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Intracellular P2X receptors as novel calcium release channels and modulators of osmoregulation in *Dictyostelium*

A comparison of two common laboratory strains

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P2X receptors are calcium permeable ligand-gated ion channels activated by ATP. Their role as cell surface receptors for extracellular ATP released physiologically by mammalian cells is well established. However, the cellular function of P2X receptor subtypes that populate the membranes of intracellular compartments is not defined. An initial report described how intracellular P2X receptors control the function of the contractile vacuole, an osmoregulatory organelle in *Dictyostelium* and other protists, and that genetic disruption of P2X receptors severely impaired cell volume control during hypotonic stress. However, later studies refuted a functional role of intracellular P2X receptors in *Dictyostelium*. Here we provide evidence that the discrepancies reported between the studies are due to the laboratory strain of *Dictyostelium* employed, which display different phenotypes in response to hypotonic stress and a varied dependency upon P2X receptors for osmoregulation. We use the recent discovery that intracellular P2X receptors are novel calcium release channels to provide some mechanistic insight in an effort to explain why the strain variance may exist.

Keywords: Calcium signalling, P2X receptor, ATP, ion channel, intracellular, *Dictyostelium*

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Introduction

P2X receptors (P2XRs) comprise a family of cation-selective ligand-gated ion channels activated by micromolar adenosine 5'-triphosphate (ATP).[1] Functional receptors assemble as trimers of pore-forming units of which the human genome encodes seven ($P2X_{1–7}$). Homo- and heteromeric assembly of receptor is documented and allows fine-tuning of biophysical and cellular responses to ATP. Initially cloned in mammals, the dogmatic view of P2XRs is that of cell surface receptors serving to respond to extracellular ATP secreted by cells in processes of cell stress, pain, inflammation and chemotransduction. ATP is omnipresent in biological systems with a major role as an energy source and substrate for enzymatic reactions. Despite the wide distribution of ATP, its role as a signaling molecule appears to be somewhat restricted, represented by the unusual phylogeny of P2XRs.[2] Phylogenetic analysis of P2XRs outside mammals reveals expression by amoeba,[3] single-celled green algae,[4] tick[5] and schistosome;[6] yet P2XRs homologs are not present in Drosophila, *C. elegans*, yeast or higher plants.[2]

In addition to a cell surface residency, some P2XR subtypes are localized to intracellular compartments of mammalian and other eukaryotic cells, including lysosomes[7-9] and phagosomes.[10] In 2007 we cloned the first P2XR from a unicellular organism, from the amoeba *Dictyostelium*.[3] In contrast to the recognized cell surface role of P2XRs, the receptors of *Dictyostelium* are exclusively intracellular.[3,11,12] *Dictyostelium* P2XRs ($P2X_{A-E}$) are localized to the contractile vacuole (CV), an osmoregulatory organelle and acidic calcium store. The receptors are orientated such that the receptor is positioned to sense changes in luminal not cytosolic ATP.[11,12] Our initial study[3] demonstrated that genetic disruption of the $P2X_A$ receptor compromised osmoregulatory

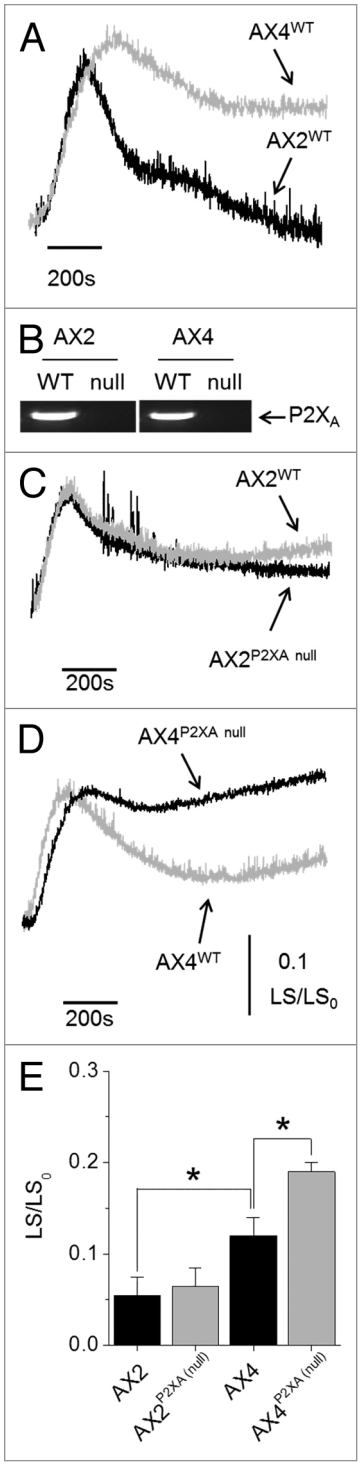

Figure 1. Dependency on $P2X_A R$ for normal osmoregulation is strain variant. (**A**) Time-dependent changes in cell volume following hypotonic challenge for wild-type AX2 and AX4 strain *Dictyostelium* (n = 10). (**B**) Generation of $P2X_A$ null cells verified by RT-PCR. (**C and D**) Effect of $P2X_A$ knockout on AX2 and AX4 hypotonic phenotype (n = 8–10). (**E**) Average light scatter for each cell type 800 s after hypotonic challenge (n = 8–10; *p < 0.05).

