PANNEXIN-1 COUPLES TO MAITOTOXIN AND NIGERICIN-INDUCED IL-1β RELEASE THROUGH A DYE-UPTAKE INDEPENDENT PATHWAY

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Running title: Pannexin-1 and IL-1β release

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Pannexin-1 is a recently identified membrane protein that can act as a non-selective pore permeable to dyes such as ethidium when ectopically expressed. Blockade of pannexin-1 in macrophage endogenously expressing the ATP-gated $P2X_7$ receptor ($P2X_7R$) blocks the initial dye uptake, but not the ionic current, and also blocks processing and release of interleukin-1β (IL-1β) in response to $P2X_7R$ activation. These results suggest that pannexin-1 may be a hemichannel activated by the $P2X_7R$ to provide the conduit for dye uptake and downstream signalling 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 $P2X_7R$ activation and to maitotoxin and nigericin, two agents considered to evoke IL-1β release via the same mechanism. Experiments were carried out over time periods during which no LDH was released from cells in order to examine only non-cytolytic pathways. $P2X_7R$ 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, while 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.

INTRODUCTION

Interleukin-1β (IL-1β) is the crucial pro-inflammatory cytokine whose early release from activated macrophage leads to the generation of the host defence response to bacterial toxins, injuries, and other inflammatory challenges (1-3). IL-1β is synthesized as a 34kD pro-peptide which has no biological activity until it is cleaved by activated caspase-1 resulting in its mature, bioactive 17 kD 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). While 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 $P2X_7$ receptor channel which itself is up-regulated in response to LPS and other inflammatory stimuli (2).

The $P2X_7$ 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 dalton (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 $P2X_7R$ ion channel opens with kinetics generally similar to other ligand-gated ion channels (ie. within milliseconds) while 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 $P2X_7R$ 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 $P2X_7R$ is directly responsible for the dye uptake (5, 6-8). We have recently identified this accessory protein as pannexin-1 (9).

Pannexins are a three-membered family of mammalian membrane proteins (Panx1, 2 and 3) about which little is currently known. They were identified in 2003/4 by low stringency homology to invertebrate gap junction proteins, innexins, (10, 11) and Panx1 can form gap junction-like connections which allow intercellular passage of dyes when over-expressed in two adjacent oocytes or mammalian epithelial cell lines (10, 12). However, there is no evidence to date that endogenous Panx1 forms gap junctions. Over-expression of Panx1 in single oocytes or mammalian cells also results in the formation of 'hemichannel-like' non-selective pores (9, 10, 13, 14), and we have obtained evidence that endogenous Panx1 forms the dye-permeable uptake path upon activation of $P2X_7Rs$ (9). We found that Panx1 and $P2X_7R$ co-immunoprecipitate in $P2X_7R$-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 $P2X_7R$-mediated processing of caspase-1 and subsequent processing and release of IL-1β (9). These results suggest a direct functional link between $P2X_7R$-mediated dye uptake and activation of caspase-1/IL-1β processing and release.

In order 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 non-physiological secondary stimuli which have been thought to act via the same mechanism(s) as $P2X_7R$, 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 $P2X_7R$ and both dye uptake and caspase-1/IL-1β processing/release in mouse J774

macrophage which natively express $P2X_7R$ before and after selective blockade of Panx1. We find that all three stimuli require signaling through Panx1 to induce caspase-1/IL-1β processing and release but Panx1 is involved only with an initial phase of $P2X_7R$-mediated dye uptake while dye-uptake pathways are not involved in IL-1β processing/release induced by maitotoxin or nigericin.

EXPERIMENTAL PROCEDURES

Matherials Carbenoxolone (CBX), ethidium bromide, ATP, nigericin and *E. coli* lipopolysaccharide (LPS) were from Sigma, maitotoxin from Alexis. The Panx1 mimetic blocking peptide ^{10}panx1 (WRQAAFVDSY), the connexin 32 blocking peptide ^{32}gap27 (SRPTEKTVFT) and the other Panx1 peptides (GTQISCFSPS; CFSPSSFSWRQAA; QKNSLQSESGNLP; YCWAAVQQKNSLQSESGNLP; LRNDSTVPDQFQ; SGILRNDSTVPDQF) 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 media, all supplemented with 10% fetal calf serum (Gibco).

RNA interference Small interference RNAs against human Panx1 was carried out as described previously (9). Briefly, HEK293 cells stably expressing $P2X_7$ receptors were transfected using Lipofectamine2000 (Invitrogen) and 200 pmol of Panx1-siRNA (5' GCUCCAAGGUAUGAACAUAtt 3') or *Silencer*® Negative Control #1 siRNA (Ambion) as the negative scrambled control. Cells were cultured for 48 h at 37°C before experiments and mRNA expression levels for Panx1, $P2X_7R$ and β-actin were determined by RT-PCR.

Reverse Transcriptase-PCR Analysis Total RNA was isolated using RNeasy Mini kit (Qiagen), followed by reverse transcription using SUPERSCRIPT™ III (Invitrogen) RNase H-reverse transcriptase with oligo-dT. Specific primers for Panx1, rat $P2X_7R$ or β-actin were previously reported (9) and used in PCR. The obtained product sizes for Panx1 (610 bp), rat $P2X_7R$ (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 20x objective and ethidium fluorescence was measured at 543/590 nm excitation/emission. Cells were pre-incubated for 15 min at 37°C with 500 μM of ^{10}panx1 or 1 mM of other Panx1 peptides or ^{32}gap27 peptide, following addition of 20 μM ethidium. Images were recorded at 5-10 sec 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 in mM: NaCl 147, HEPES 10, glucose 13, $CaCl_2$ 2, $MgCl_2$ 1 and KCl 2. 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, 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 $2x10^6$ cells per well in a 6-well dish on the night before treatment. Cells were stimulated with LPS (1 μg/ml) for 4 hours, washed twice with the same solution used for dye uptake experiments and pre-incubated for 30 min with 500 μM ^{10}panx1 or 1 mM of other Panx1 peptides or ^{32}gap27 peptide and 5mM ATP, 0.2 nM maitotoxin or 5 μM nigericin applied for 10-20 min. Cells were lysed in RIPA buffer (20 mM TrisHCl pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$) supplemented with

1% Triton X-100 and Complete protease inhibitor cocktail (Roche) for 1 h at 4°C and centrifuged to remove particulate matter. Supernatants were clarified by brief centrifugation and 60% of the volume was concentrated using 10-kDa nominal molecular weight cut-off filters (Millipore). 15 μg of total protein and the concentrated supernatants were resolved in 4-15% polyacrylamide gels, transferred to PVDF membranes (Millipore) by electro-blotting and immunobloted 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 HRP-conjugated secondary antibody (DA-KOCytomation) at 1:2000 dilution and detection using the ECL-plus kit (Amersham) and Kodak Bio-Max MS film (Sigma). Results were analysed by densitometry measurements using GeneSnap/GeneTools software (Syngene).

