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EVIDENCE FOR EXTRACELLULAR ATP AS A STRESS SIGNAL IN A SINGLE CELLED ORGANISM

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ABSTRACT

ATP is omnipresent in biology and acts as an extracellular signalling molecule in mammals. Information regarding the signalling function of extracellular ATP in single celled eukaryotes is lacking. Here we explore the role of extracellular ATP in cell volume recovery during osmotic swelling in the amoeba *Dictyostelium*. Release of micromolar ATP could be detected during cell swelling and regulatory cell volume decrease (RVD) phases during hypotonic challenge. Scavenging ATP with apyrase caused profound cell swelling and loss of RVD. Apyrase-induced swelling could be rescued by 100μM βγ-imidoATP. N-ethylmalemide (NEM), an inhibitor of vesicular exocytosis, caused heightened cell swelling, loss of RVD and inhibition of ATP release. Amoeba with impaired contractile vacuole (CV) fusion (drainin KO cells) displayed increased swelling but intact ATP release. 100μM Gd^{3+} caused cell swelling while blocking any recovery by βγ-imidoATP. ATP release was 4-fold higher in the presence of Gd^{3+}. Cell swelling was associated with an increase in intracellular nitric oxide (NO), with NO scavenging agents causing cell swelling. Swelling-induced NO production was inhibited by both apyrase and Gd^{3+}, while NO donors rescued apyrase- and Gd^{3+}-induced swelling. These data suggest extracellular ATP released during cell swelling is an important signal that elicits RVD. Though the cell surface receptor for ATP in *Dictyostelium* remains elusive, we suggest ATP operates through a Gd^{3+}-sensitive receptor that couples to intracellular NO production.

INTRODUCTION

The ability to control cell volume is an essential function for cell survival in the face of osmotic challenge. Perturbations in cell volume evoke wide ranging signalling events leading to acute protective responses (e.g. rearrangement of the cytoskeleton) and longer-term adaptive responses (e.g. alteration in osmolyte transport and gene expression) [1]. During acute swelling, cells can respond by a process of regulatory cell volume decrease (RVD). Under normal physiological conditions, mammalian cells are exposed to extracellular fluid osmolarity of approximately 285 mOsm, which is kept constant by normal body fluid homeostasis. Cell swelling often occurs as a consequence of changes to the intracellular composition of osmolytes, which results in intracellular hypotonicity and the influx of water. Compositional changes may occur during increase cellular

transport or accumulation of nutrients or metabolic waste. Osmotically swollen mammalian cells release K^+, Cl^- and non-essential organic osmolytes in an effort to reverse the flow of water by osmosis. In contrast to mammalian cells, free-living eukaryotic single cells can be subject to rapid and harsh changes in osmolarity of the extracellular environment. As a consequence, the majority of single celled organisms have evolved a specialised organelle called the contractile vacuole, a bladder-like structure that plays a major role in extruding water from the cytoplasm and expelling it into the extracellular space [2].

ATP is a ubiquitous molecule, used as an energy currency by cells, and as a substrate for protein phosphorylation inside the cell. In mammalian cells, extracellular ATP acts as a potent signalling molecule via activation of cell surface ionotropic (P2X) and metabotropic (P2Y) receptors. ATP release and signalling is involved in diverse physiological and pathophysiological events including pain, inflammation and control of blood vessel tone. Molecular mechanisms of ATP release in mammalian cells are also diverse and further work is required to understand how cellular events couple to ATP release. ATP is released from mammalian cells when cells are subject to different types of mechanical force including stretch [3-4], flow [5] and shear stress [6]. ATP is also released in response to osmotic swelling, acting as an early extracellular stress signal to initiate RVD via P2 receptor activation [7-9]. Early studies demonstrate the presence of extracellular ATP in cultures of single celled eukaryotes [10-12], but a role of extracellular ATP as a signal molecule in primitive organisms has not been defined. Parish & Weibel (1980) [10] made an early report demonstrating intracellular calcium responses evoked in the amoeba *Dictyostelium* by exogenous ATP. A more recent study by Ludlow et al (2008) [13] also showed calcium response evoked by extracellular ATP. Both studies suggest the existence of cell surface receptor capable of responding to extracellular ATP, though the molecular basis for ATP reception and evidence for the extracellular signalling by endogenous ATP are lacking. To this end, we sought to investigate the role of extracellular ATP signalling during osmotic swelling in *Dictyostelium*.

