP, 0.2 mM maitotoxin, or 5 mM nigericin applied for 10–20 min. The cells were lysed in radioimmune precipitation assay buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM MgCl2, 1 mM CaCl2) supplemented with 1% Triton X-100 and Complete protease inhibitor mixture (Roche Applied Science) for 1 h at 4 °C and centrifuged to remove particulate matter. The supernatants were clarified by brief centrifugation and 60% of the volume was concentrated using 10-kDa nominal molecular mass cut-off filters (Millipore). 15 μg of total protein and the concentrated supernatants were resolved in 4–15% polyacrylamide gels, transferred to polyvinylidene difluoride membranes (Millipore) by electroblotting, and immunoblotted with 1:1000 of anti-IL-1β mouse monoclonal antibody (3ZD; Biological Resources Branch, NCI) or with 1:500 of anti-caspase-1 p10 rabbit polyclonal antibody for mouse (Santa Cruz), following horseradish peroxidase-conjugated secondary antibody (DA-KOCytomation) at 1:2000 dilution and detection using the ECL Plus kit (Amersham Biosciences) and Kodak Bio-Max MS film (Sigma). The results were analyzed by densitometry measurements using GeneSnap/GeneTools software (Syngene).

**LDH Release Assay**—The presence of LDH in the media was detected in all of the experiments using a cytotoxicity detection kit (Roche Applied Science) following the manufacturer’s instructions and compared with the total amount of LDH in the cells. In all of the experiments described in this study, the percentage of LDH present in the media was <8%.

**K+ Release Assay**—Cellular and released K+ were measured against standards using the Ciros Vision inductively coupled plasma-atomic emission spectrometer (Spectro Analytical UK Ltd). For these experiments, the cells were seeded at 1 × 10⁶ cells/well in a 12-well dish on the night before treatment. The cells were treated with 1 μg/ml LPS for 4 h, washed twice, and preincubated for 30 min with 500 μM of 10panx1, following 20 min with 5 mM ATP, 0.2 mM maitotoxin, or 5 mM nigericin. The
medium was removed, and the cells were lysed in 1 ml of 10% nitric acid.

Statistical Analysis—The average results are expressed as the means ± S.E. from the number of assays indicated. The data were analyzed by an unpaired Student’s t test to determine difference between groups using Instat (GraphPad) and Excel (Microsoft) software.

RESULTS

Panx1-dependent and -independent Dye Uptake—We first compared the kinetics of ethidium uptake evoked by ATP, maitotoxin, and nigericin in HEK cells stably expressing P2X7-R. Because all of these agents can result in cell death by apoptosis and/or necrosis if the duration and/or concentration of stimulus is sufficient (7, 15–22), we titrated both of these variables to limit our experiments to conditions where cytolysis could be ruled out. We did this by measuring LDH release as a function of duration and concentration of agonist; all of the data presented in this study were obtained under conditions where LDH release was <8% of total cellular LDH. The conditions for HEK cells were 3 mM ATP/10 min (LDH release < 8%, n = 8), 0.5 nM maitotoxin/20 min (LDH release < 2%, n = 8), and 5 μM nigericin/20 min (LDH release < 2%, n = 8). The basal levels of LDH release varied from 0.2 to 1.6% (n = 16).

Fig. 1A illustrates the markedly different rates of ethidium uptake induced by ATP, maitotoxin, and nigericin. ATP induced rapid dye uptake, which saturated the system optics within 3–5 min; dye uptake to maitotoxin was 1–2 orders of magnitude slower, whereas no dye uptake occurred in response to nigericin. In particular, no significant dye uptake occurred in response to maitotoxin during the same period (1–5 min) over which ATP evoked its maximum response (Fig. 1, B and C). In agreement with our previous study (9), inhibition of Panx1 using Panx1-targeted siRNA decreased ATP-evoked ethidium uptake by 70–90% over the initial 3–5 min of agonist application (Fig. 1, B and E). However, significant ethidium uptake was observed when agonist application was prolonged to 10–15 min; this delayed uptake can be seen most clearly in Fig. 1C in which dye uptake has been plotted on a logarithmic time scale. The kinetics of the Panx1-insensitive component of the ATP-evoked response were similar to the kinetics of the maitotoxin-evoked ethidium uptake (Fig. 1, C and E). Panx1 siRNA did not alter maitotoxin-induced dye uptake in the same experiments in which this treatment effectively inhibited the initial phase of the ATP-evoked dye uptake and in which reverse transcriptase-PCR analysis showed complete absence of Panx1 mRNA (Fig. 1D). The results from all of the experiments as illustrated in Fig. 1 (A–C) are summarized in the histogram shown in Fig. 1E where the slope of the ethidium uptake is plotted.