LDH release assay The presence of LDH in the media was detected in all the experiments using the Cytotoxicity Detection kit (Roche) following manufacturer instructions and compared to the total amount of LDH in the cells. In all 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, cells were seeded at $1x10^6$ cells per well in a 12-well dish on the night before treatment. Cells were treated with 1 μg/ml LPS for 4 hours, washed twice and pre-incubated for 30 min with 500 μM of ^{10}panx1, following 20 min with 5mM ATP, 0.2 nM maitotoxin or 5 μM nigericin. The medium was removed and the cells lysed in 1 ml of 10% nitric acid.

Statistical analysis Average results are expressed as the mean ± s.e.m. from the number of assays indicated. 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 $P2X_7R$. 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 data presented in this study were obtained under conditions where LDH release was < 8% of total cellular LDH. 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); 5 μM nigericin/20 min (LDH release < 2%, n = 8). Basal levels of LDH release varied from 0.2 - 1.6% (n = 16).

Figure 1A illustrates the markedly different rates of ethidium uptake induced by ATP, maitotoxin and nigericin: ATP induced rapid dye uptake which saturated the system's optics within 3-5 min, dye uptake to maitotoxin was one to two orders of magnitude slower while 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 (Figure 1, B and C). In agreement with our previous study (9), inhibition of Panx1 using Panx1-targetted siRNA decreased ATP-evoked ethidium uptake by 70 - 90% over the initial 3-5 min of agonist application (Figure 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 Figure 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 (Figure 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 RT-PCR analysis showed complete absence of Panx1 mRNA (Figure 1D). Results from all experiments as illustrated in Figure 1A-C are summarized in the histogram shown in Figure 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, ^{10}panx1, that effectively and selectively inhibited ATP-mediated dye uptake but not $P2X_7R$ activation (9). In order to gain further confidence in its function as a selective Panx1 inhibitor, we compared in more detail the actions of ^{10}panx1 and six other Panx1-mimetic peptides corresponding to other putative ectodomain residues in Panx1 (Figure 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 while ^{10}panx1 (0.5 mM) reduced the slope of the ATP-evoked response by 66.2 ± 5.4% (n = 9; Figure 2, A and E). Similar to results obtained with Panx1 siRNA, ^{10}panx1 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 Figure 2D) and maitotoxin-induced ethidium uptake was unaffected by ^{10}panx1 (Figure 2, C, D and E). We also examined the actions of carbenoxolone (CBX) on ATP and maitotoxin-evoked dye uptake . CBX is a licorice derivative which 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 30 - 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 ^{10}panx1 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 (Figure 2E).

We carried out identical experiments on J774 macrophage after establishing non-cytolytic conditions. These were: 5 mM ATP/20 min (LDH release 6.5 ± 0.6%, n=8), 0.2 nM maitotoxin/20 min (8 ± 0.7%, n=9) and 5 μM 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 $P2X_7R$: maitotoxin-evoked dye uptake was approximately ten times slower than ATP-evoked dye uptake (Figure 3, A and C) while nigericin did not evoke any dye uptake (Figure 3E). ^{10}panx1 reduced the initial dye uptake induced by ATP and revealed a slower component that was the same as the maitotoxin-induced response; neither (Figure 3, B, C and 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 non-cytolytic 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, while pro-IL-1β (34 kD band, as well as an often-observed 22 kD band) was highly expressed in cell lysates 4 hr after LPS addition with neither pro nor mature IL-1β present in the medium (Figure 4, A, B and C). Nigericin (Figure 4A), maitotoxin (Figure 4B) and ATP (Figure 4, A, B and C) all induced the release of mature IL-1β (17 kD band) into the medium. When ^{10}panx1-mimetic inhibitory peptide was present, none of these compounds resulted in release of any form of IL-1β (Figure 4, A, B and C). Figure 4C also shows further evidence for the selective inhibition by ^{10}panx1 where it can be seen that another Panx1-mimetic peptide (^{14}panx1) did not alter the ATP-induced release of mature IL-1β (Figure 4C, lane 4, nor did incubation with the connexin-mimetic gap juction inhibitory peptide, ^{32}gap27 (27) (Figure 4C, lane 5).

Based on semi-quantitative densitometry measurements, we calculated the amount of mature IL-1β in the medium at 20 min of stimulation as a percent of total IL-1β to be 21.5 ± 2.6% with ATP, 39.7 ± 10.8% with maitotoxin and 11 ± 0.9% with nigericin (n = 3 to 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 which previously have been reported (15-20, 26) because the concomitant LDH release increased by only a few percent. However, in order to unequivocally rule out 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% vs 1.9 ± 0.2%, n = 5). Even under these quite stringent conditions ^{10}panx1-mimetic peptide abolished agonist-stimulated processing and release of IL-1β (Figure 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 kD, p10 band) in LPS-primed J774 cells by both maitotoxin and nigericin (Figure 5B, lanes 3 and 5); this was completely blocked by incubation with ^{10}panx1 (Figure 5B, lanes 4 and 6). We have previously obtained similar results from LPS primed macrophage in response to $P2X_7R$ 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 unanswered (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 re-confirmed in the present series of experiments on J774 macrophage (Figure 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 (Figure 6B) and ATP (9) all reduced intracellular K^+ by 50 – 75% with concomitant increased extracellular K^+, values which were not altered by incubation with ^{10}panx1 inhibitory peptide (Figure 6B). Interestingly, ethidium uptake evoked by ATP or maitotoxin was unaltered in high extracellular K^+, and ^{10}panx1 inhibitory

peptide inhibited ATP (but not maitotoxin)-induced ethidium uptake to the same degree as in normal extracellular solution (Figure 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 $P2X_7R$ 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 (Figure 8). Selective (siRNA silencing or ^{10}panx1 inhibitory peptide) and non-selective (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 $P2X_7R$ activation after inhibition of Panx1 be explained more simply by incomplete block of Panx1 protein by the siRNA or its function by ^{10}panx1 and CBX? This seems highly unlikely for the following reasons: We used supramaximal concentrations of ^{10}panx1 and CBX in the present experiments, concentrations which 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 ^{10}panx1 or CBX was the same in J774 macrophage natively expressing $P2X_7R$ and in HEK cells stably expressing $P2X_7R$ 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 ^{10}panx1 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 Abs proven sufficiently specific in our hands (9 and personal observations); 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 $P2X_7R$: a Panx1 dependent initial rapid phase and a Panx1 independent slower phase identical to the maitotoxin-induced ethidium uptake (Figure 8).

The slower Panx1-insensitive dye uptake path was not associated with $P2X_7R$ 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 $P2X_7R$ 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 are 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 stimulation) to no

release (10 min 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 hr after removing the stimuli used in these experiments, we have not observed any decrease in cell number or subsequent significant increase in LDH release (unpublished observations). In any event, because maitotoxin and $P2X_7R$ activation has been shown to mediate IL-1β processing and release through both regulated (non-cytolytic) and active cell death processes (2, 17, 26), it will be important to determine whether Panx1 signaling is also required for $P2X_7R$ or maitotoxin-induced cell death.

The most physiologically significant result from the present study was the finding that Panx1 inhibition abolished capase-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 $P2X_7Rs$ couple to Panx1 hemichannel opening which does provides the functional link to caspase-1/IL-1β processing and release while 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 favour the latter possibility.