MATERIALS & METHODS

Cells and cell size measurement

AX4 wild-type and drainin knockout AX4 *Dictyostelium discoideum* cells were cultured in shaking flasks containing HL5 medium with glucose at 22°C. Time resolved measurement of changes in cell size were performed by right-angled light scattering (LS) at 600nm using a Hitachi F2000 spectrophotometer. This photometric technique allows measurement of macroscopic cell size changes in populations of cells, where the intensity of scattered light corrects in a near-linear fashion with cell size [14].

Cells in culture were sedimented at 500x*g* for 5mins at 22°C. Cells were re-suspended at $2x10^6$ cells/mL in HL5 medium or 2mM HEPES-KOH (pH7.2) for hypotonic challenge. Cells were continuously stirred in a quartz cuvette and light at 600nm collected every 2 seconds. All compounds were injected manually. Experiments using the temperature-sensitive *N*-ethyl maleimide-sensitive factor (NSF) conditional mutant strain were performed at 28°C.

ATP detection

150µL of sample was withdrawn from cell suspensions at regular intervals. Samples were spun immediately at 500x*g* for 5 mins at 4°C to produce a cell-free supernatant and limit cell-dependent ATP breakdown. ATP was quantified by luciferase-luciferin assay as described previously [15].

Nitric oxide (NO) assay

NO_2 and NO_3 metabolites of NO were quantification by the modified Griess assay [16]. Briefly, 2,3-diaminonaphthalene was reacted with sample under acidic conditions for 1 hour at 37°C to form the fluorescent product 1-H-naphthotriazole. Accumulation of 1-H-naphthotriazole was measured using a fluorescence plate reader with excitation at 365nm and emission at 450nm.

Statistical analysis

Hypothesis testing was performed by one-way ANOVA analysis.

RESULTS

Extracellular ATP is required for cell volume recovery following swelling in *Dictyostelium* amoeba

Dictyostelium amoeba exposed to hypotonic stress underwent cell swelling that peaked around 400 seconds post challenge and was followed by a progressive cell volume recovery phase (**Figure 1A**). At plateau, the average volume recovery was around 40% of peak (**Figure 1A**). No significant changes in cell volume were observed in isotonic conditions (**Figure 1A**). During cell swelling, extracellular ATP increased significantly peaking at 8.1±1.5 µM (*N*=5) from a baseline of 2.2±0.8 µM (*N*=5) (**Figure 1C**). No significant changes in extracellular ATP were observed in isotonic conditions (**Figure 1B**). Application of apyrase to scavenge ATP during hypotonic challenge lead to profound cellular swelling and loss of the cell volume recovery phase (**Figure 1D**), detectable ATP was negligible following apyrase treatment (data not shown). Peak swelling observed in the presence of apyrase was 13.6±1.5 LS ($P<0.05$ *vs* control) compared to an average swelling of 6.8±0.8 LS in the absence of apyrase. The

non-hydrolysable ATP analogue βγ-imidoATP, but not ATP (data not shown), could rescue (97±0.8%, *N*=4; *P*<0.01 *vs* control) the volume recovery response in the presence of apyrase (**Figure 1D**). Application of apyrase under isotonic conditions had no effect on cell size (**Figure 1E**). Heat-inactivated apyrase had no effect of cell size (data not shown). These data suggest that extracellular ATP is released during cell swelling and is important for cell volume recovery.