We previously generated a series of peptides corresponding to residues within the putative extracellular loops of Panx1 and found one, 10panx1, that effectively and selectively inhibited ATP-mediated dye uptake but not P2X7-R activation (9). To gain further confidence in its function as a selective Panx1 inhibitor, we compared in more detail the actions of 10panx1 and six other Panx1-mimetic peptides corresponding to other putative ectodomain residues in Panx1 (Fig. 2A). Here we found that kinetics of ATP-evoked ethidium uptake (up to 20 min agonist application) were not significantly changed by any of the six Panx1-mimetic peptides (1 mM) targetted to other regions of Panx1, whereas 10panx1 (0.5 mM) reduced the slope of the ATP-evoked response by 66.2 ± 5.4% (n = 9; Fig. 2, A and E). Similar to results obtained with Panx1 siRNA, 10panx1 revealed a slower component to the ATP-mediated dye uptake whose kinetics were similar to those of the maitotoxin-induced dye uptake (seen on logarithmic time scale in Fig. 2D); and maitotoxin-induced ethidium uptake was unaffected by 10panx1 (Fig. 2, C–E). We also examined the actions of CBX on ATP and maitotoxin-evoked dye uptake. CBX is a licorice derivative that is a potent inhibitor of 11β-hydroxysteroid dehydrogenase (at nanomolar concentrations), an effective inhibitor of Panx1-evoked currents in Panx1-overexpressing cells (at 1–20 μM), and a gap junction inhibitor via blockade of connexin (at 10–500 μM) (9, 13, 23, 24). Thus, although it cannot be considered selective, it provides useful indirect support
for the involvement of Panx1. CBX (20 μM) had identical actions to 10panx1 inhibitory peptide or Panx1 siRNA; it inhibited the initial component of the ATP-evoked dye uptake but was without effect on the maitotoxin-induced response (Fig. 2E).

We carried out identical experiments on J774 macrophage after establishing noncytolytic conditions. These were 5 mM ATP/20 min (LDH release 6.5 ± 0.6%, n = 8), 0.2 mM maitotoxin/20 min (7.8 ± 0.7%, n = 9), and 5 mM nigericin/20 min (7.5 ± 1.8%, n = 9), with control LDH levels of 1.4 ± 0.2% (n = 26). We found essentially identical results to those obtained from HEK cells ectopically expressing P2X7R; maitotoxin-evoked dye uptake was approximately 10 times slower than ATP-evoked dye uptake (Fig. 3, A and C), whereas nigericin did not evoke any dye uptake (Fig. 3E). 10panx1 reduced the initial dye uptake induced by ATP and revealed a slower component that was the same as the maitotoxin-induced response (Fig. 3, B–D). CBX (20 μM) gave identical results (data not shown).

**Panx1 Signaling Is Required for Maitotoxin-, Nigericin-, and ATP-mediated IL-1β Release**—We next carried out Western blot analysis of IL-1β processing and release after the same 20-min stimulation period under noncytolytic conditions with each of these releasers. As observed in several previous studies (2, 17, 19, 25, 26), no IL-1β was detected in cell lysates or medium in the absence of LPS priming, whereas pro-IL-1β (34-kDa band, as well as an often-observed 22-kDa band) was highly expressed in cell lysates 4 h after LPS addition with neither pro nor mature IL-1β present in the medium (Fig. 4). Nigericin (Fig. 4A), maitotoxin (Fig. 4B), and ATP (Fig. 4) all induced the release of mature IL-1β (17-kDa band) into the medium. When 10panx1-mimetic inhibitory peptide was present, none of these compounds resulted in the release of any form of IL-1β (Fig. 4). Fig. 4C also shows further evidence for the selective inhibition by 10panx1 where it can be seen that another Panx1-mimetic peptide (14panx1) did not alter the ATP-induced release of
Pannexin-1 and IL-1β Release