The evidence for $P2X_7R$-induced Panx1 hemichannnel 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 which are dye permeable and these currents and associated dye uptake are blocked by ^{10}panx1, CBX and Panx1 siRNA silencing (9, 10, 13). Thus, because Panx1 inhibition blocks $P2X_7R$ 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 - $P2X_7R$-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-1β 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-1β. 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-1β 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 (9 and unpublished observations). Whether LPS priming may lead to the expression of such currents remains to be determined. Indeed, if $P2X_7R$-mediated Panx1-dependent dye uptake is unrelated to downstream signaling to IL-1β release, its functional significance becomes as unclear as the Panx-1

independent dye uptake. Nevertheless, it is just this $P2X_7R$-induced dye uptake that has been the primary cellular assay used in drug discovery programs to identify several highly selective $P2X_7$ receptor antagonists (29), thereby making further studies of this signaling process of fundamental physiological significance.

Reasons for favouring a common mechanism of action of Panx1-mediated processing and release of IL-1β induced by maitotoxin, nigericin and $P2X_7R$ 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 (9, 17, 19, 20, 28 and this study) that remains unaltered after Panx1 inhibition (9 and this study), they all initiate caspase-1 cleavage which is the key event required for inflammasome activation and consequent IL-1β processing and release (1-4), they all couple to the cyropyrin-specific, but not the ICE-protease activating factor (IPAF)-dependent/NALP1 inflammasome (4, 30), 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 hemichannnel 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 (personal observations). But, until Panx1 Abs 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. Figure 8 provides a schematic outlining 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 $P2X_7R$, (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 $P2X_7R$ activation but this dye uptake path is not directly involved in caspase-1/IL-1β processing and release, (iv) and that a slower Panx1-independent dye-uptake path can be activated by both $P2X_7R$ and maitotoxin, but not nigericin, and this dye-uptake path is not involved in the regulated (non-cytolytic) processing and release of IL-1β.

Concepts regarding cellular mechanisms of action of $P2X_7R$, 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 macrophage, these early non-cytolytic events converge onto rapid caspase-1 proteolysis and subsequent processing and release of IL-1β. Exactly how and where Panx1 is involved in the IL-1β 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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Footnotes

This work was supported by the Wellcome Trust and a postdoctoral fellowship to PP from AstraZeneca Charnwood UK. The authors declare no conflict of interest with regard to this study. We thank Elizabeth Martin and Weihong Ma for tissue culture assistance. We acknowledge the Centre for Chemical Instrumental Analysis and Services, Department of Chemistry, University of Sheffield for analysing ion content of our samples.

The abbreviations used are: ATP, adenosine 5'-triphosphate; NIG, nigericin; MTX, maitotoxin; LDH, lactate dehydrogenase; CBX, carbenoxolone; LPS, lipopolysaccharide; IL-1β, interleukin-1β; siRNA, small interfering RNA; HEK, human embryonic kidney 293 cell line; PVDF, polyvinylidene difluoride; HRP, horseradish peroxidase; RT-PCR, reverse transcriptase-polymerase chain reaction.

FIGURE LEGENDS

FIGURE 1. **Panx1 is involved in only an initial phase of ATP-induced dye uptake and not involved in MTX-induced dye-uptake.** A, kinetics of dye uptake in HEK cells expressing $P2X_7R$ in response to 3 mM ATP, 0.5 nM MTX or 5 μM nigericin (NIG) (added at arrow). Nigericin did not evoke dye uptake; digitonin added at end of experiment. B and C, siRNA silencing of Panx1 (red traces) did not alter MTX induced dye uptake but inhibited the early phase of ATP-induced dye uptake. B shows linear time scale over 300 s when ATP-induced dye uptake is maximum and C shows logarithmic time scale over 1200 s when MTX-induced dye uptake is maximum. D, RT-PCR shows Panx1, $P2X_7R$ and β-actin cDNA detection after transfection with Panx1-siRNA or scramble-siRNA as indicated. E, summary of the slope of dye uptake of all results from experiments as in B and C; $n = 3$ independent experiments in each case; asterisks indicate p value < 0.001.

FIGURE 2. **Panx1-sensitive and insensitive dye-uptake revealed by ^{10}panx1 inhibitory peptide.** A, normalized slope of ethidium uptake fluorescence recorded in HEK cell expressing $P2X_7R$ in response to 3 mM ATP after incubation with peptides corresponding to residues in the putative extracellular loops of Panx1 shown schematically (1: GTQISCFSPS; 2: WRQAAFVDSY (^{10}panx1); 3: CFSPSSFSWRQAA; 4: QKNSLQSESGNLP; 5: YCWAAVQQKNSLQSESGNLP; 6: LRNDSTVPDQFQ; 7: SGILRNDSTVPDQF). B, C and D, ethidium uptake evoked by 3mM ATP (B and D) and 0.5 nM maitotoxin (C and D) in the absence (black) and presence (red) of ^{10}panx1; B and C show dye-uptake over the initial 5 min of application on linear time scale and D shows response over 20 min on logarithmic time scale. E, summary of the slope of dye uptake of all results from experiments using ^{10}panx1 peptide as indicated in B, C and D or using 20 μM of CBX, $n = 3$ independent experiments in each case; asterisks indicate p value < 0.001.

FIGURE 3. **Rapid Panx1-dependent and slow Panx1-independent dye uptake is also present in J774 macrophages.** A, B and C, effect of ^{10}panx1 inhibitory peptide on 5 mM ATP (A) and 0.2 nM MTX (B) induced dye uptake recorded in J774 macrophages. A and B show the initial 400 s of agonist application when ATP response is maximum on linear time scale and C shows dye-uptake over 20 min on logarithmic time scale. In the presence of 0.5 mM ^{10}panx1 inhibitory peptide (red traces), the ATP-evoked dye uptake is the same as the MTX-induced response. D, summary of the slope of dye uptake of all results from experiments using ^{10}panx1 peptide as indicated in A, B and C, $n = 3$ independent experiments in each case; asterisks indicate p value < 0.001. E, normalized slope of ethidium uptake fluorescence recorded in J774 macrophages, HEK cells expressing $P2X_7R$ or plain HEK cells in response to 3 mM ATP (HEK-$P2X_7R$) or 5 mM ATP (J774 and HEK) or 5 μM nigericin (NIG); $n = 4$ independent experiments in each case.

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, B and C), 5 μM nigericin (NIG) (A) or

0.2 nM MTX (B). No processed 17kD IL-1β was observed in the medium after 20 min incubation with 0.5 mM of ^{10}panx1 inhibitory peptide. Histograms below each blot show the dye uptake slope normalized to the ATP response, n = 3 to 5 independent experiments in each case. C, only ^{10}panx1 peptide was effective in blocking ATP induced IL-1β processing/release and dye uptake, confirming the selectivity of the peptide (^{14}panx1: SGILRNDSTVPDQF). LDH release varied from <2 - 8% at this time point.