The contractile vacuole is the major osmoregulatory organelle in *Dictyostelium* and other protists. It serves to accumulate water during hypotonic stress and release it via an atypical exocytosis event. We have previously identified that the contractile vacuole system in *Dictyostelium* can accumulate ATP via a translocation mechanism of unknown molecular basis [17]. Furthermore, vesicular ATP release is one of numerous mechanisms proposed as modes of ATP secretion in mammalian cells [18]. To this end, we sought to employ *N*-ethylmaleimide (NEM) that blocks vesicular exocytosis and ATP release in mammalian cells [15]. NEM caused profound swelling and loss of regulatory cell volume decrease under hypotonic conditions (Figure 2A). Peak swelling in the presence of NEM was 46.5±2.2 (*N*=6; P<0.05 *vs* control) compared to swelling in the absence of NEM (8.4±1.2; *N*=6). Interestingly, despite the heightened swelling caused by NEM, extracellular ATP accumulation was blocked (7.7±1μM control *vs* 2.1±0.5μM NEM; *N*=6, *P*<0.05) (**Figure 2B and 2C**). In the presence of NEM, the peak ATP concentration was not significantly difference than baseline, suggesting no ATP release (**Figure 2C**). To further explore a role of exocytosis, we utilised a temperature-sensitive *N-ethyl* maleimide-sensitive factor (NSF) conditional mutant strain, which has been extensively studied and has impaired exocytosis [19-20]. NSF mutant cells swelled profoundly during hypotonic stress (**Figure 2D**), with peak swelling of 40±2.6 (*N*=6, P<0.05 *vs* control). As for NEM-treated wild-type cells, NSF mutant also displayed impaired ATP secretion during hypotonic challenge (1.8±0.2μM wild-type *vs* 9.1±0.8μM NSF mutants; *N*=6, *P*<0.05) (**Figure 2E and 2F**). These data supported a vesicular exocytotic event as a possible route to ATP release and extracellular accumulation. In an effort to explore the role of contractile vacuole voiding in ATP release, we used drainin knockout amoeba that display severely impaired membrane fusion of the contractile vacuole [21]. Drainin knockout cells exhibited extensive swelling during hypotonic stress (25±1.2 LS *vs* 9.4±0.8 LS control; *N*=5, *P*<0.05) with total absence of any volume recovery phase (**Figure 3A**), supporting a role of contractile membrane fusion in recovery from hypotonic swelling [21-23]. Drainin knockout cells are null for DDB_G0269130 gene that encodes a rabGAP [21]. NEM-treated cells, extracellular ATP accumulation was still observed (**Figure 3B**). Indeed the peak concentrations of extracellular ATP were 2-fold that of wild-type cells (**Figure 3C**). Taken together these data support a role for vesicular release of ATP during cell swelling but negate membrane fusion of the contractile vacuole as a potential source.

Previous work on ATP receptors in *Dictyostelium* has identified five P2X receptor homologs ($P2X_A$ – $P2X_E$) that encode ATP-activated ion channels [22-23]. In mammalian cells, P2X receptors are traditionally viewed as cell surface receptors for ATP, but our own work and that of others, demonstrates an exclusively intracellular localisation of *Dictyostelium* P2X receptors. Despite this, cellular responses to extracellular ATP have been reported [13], though the molecular basis for cell surface reception of ATP in *Dictyostelium* remains elusive. Ludlow et al (2008) [13] reported that cellular responses to exogenous ATP were completely blocked by Gd^{3+}. To this end, we sought to examine the effect of micromolar Gd^{3+} on the cell swelling response and ATP release. Exposure to Gd^{3+} mimicked the effect of apyase, resulting in loss of the cell volume recovery phase and extensive cell swelling under hypotonic conditions (**Figure 4A**). Unlike NEM, which uncoupled cell swelling from ATP release (**Figure 2B**), ATP release was greatly heightened in the presence of Gd^{3+}, with peak concentrations rising 4-fold ($P<0.05$; $N=5$) over control conditions (**Figure 4B and 4C**). Interestingly, Gd^{3+} blocked any recovery from apyrase-induced swelling by addition of βγ-imidoATP (**Figures 4D and 4E).** These data are suggestive of a Gd^{3+}-sensitive ATP-dependent mechanism evoked during volume regulation under hypotonic stress.

Nitric oxide (NO) is produced by many cells during stress or trauma. To better understand possible signal transduction events that may result from ATP sensing during cell swelling, we investigated NO production as a feasible messenger. As for extracellular ATP, intracellular NO increased during cell swelling (**Figure 5A**), reaching a steady-state phase after several minutes post hypotonic challenge. Changes in NO levels were not observed under isotonic conditions (**Figure 5A**). Next we employed a NO scavenger to test whether the NO produced was important for the volume recovery process. Pre-incubation with the scavenger PTIO resulted in a loss of the cell volume recovery phase and profound swelling (85±2.1 LS PTIO *vs* 10.2±0.6 LS control; $N=5$, $P<0.05$) (**Figure 5B**), suggesting NO is required for volume recovery. Similar results were observed for the chemically unrelated NO scavenger SDTC (data not shown). Application of the cell-impermeable NO scavenger haemoglobin had no effect (data not shown). To test for a requirement of either extracellular ATP or extracellular ATP sensing for NO production, we tested the effects of apyrase and Gd^{3+} on swelling-induced NO production, respectively. Both apyrase and Gd^{3+} ablated NO production during cell swelling ($N=5$; $P<0.05$) (**Figure 5C**). In reciprocal experiments we examined the ability of the NO donor SNP to rescue swelling induced by either apyrase or Gd^{3+}, in an effort to determine if NO lay downstream of ATP-sensing mechanisms at the cell surface. Strikingly, SNP could attenuate both apyrase- and Gd^{3+}-induced swelling (**Figures 5D and 5E**). SNP had no effect on cell volume under isotonic conditions (data not shown). These data demonstrate that NO donation can rescue the effects of apyrase and Gd^{3+} suggesting that NO acts downstream on pathways attenuated by apyrase and Gd^{3+}.