FIGURE 4. Panx1 is required for IL-1β processing and release in response to nigericin, MTX, and ATP. IL-1β Western blot analysis from cell lysate and medium from LPS-primed J774 macrophages after stimulation with 5 mM ATP (A–C), 5 μM nigericin (NIG) (A), or 0.2 mM MTX (B). No processed 17-kDa IL-1β was observed in the medium after 20 min of incubation with 0.5 mM of 10panx1 inhibitory peptide. Histograms below each blot show the dye uptake slope normalized to the ATP response (n = 3–5 independent experiments in each case). C, only 10panx1 peptide was effective in blocking ATP-induced IL-1β processing/release and dye uptake, confirming the selectivity of the peptide (14panx1, SGILRNDSTVPDQF). LDH release varied from <2 to 8% at this time point.

mature IL-1β (Fig. 4C, fourth lane, nor did incubation with the connexin-mimetic gap junction inhibitory peptide, 32gap27 (27) (Fig. 4C, fifth lane).

Based on semi-quantitative densitometry measurements, we calculated the amount of mature IL-1β in the medium at 20 min of stimulation as a percentage of total IL-1β to be 21.5 ± 2.6% with ATP, 39.7 ± 10.8% with maitotoxin and 12 ± 0.9% with nigericin (n = 3–11). These high levels of mature IL-1β released make it improbable that any significant amount resulted from possible active processes associated with cell death that previously have been reported (15–20, 26), because the concomitant LDH release increased by only a few percent. However, to unequivocally rule out the involvement of any cytolytic process in our experiments, we also examined IL-1β processing and release at times where LDH release differed not at all from basal levels; in this series of experiments, the shortest time at which we could reliably detect mature IL-1β in the medium by Western blot was 10 min, and LDH levels at this time point were not different from basal levels (1.2 ± 0.3% versus 1.9 ± 0.2%, n = 5). Even under these quite stringent conditions 10panx1-mimetic peptide abolished agonist-stimulated processing and release of IL-1β (Fig. 5A).

Blockade of Panx1 not only blocked processing and release of IL-1β by all three agonists but also prevented the upstream intracellular processing of caspase-1. Western blot analysis of cell lysates using the caspase-1 antibody revealed the processing of caspase-1 (10-kDa, p10 band) in LPS-primed J774 cells by both maitotoxin and nigericin (Fig. 5B, third and fifth lanes); this was completely blocked by incubation with 10panx1 (Fig. 5B, fourth and sixth lanes). We have previously obtained similar results from LPS-primed macrophage in response to P2X7R activation (9).

Panx1 Signaling Is Downstream from Intracellular K+ Depletion—Loss of intracellular K+ homeostasis has long been considered a key upstream event leading to caspase-1/IL-1β processing and release, although its precise mechanism remains unexplained (2, 19, 26). Certainly, cells bathed in a high external K+ concentration no longer process or release IL-1β (2, 19, 26), a phenomenon we have reconfirmed in the present series of experiments on J774 macrophage (Fig. 6A), and, as expected, direct measurements of intracellular K+ from parallel wells of cells using atomic emission spectrometry showed no change in K+ (data not shown). In contrast, when normal extracellular K+ (2 mM) was present, maitotoxin, nigericin (Fig. 6B), and ATP (9) all reduced intracellular K+ by 50–75% with concomitant increased extracellular K+, values that were not altered by incubation with 10panx1 inhibitory peptide (Fig. 6B). Interestingly, ethidium uptake evoked by ATP or maitotoxin was unaltered in high extracellular K+, and 10panx1 inhibitory peptide inhibited ATP (but not maitotoxin)-induced ethidium uptake to the same degree as in normal extracellular solution (Fig. 7, A and B).

DISCUSSION

This study reveals that pannexin-1 is required for caspase-1/IL-1β processing and release induced by all three secondary stimuli that are known to activate the cryopyrin-specific inflammasome: maitotoxin, nigericin, and P2X7R activation. However, the present results also force us to conclude that the hypothesis that this results from an opening of a dye-permeable plasma membrane hemichannel pore (9) cannot be considered a general mechanism by which Panx1 signals to inflammasome activation (Fig. 8). Selective (siRNA silencing or 10panx1 inhibitory peptide) and nonselective (CBX) block of Panx1 inhibited
only an initial phase of the ATP-mediated dye uptake without altering maitotoxin-induced ethidium uptake. Moreover, nigericin did not evoke dye uptake, thus ruling out involvement of any dye-permeable pore in its actions.