FIGURE 5. **Panx1 signaling is independent of cell death and required for caspase-1 activation by nigericin and MTX.** A, IL-1β western blot analysis from LPS-primed J774 macrophages after 10 min stimulation with 5 mM ATP or 0.2 nM MTX; no LDH was detected in medium at this time point. Histogram below blot shows ethidium fluorescent units (f.u.) at 10 min normalized to digitonin-maximum; ATP-induced dye-uptake in the presence of ^{10}panx1 was not significantly different from kinetics of MTX-induced ethidium uptake; n = 5 independent experiments in each case; asterisks indicate p value < 0.001. B, Western blot analysis of caspase-1 from mouse J774 macrophage. After LPS priming, 5 μM nigericin (NIG) or 0.2 nM MTX was present for 20 min to induce caspase-1 processing (lanes 3 and 5), which was prevented by 20 min of incubation with the ^{10}panx1 inhibitory peptide (0.5 mM, lanes 4 and 6).

FIGURE 6. **Panx1 signaling is downstream from intracellular K^+ depletion.** A, western blot analysis for IL-1β from LPS-primed J774 macrophages (cell lysate and medium) after stimulation for 20 min with 5 mM of ATP, 5 μM nigericin (NIG) or 0.2 nM MTX in normal extracellular K^+ solution or in high K^+ extracellular solution (130 mM KCl). No processed 17kD IL-1β was observed in the medium containing high K^+. B, Intracellular and released K^+ (plotted as fraction of control) from LPS primed J774 cells treated with 5 μM nigericin (NIG) or 0.2 nM MTX in the presence or absence of 0.5 mM ^{10}Panx1 peptide, n = 4.

FIGURE 7. **High extracellular K^+ does not affect dye uptake.** A and B, dye uptake induced by 3 mM ATP in HEK expressing $P2X_7R$ (A) or by 5 mM ATP in J774 cells (B) in normal extracellular K^+ solution or in high K^+ extracellular solution (130 mM KCl). 0.5 mM of ^{10}panx1 inhibitory peptide blocks dye uptake in high K^+ extracellular solution (B). C, summary of the slope of dye uptake of all results from experiments using high external K^+ in response to ATP and MTX, n = 3 independent experiments in each case.

FIGURE 8. **Panx1 dependent and independent pathways in J774 macrophage.** Schematic based on results from this study indicating the Panx1-independent dye-uptake induced by both maitotoxin and $P2X_7R$; this path is not involved in IL-1β release. Panx1 is involved in the initial rapid dye uptake induced by $P2X_7R$ activation but this path does not appear to be directly involved in IL-1β release. We suggest a common Panx1-mediated pathway may be activated by all three agents; this pathway is downstream from K^+ efflux and upstream to caspase-1 proteolysis.

Figure 1

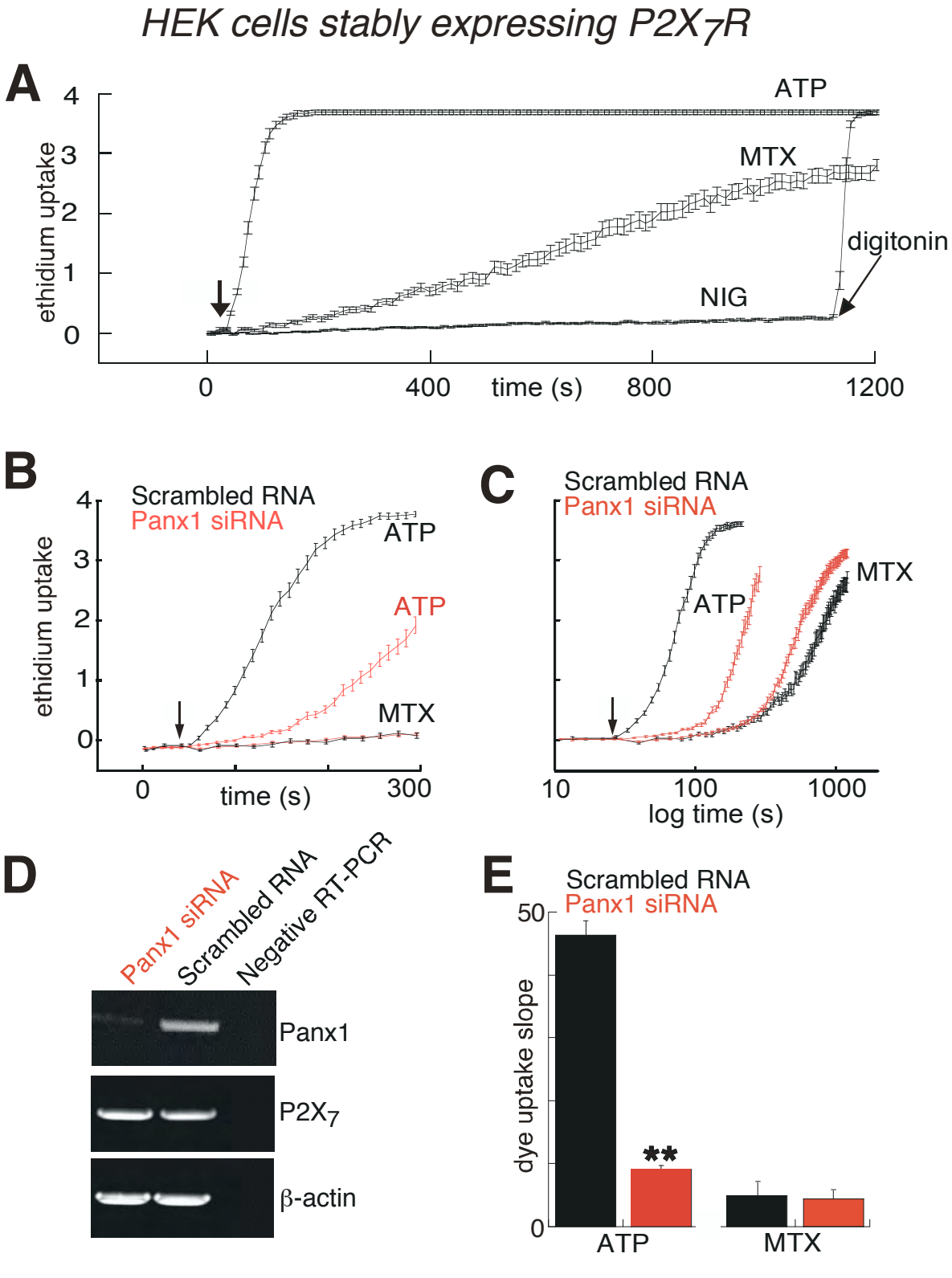
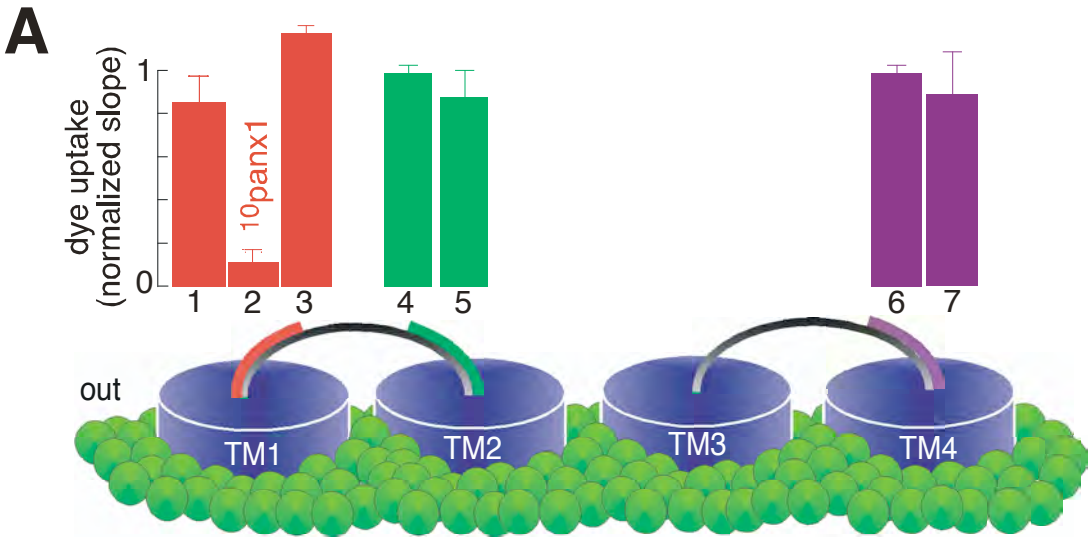
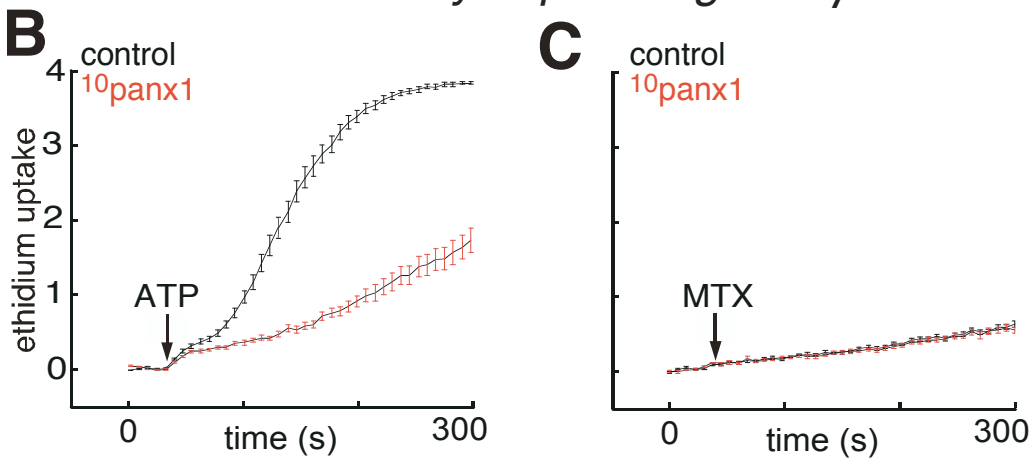