function and loss of regulated cell volume decrease (RVD) in response to hypotonic swelling.[3] These data suggested a functional role for intracellular P2XRs. However, a consequent study by Ludlow et al. (2009) revealed near normal osmoregulation in cells void of P2XRs. This controversy has left the functional role of intracellular P2XRs unclear. The factors underlying the discrepancy between the two studies is important to address in an effort to: (1) validate *Dictyostelium* as a model with which to study intracellular P2XR signaling; and (2) to further study how ATP dependent signaling evolved. One striking difference between the study of Fountain et al. (2007) and Ludlow et al. (2009) is the use of laboratory strain, AX4 and AX2, respectively. Although one might expect phenotypic differences between laboratory strains to be subtle, extensive differences have been previously reported[13-15] with genetic variability a likely contributing factor.[16]

Our recent study[12] has gone someway to demonstrate strain variance as a factor between the two studies, but here we provide a direct and definitive analysis of the effect of strain on the dependency for P2XRs during normal osmoregulation.

Results

Proficiency of osmoregulation varies between laboratory strains of *Dictyostelium*. Hypotonic challenge caused cell swelling in both wild-type AX2 and AX4 laboratory strains (**Fig. 1**). Time to peak and the magnitude of peak swelling was similar between strains. However, regulated cell volume decrease (RVD) was markedly different with AX2 recovering much greater volume compared with swollen AX4 cells (**Fig. 1**). RVD in AX4 was approximately 2-fold less than AX2 strain cells. This direct comparison of wild-type AX2 and AX4 demonstrates distinct strain variance in response to hypotonic challenge and suggests AX2 cells are far more adept at volume recovery following swelling (**Fig. 1**).

Differences in P2XR dependency and ATP evoked vacuolar Ca^{2+} release. Blasticidin resistant clones were identified following transformation with the $P2X_A$ receptor targeting vector.[11] $P2X_A$ null cells were verified by RT-PCR (**Fig. 1**). AX2 $P2X_A$ null cells behaved as wild-type (**Fig. 1**) exhibiting no differences in neither peak swelling nor RVD. In stark contrast, disruption of $P2X_A$ in AX4 ablated RVD (**Fig. 1**) with cells exhibiting persistent swelling after peak. Highly purified vacuoles isolated from AX2 and AX4 wild-type cells both released calcium into the extravacuolar space in response to 4 mM ATP (**Fig. 2**). The magnitude of calcium release was significantly smaller in vacuoles isolated from AX2 cells vs. AX4 cells (**Fig. 2**). The magnitude of release was approximately 2-fold less in AX2 cells. Knockout of $P2X_A$ significantly reduced ATP evoked calcium release in vacuoles isolated from both AX2 and AX4 strains (**Fig. 2**).

Discussion

This study demonstrates that two commonly used laboratory strains of *Dictyostelium* display different phenotypes in response to hypotonic stress. We also provide direct evidence that the magnitude of CV calcium released in response to ATP differs substantially between AX2 and AX4, with the magnitude of release being significantly smaller in AX2 cells. Our data agree with those of Ludlow et al. (2009) in that AX2 cells are not dependent upon the $P2X_A$ receptor for normal volume regulation. However this current study, which employs the $P2X_A$ receptor targeting vector described by Ludlow et al. (2009), substantiates our original findings[3] that intracellular P2XRs are required for normal osmoregulation in AX4 cells. However, the molecular basis for the differences in proficiency of osmoregulation displayed between AX2 and AX4 wild-type strains remains unclear, and is likely to be a fruitful line of investigation to fully understand the role of intracellular P2XR function in cell volume control. One apparent difference is in the magnitude of ATP evoked calcium release from the CV, the organelle underlying RVD. We recently described that intracellular P2X receptors mediate calcium release in response to ATP, and this is true for both AX2 and AX4 strains.[12] Indeed the $P2X_A$ receptor contributes around 20–30% of total calcium release in response to ATP in