Might the slow dye uptake observed with maitotoxin and revealed with P2X7R activation after inhibition of Panx1 be explained more simply by incomplete block of Panx1 protein by the siRNA or its function by 10panx1 and CBX? This seems highly unlikely for the following reasons. We used supramaximal concentrations of 10panx1 and CBX in the present experiments, concentrations that completely abolish hemichannel currents induced by overexpression of Panx1 in oocytes and HEK cells (9, 13). Inhibition of the initial phase of the ATP-mediated dye uptake by either 10panx1 or CBX was the same in J774 macrophage natively expressing P2X7R and in HEK cells stably expressing P2X7R at much higher levels, thus indicating a saturation of Panx1 sites of action by these inhibitors. In HEK cells in which high levels of siRNA transfection could be achieved and which resulted in complete absence of Panx1 mRNA, the initial phase of the ATP-mediated ethidium uptake was reduced to the same degree as with inhibition by 10panx1 or CBX. We have not yet been able to verify the degree to which endogenous Panx1 protein is reduced under siRNA silencing, because we have not yet successfully generated adequate anti-Panx1 antibodies, nor have the currently available Panx1 antibodies proven sufficiently specific in our hands (9); nevertheless, this treatment does abolish all ectopically expressed Panx1 protein assayed by Western blot after expression of epitope-tagged Panx1 protein (9). Taken together, these results strongly support the conclusion that there are (at least) two dye uptake pathways activated by P2X7R: a Panx1-dependent initial rapid

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phase and a Panx1-independent slower phase identical to the maitotoxin-induced ethidium uptake (Fig. 8).

The slower Panx1-insensitive dye uptake path was not associated with P2X7R or maitotoxin-induced IL-1β processing or release, and its functional significance, if any, remains to be determined. However, it is worth considering whether this may represent the cytolytic actions of these agents, that is, the induction of a colloid-osmotic pore that can be induced by high levels of both maitotoxin and P2X7R activation (7, 17, 21, 22). Our results suggest this is not the case, at least over the time period of our experiments, because the appearance of such colloid-osmotic pores is associated with substantial LDH release (>30% total LDH) and a sharp increase in ethidium or YoPro1 uptake subsequent to the slow linear increase in the case of maitotoxin (7, 22). We convincingly ruled out cell death events in our experiments by titrating the concentration and duration of agonist application to maintain minimal (8% maximum at 20 min of stimulation) to no release (10 min of stimulation) of LDH, but the Panx1-independent dye uptake was still observed. We consider it possible that this slow dye uptake path may be an indicator of cells that will follow through to eventual cell death by colloid-osmotic necrosis. However, in preliminary experiments in which we followed activated macrophage for up to 24 h after removing the stimuli used in these experiments, we have not observed any decrease in cell number or subsequent significant increase in LDH release.³ In any event, because maitotoxin and P2X7R activation has been shown to mediate IL-1β processing and release through both regulated (nontoxic) and active cell death processes (2, 17, 26), it will be important to determine whether Panx1 signaling is also required for P2X7R- or maitotoxin-induced cell death.

The most physiologically significant result from the present study was the finding that Panx1 inhibition abolished caspase-1/IL-1β processing and IL-1β release by all three agonists without similar correlation in activation or inhibition of dye uptake. Do these results rule out the possibility that Panx1 acts as a dye-permeable hemichannel pore in response to stimuli which require Panx1 signaling for processing and release of IL-1β? There appear to be two possible explanations. One is that P2X7Rs couple to Panx1 hemichannel opening, which provides the functional link to caspase-1/IL-1β processing and release, whereas maitotoxin and nigericin couple to Panx1 activation of IL-1β signaling through a separate and independent mechanism. The other is that all three agonists couple to Panx1-mediated IL-1β release through the same mechanism, one that is independent from plasma membrane hemichannel function. We favor the latter possibility.