HEK cells stably expressing P2X7R
A
ethidium uptake
ATP
MTX
digitonin
NIG
time (s)
B
Scrambled RNA
Panx1 siRNA
ATP
ATP
MTX
ethidium uptake
time (s)
C
Scrambled RNA
Panx1 siRNA
ATP
MTX
log time (s)
D
Panx1 siRNA
Scrambled RNA
Negative RT-PCR
Panx1
P2X7
β-actin
E
Scrambled RNA
Panx1 siRNA
dye uptake slope
**
ATP
MTX

Figure 2



HEK cells stably expressing P2X7R



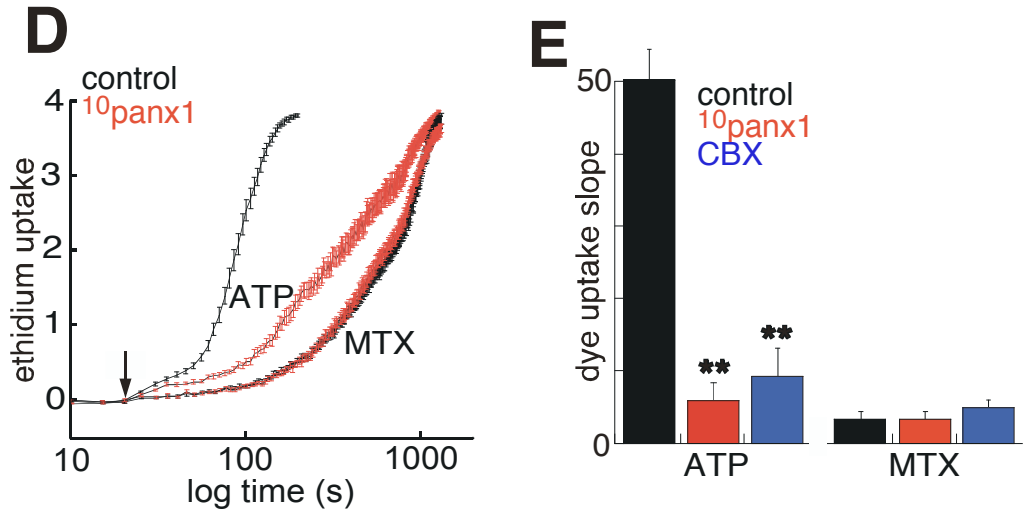


Figure 3