both strains.[12] However, one striking difference is the magnitude of ATP evoked calcium release observed in CVs isolated from AX2 and AX4 strains, with AX2 vacuoles release significantly (approximately 2-fold) less calcium in response to ATP. Genetic disruption of $P2X_A$ significantly reduced ATP evoked calcium release in AX2 and AX4, approximately 70% for both strains. Interestingly, $P2X_A$ disruption does not ablate ATP evoked calcium release as for disruption of all P2XRs ($P2X_A$–$P2X_E$),[12] and suggests $P2X_A$ is the major component of calcium release in both *Dictyostelium* strains. CV calcium is important for normal osmoregulation as depleting it results in total loss of RVD.[12] If RVD in *Dictyostelium* was completely dependent upon P2XR-dependent calcium release from the CV, one might expect that AX2 would be less adept at osmoregulation than AX4 cells, owing to the smaller magnitude of ATP evoked calcium release. CV calcium is important for normal osmoregulation as depleting it results in total loss of RVD.[12] One interpretation is that P2XR-dependent calcium release is less important for RVD or redundant in AX2, and that another calcium release pathways exist and predominant. Other signals such as calmodulin antagonism, arachidonic and calcium itself mobilise CV calcium.[17] The presence of P2XR redundant mechanisms in AX2 cells and how different signaling pathways interact merits further investigation. This current study and our previous study[12] support a role for intracellular P2XRs as novel calcium release channels which release stored calcium in response to elevated luminal ATP. We also validate *Dictyostelium* as a genetically amenable model eukaryote with which to study signaling by intracellular P2XRs, with the hope to understand how P2XRs may regulate the function of intracellular compartments in mammalian cells.

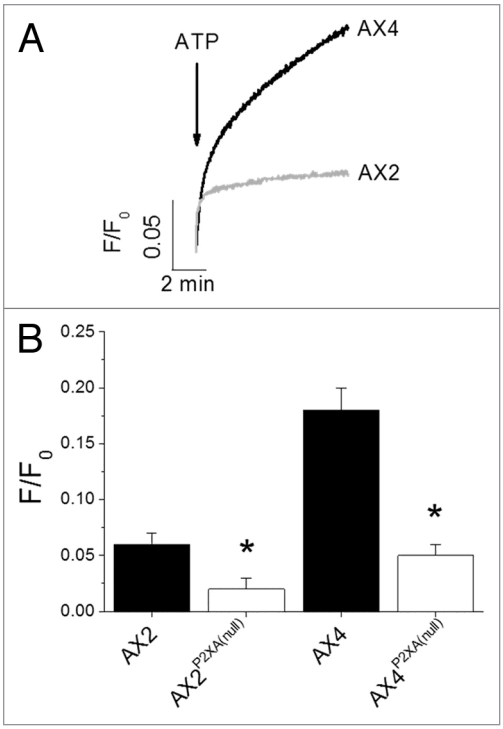

Figure 2. Comparison of ATP evoked vacuolar Ca^{2+} release in different strains. (**A**) ATP (4 mM) evoked calcium release measured in highly purified contractile vacuoles isolated from wild-type AX2 and AX4 *Dictyostelium*. (**B**) Mean peak ATP evoked vacuolar Ca^{2+} release for both wild-type strains and $P2X_A$ null strains n = 6–8; *p < 0.01).

Methods

Cell culture and gene disruption. Wild-type AX2 (Rob Kay laboratory strain) and wild-type AX4 (Chris Thompson laboratory strain) were cultivated in shaking culture at 21°C in HL5 medium containing glucose. Cells were maintained at a density less than 1×10^6 cells/mL. $P2X_A$ knockouts were generated using the targeting vector used previously by Ludlow et al. (2009). Briefly, cells were transformed by electroporation followed by selection with 10 μg/mL blasticidin for 14 d. Loss of $P2X_A$ was confirmed by RT-PCR using 5'-GCA GTC GAT TTA CAT GGT TAC-3' sense and 5'-AGT TTG GAA ATG GAA AGA ACC-3' antisense primers.

Vacuole purification and calcium release assay. Purification and real-time measurement of calcium release were performed as described previously.[12] Calcium release was followed using membrane impermeable Fluo-3 (ex λ 505-nm; em λ 526-nm).

Osmoregulation assay. All cells were suspended in fresh HL5 medium for 2 h prior to experimentation in an effort to avoid any adverse effects of conditioned media on cell performance. Changes in cell size were measured by right-angled scatter of light at 600 nm using a Hitachi F-2000 spectrophotometer. Hypotonic stress was induced by replacing HL5 medium with distilled water (1×10^6 cells/mL).

Statistics. Average results are expressed as mean ± SE from the number of experiments indicated. Hypothesis testing employed unpaired two-tailed Student's t-test.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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