The evidence for P2X7R-induced Panx1 hemichannel opening and consequent IL-1β release is primarily based on correlative data from ectopically expressed Panx1. That is, overexpression of Panx1 in oocytes and mammalian cells clearly results in the appearance of hemichannel-like currents that are dye-permeable, and these currents and associated dye uptake are blocked by 10panx1, CBX, and Panx1 siRNA silencing (9, 10, 13). Thus, because Panx1 inhibition blocks P2X7R-mediated dye uptake (the initial phase) and IL-1β release, it is...
reasonable to suggest a hemichannel function for Panx1 in this process. However, new data obtained in the present study makes this idea less likely. In high extracellular K\(^+\), and thus under conditions of zero net current flow/zero K\(^+\) flux, P2X,R-induced dye uptake was the same as in normal solution and showed the same Panx1-dependent and -independent dye uptake kinetics. This study and several previous ones (17, 19, 28) have shown that blockade of K\(^+\) efflux by incubating cells with high extracellular K\(^+\) abrogates caspase 1/IL-1\(\beta\) processing and release induced by all three releasers, but intracellular K\(^+\) depletion per se is not sufficient to activate the inflammasome, because Panx1 inhibition had no effect on K\(^+\) efflux while completely blocking IL-1\(\beta\). These results imply that current flow/K\(^+\) efflux is required for activation of the Panx1-dependent inflammasome but not for activation of the Panx1 dye uptake path, thus making it unlikely this dye uptake pathway is responsible for ATP-mediated IL-1\(\beta\) release. It should also be emphasized that no endogenous currents with the same properties as ectopically expressed Panx1 currents have yet been reported, and we have not recorded similar currents from unactivated macrophage (Ref. 9). Whether LPS priming may lead to the expression of such currents remains to be determined. Indeed, if P2X,R-mediated Panx1-dependent dye uptake is related to downstream signaling to IL-1\(\beta\) release, its functional significance becomes as unclear as the Panx1-independent dye uptake. Nevertheless, it is just this P2X,R-induced dye uptake that has been the primary cellular assay used in drug discovery programs to identify several highly selective P2X7 receptor antagonists (29), thereby making further studies of this signaling process of fundamental physiological significance.

Reasons for favoring a common mechanism of action of Panx1-mediated processing and release of IL-1\(\beta\) induced by maitotoxin, nigericin, and P2X,R include data from the present study in addition to much previous evidence for common signaling by these agents: they all lead to rapid intracellular K\(^+\) depletion (Refs. 9, 17, 19, 20, and 28 and this study) that remains unaltered after Panx1 inhibition (Ref. 9 and this study); they all initiate caspase-1 cleavage, which is the key event required for inflammasome activation and consequent IL-1\(\beta\) processing and release (1–4); they all couple to the cyropyrin-specific, but not the ICE-protease activating factor-dependent/NALP1 inflammasome (4, 25); and they all require Panx1 signaling to activate this inflammasome (this study). If a common mechanism does underlie Panx1 signaling, clearly it cannot involve plasma membrane hemichannel activity based on data from the present study, as discussed above. A recent study has suggested a role for Panx1 as an intracellular calcium-leak channel in the endoplasmic reticulum, although this conclusion is also based primarily on results from Panx1 overexpression experiments (12), as has been the case for Panx1 plasma membrane hemichannels (see above). Like Vanden Abeele et al. (12), we also observe dense expression of epitope-tagged ectopically expressed Panx1 in the endoplasmic reticulum as well as plasma membrane. But, until Panx1 antibodies of reliable specificity become available to delineate the subcellular distribution of native Panx1 protein, speculation as to a possible role for intracellular Panx1 signaling to inflammasome activation remains premature. Fig. 8 provides a schematic outline of what we can conclude from our results: (i) that Panx1 functions downstream from K\(^+\) efflux induced by all three agonists but does not play a role in it or in current flow via P2X,R; (ii) that Panx1 functions upstream from caspase-1 cleavage and is required for its activation; (iii) that Panx1 does form (or induce) the early dye uptake path through P2X,R activation, but this dye uptake path is not directly involved in caspase-1/IL-1\(\beta\) processing and release; and (iv) that a slower Panx1-independent dye uptake path can be activated by both P2X,R and maitotoxin, but not nigericin, and this dye uptake path is not involved in the regulated (noncytolytic) processing and release of IL-1\(\beta\).

Concepts regarding cellular mechanisms of action of P2X,R and maitotoxin poisoning have evolved rapidly: from the induction or formation of a colloid-osmotic cytolytic pore as their sole effect to a highly regulated sequence of multiple signaling events that occur prior to and independent of active cell death processes (2, 5, 7, 19, 26). In activated macrophages, these early noncytolytic events converge onto rapid caspase-1 proteolysis and subsequent processing and release of IL-1\(\beta\). Exactly how and where Panx1 is involved in the IL-1\(\beta\) cascade remains to be determined, but this study shows that Panx1 must now be considered a critical and required component in cryopyrin-specific inflammasome signaling to release this key pro-inflammatory cytokine.

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