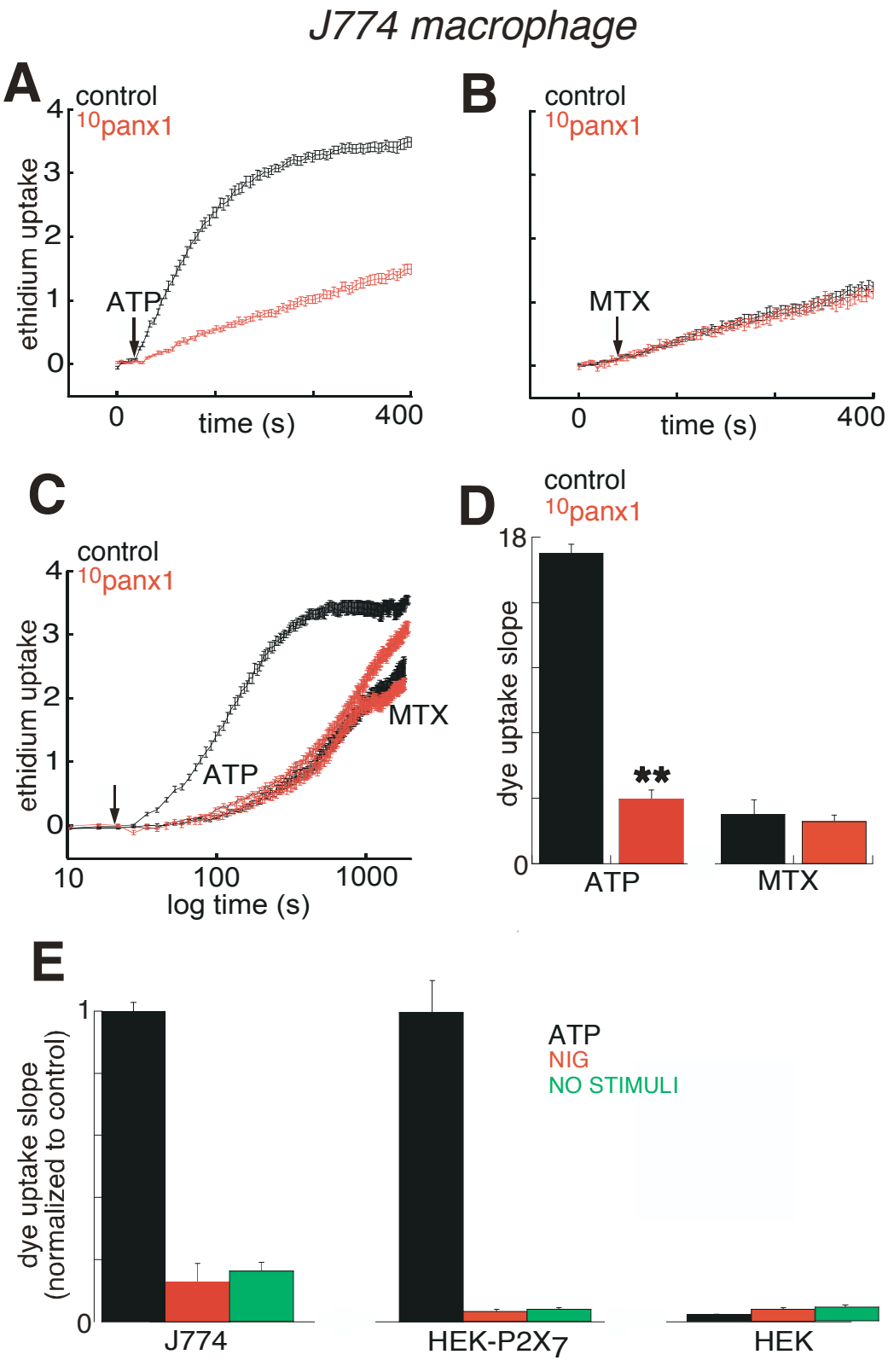

J774 macrophage
A
control
10panx1
ethidium uptake
ATP
time (s)
B
control
10panx1
MTX
time (s)
C
control
10panx1
ethidium uptake
ATP
MTX
log time (s)
D
control
10panx1
dye uptake slope
**
ATP
MTX
E
dye uptake slope
(normalized to control)
ATP
NIG
NO STIMULI
J774
HEK-P2X7
HEK

Figure 4

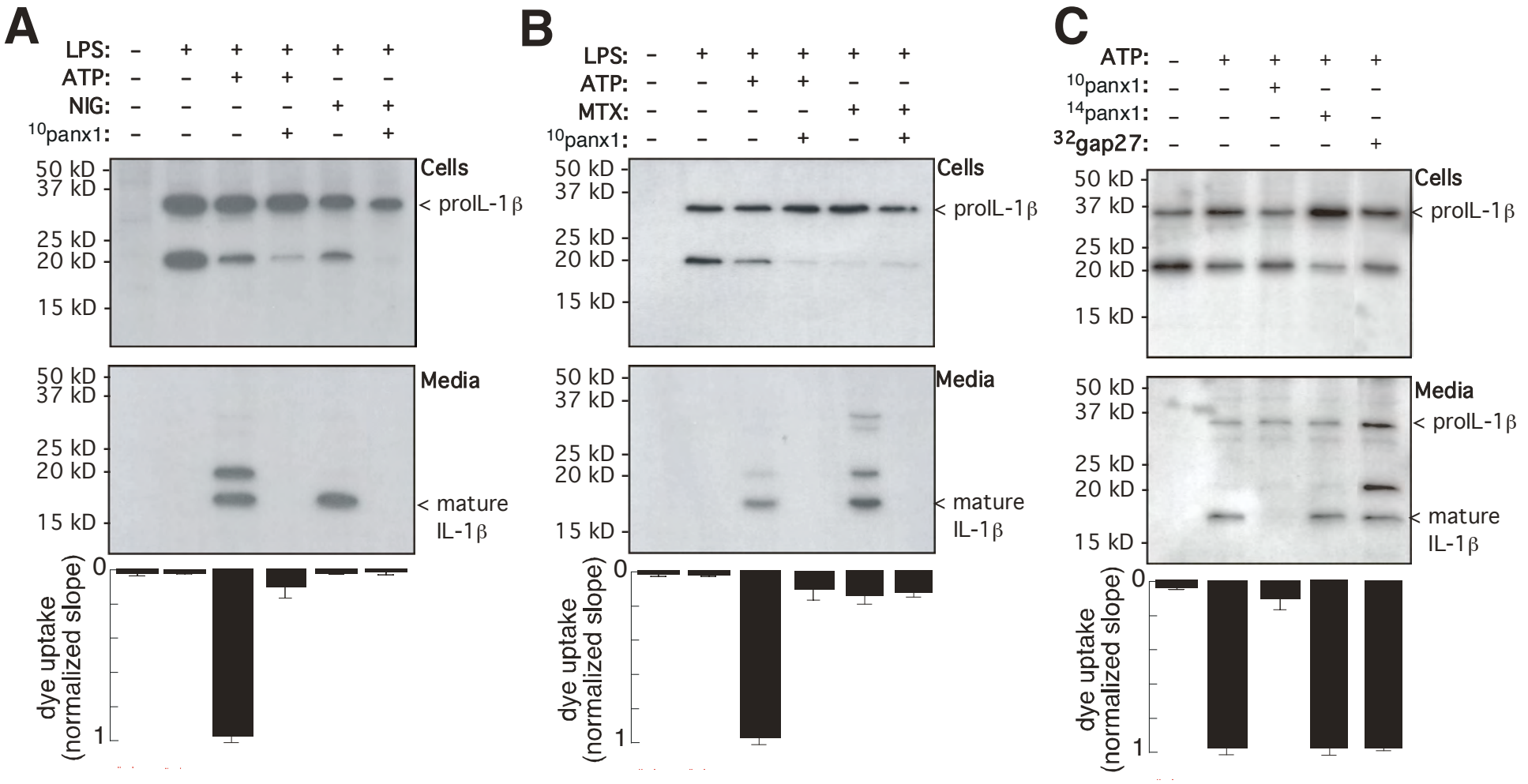
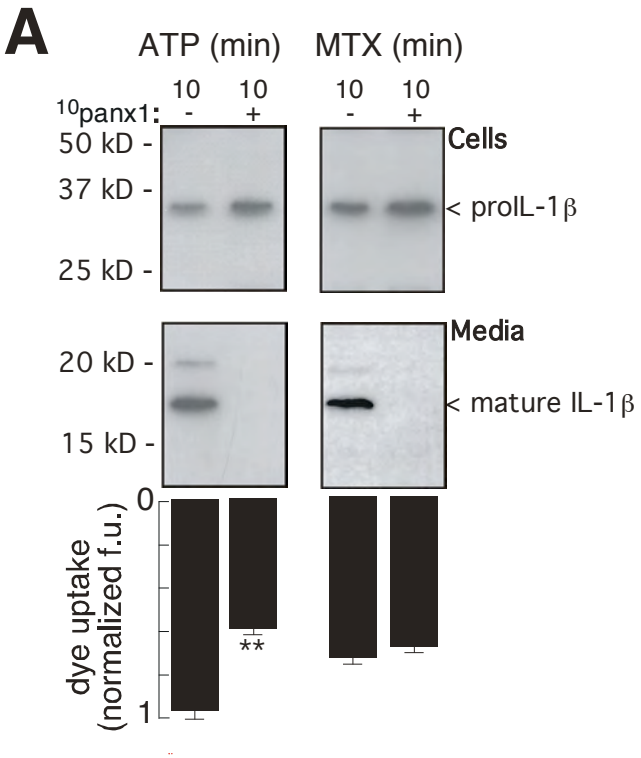

A
LPS: – + + + + +
ATP: – – + + – –
NIG: – – – – + +
10panx1: – – – + – +
50 kD
37 kD
25 kD
20 kD
15 kD
Cells
< proIL-1β
Media
< mature IL-1β
dye uptake (normalized slope)
0
1
B
LPS: – + + + + +
ATP: – – + + – –
MTX: – – – – + +
10panx1: – – – + – +
C
ATP: – + + + +
10panx1: – – + – –
14panx1: – – – + –
32gap27: – – – – +

Figure 5


A
ATP (min)
MTX (min)
10 10 10 10
10panx1: - + - +
50 kD -
37 kD -
25 kD -
Cells
< proIL-1β
20 kD -
15 kD -
Media
< mature IL-1β
0
1
dye uptake (normalized f.u.)
**

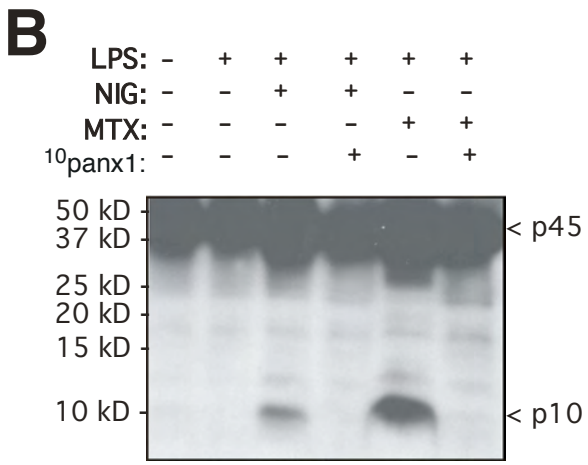

B
LPS: - + + + + +
NIG: - - + + - -
MTX: - - - - + +
10panx1: - - - + - +
50 kD
37 kD
25 kD
20 kD
15 kD
10 kD
< p45
< p10

Figure 6

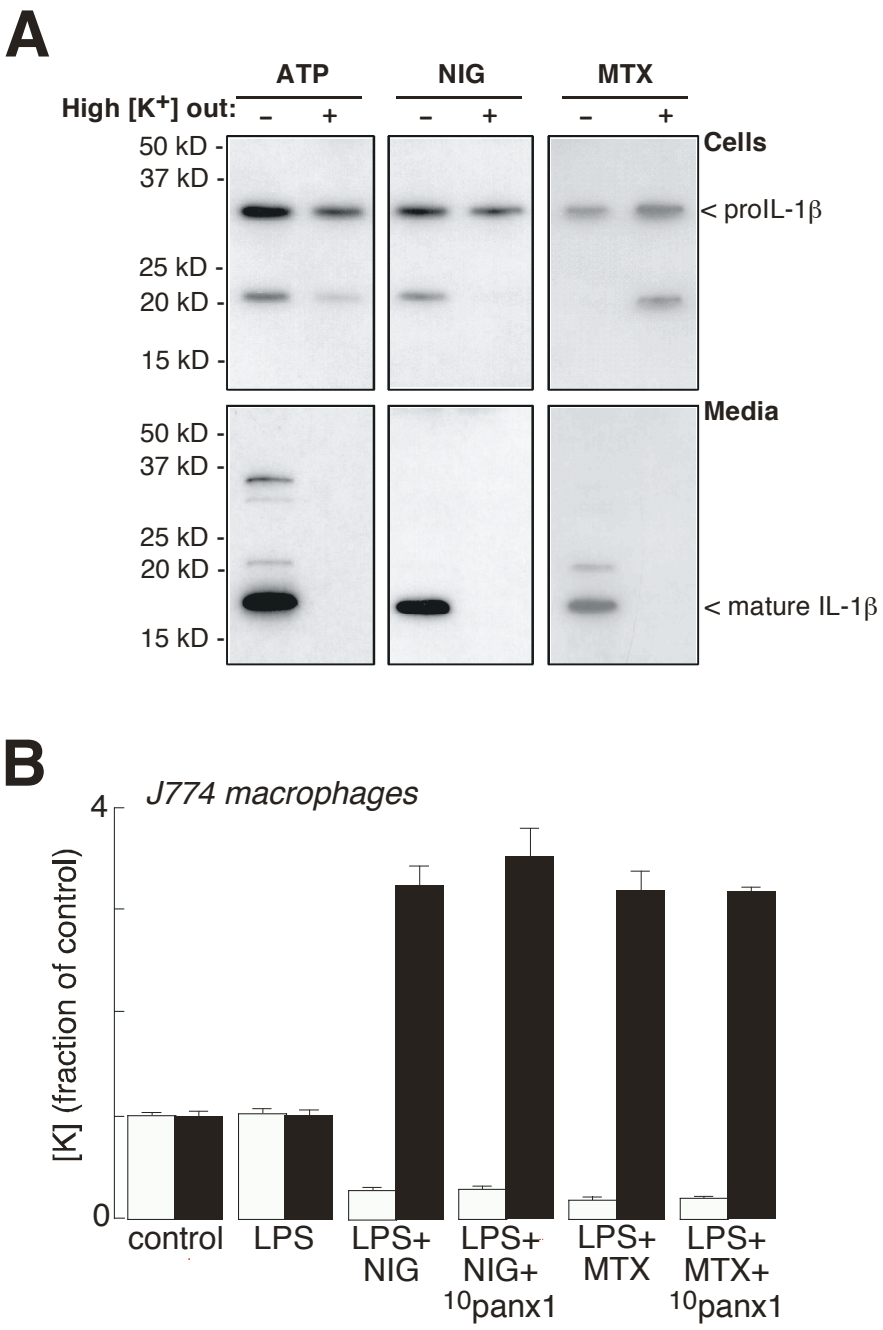

A
ATP
NIG
MTX
High [K+] out:
– + – + – +
Cells
50 kD
37 kD
25 kD
20 kD
15 kD
< proIL-1β
Media
50 kD
37 kD
25 kD
20 kD
15 kD
< mature IL-1β
B
J774 macrophages
[K] (fraction of control)
4
0
control
LPS
LPS+ NIG
LPS+ NIG+ 10panx1
LPS+ MTX
LPS+ MTX+ 10panx1

Figure 7

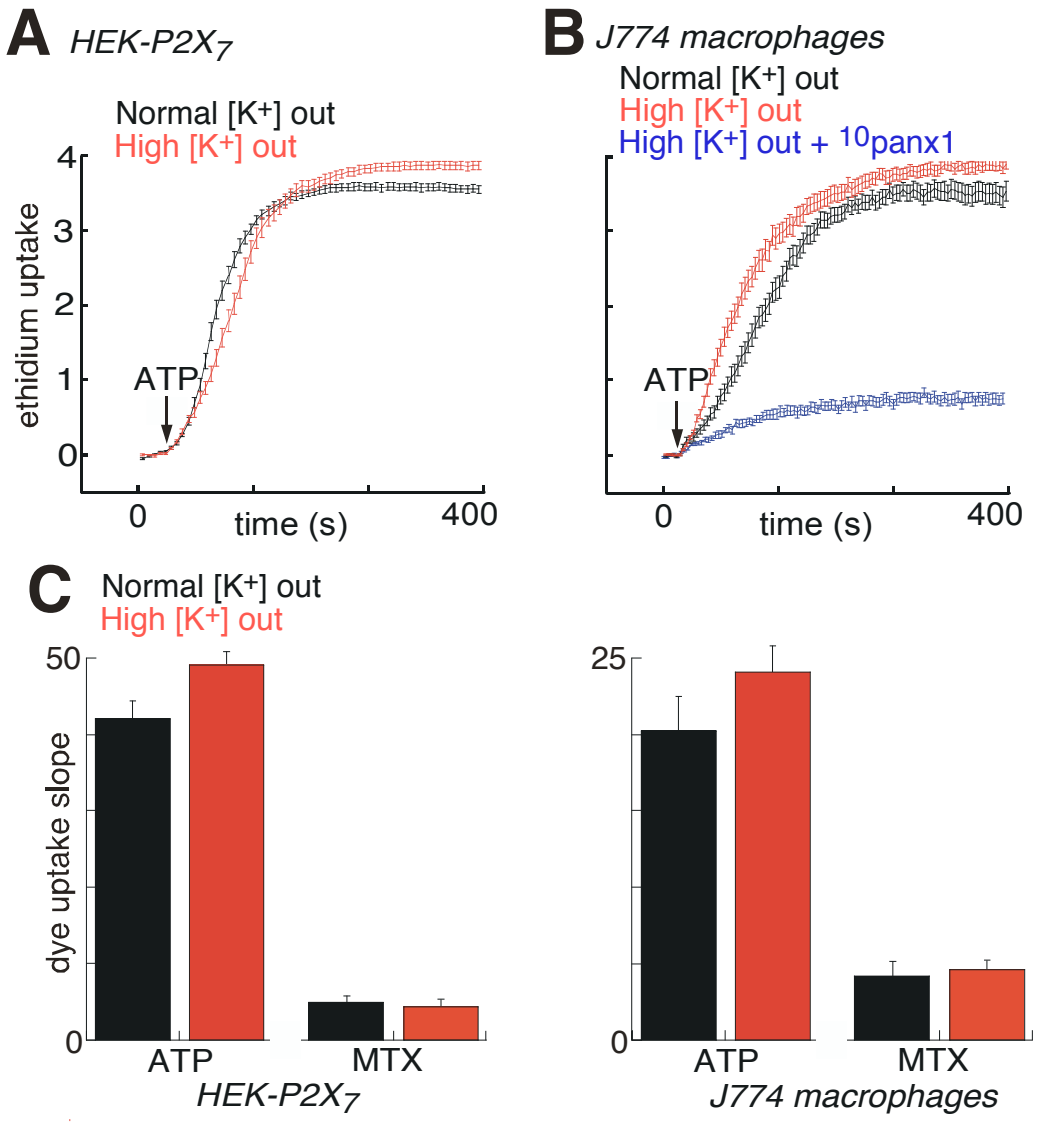
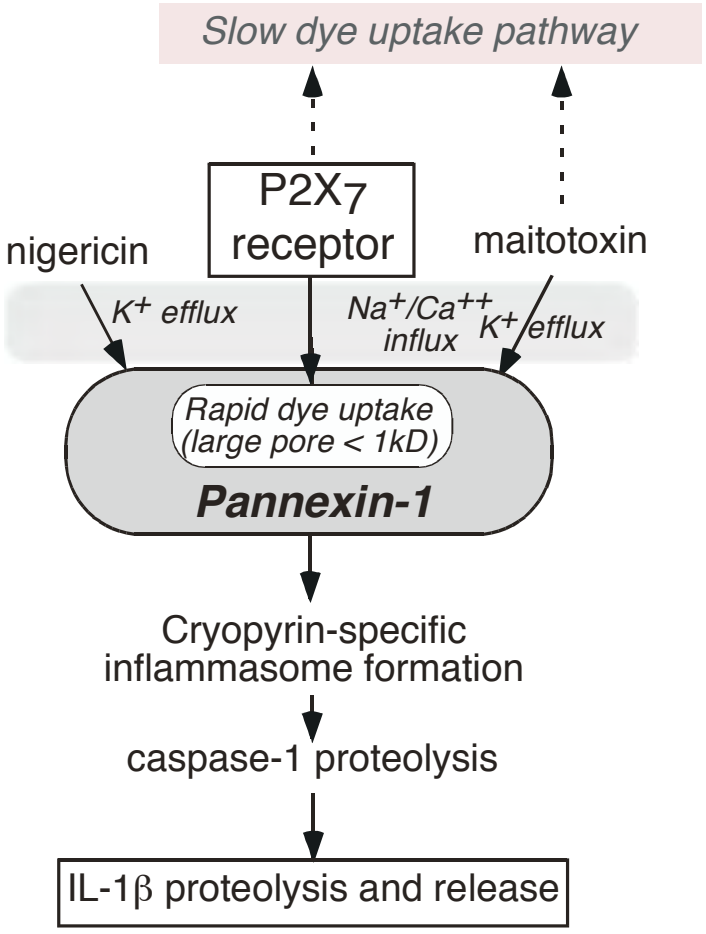

A HEK-P2X7
Normal [K+] out
High [K+] out
ethidium uptake
4
3
2
1
0
ATP
0
time (s)
400
B J774 macrophages
Normal [K+] out
High [K+] out
High [K+] out + 10panx1
ATP
0
time (s)
400
C
Normal [K+] out
High [K+] out
dye uptake slope
50
0
ATP
MTX
HEK-P2X7
25
0
ATP
MTX
J774 macrophages

Figure 8


Slow dye uptake pathway
P2X7 receptor
nigericin
maitotoxin
K+ efflux
Na+/Ca++ influx
K+ efflux
Rapid dye uptake (large pore < 1kD)
Pannexin-1
Cryopyrin-specific inflammasome formation
caspase-1 proteolysis
IL-1β proteolysis and release