DISCUSSION

In this study, we propose that ATP released during hypotonic swelling of *Dictyostelium* amoeba operates as a stress signal through an unknown cell surface receptor to stimulate NO production and recover cell volume. This represents the first report of a role for endogenous extracellular ATP in a single celled organism. P2X receptors for ATP have been cloned from several primitive species [22-29], including single cells, though the physiological role of ATP signalling in such organisms remains elusive. The subcellular distribution of P2X receptors in more primitive organisms also remains to be determined. It is highly likely that the P2X receptors identified in *Dictyostelium* do not serve as cell surface receptors for ATP due to their localisation to the contractile vacuole [22-23, 29]. Though the contractile vacuole fuses with the plasma membrane during voiding of water, no mixing of the membranes occur [30]. This would negate vacuole fusion as a route for potential P2X receptor trafficking to the plasma membrane. Furthermore, intracellular calcium responses in *Dictyostelium* elicited by exogenous ATP application are unaffected by P2X receptor knockout [13]. The well curated genome information available for *Dictyostelium* also yields no information to suggest expression of P2Y receptor homologues [31]. Recently DORN1, which bares no homology with known P2X or P2Y receptors, was identified as a receptor for extracellular ATP in plants, and is linked to stress signalling [32-33]. It is therefore possible that a novel receptor type mediates responsiveness to extracellular ATP in *Dictyostelium*.

The identification of cell surface ectonucleotidase-like activity in *Dictyostelium* provides precedence for the existence of extracellular ATP [10], possible in a signalling capacity. In addition, previous studies have demonstrated that *Dictyostelium* can condition growth medium with ATP [10]. This suggests that *Dictyostelium* amoeba secrete ATP constitutively as for some mammalian cells [15,18]. Our own bulk phase measurements suggest a relatively high basal level of extracellular ATP, between 2-4μM. In this study, inhibition of vesicular secretion with NEM or drainin knockout did not affect the level of basal ATP, though NEM strongly inhibited ATP release during swelling. Exocytosis of ATP containing vesicles such as lysosomes, has been shown to contribute to constitutive ATP secretion in mammalian cells [15], though this appears not to be the case for *Dictyostelium*. Our current study suggests that constitutively secreted ATP does not influence cell volume, as apyrase had no effect on cell size under isotonic conditions. The contractile vacuole of *Dictyostelium* harbours an ATP translocation mechanism of unknown molecular basis, facilitates ATP accumulation within the vacuole lumen [17]. Despite this, drainin knockout cells that have impaired contractile vacuole fusion exhibit heightened swelling but no inhibition of ATP release, strongly suggesting contractile vacuole voiding is not the source of ATP. As for *Dictyostelium*, Gd^{3+} inhibits RVD in various mammalian cells swollen by hypotonicity including hepatocytes [34], neuronal [35] and erythrocytes [36]. However, in

mammalian cells Gd^{3+} also blocks any swelling-induced ATP release [34,37]. This in contrast to the heightened ATP release observed in *Dictyostelium* in the presence of Gd^{3+}. In mammalian cells, the inhibitory action of Gd^{3+} on ATP release is reported to be through blockade of mechanosensitive receptors [7, 37-38], which presumably integrate swelling-induced stretching of the plasma membrane and stimulated ATP release. Gd^{3+} also blocks receptor-mediated ATP release in mammalian cells [37]. This suggests that ATP is released via a Gd^{3+}-insensitive mechanism in *Dictyostelium*, and that the increased ATP release occurs due to profound cell swelling observed in the presence of Gd^{3+}. An alternative explanation for the Gd^{3+}-induced swelling is that Gd^{3+} blocks sensing of extracellular ATP. This explanation is supported by the observation that Gd^{3+} blocks rescue by βγ-imidoATP during apyrase-induced swelling. βγ-imidoATP can activate *Dictyostelium* P2X receptors [22], but as discussed, the intracellular residency of P2X receptors makes them unlikely mediators. Ludlow et al (2008) [13] demonstrated that Gd^{3+} could block calcium responses evoked by extracellular ATP in *Dictyostelium*. Our data supports the presence of a Gd^{3+}-sensitive cell surface receptor for ATP.

In this study, we demonstrate that intracellular NO increases during cell swelling. Experiments with a NO scavenging agent suggest that the NO produced is important for the cell volume recovery process. Apyrase and Gd^{3+} which both block cell volume recovery also block NO production during cell swelling. Moreover, NO donation can rescue cell volume recovery in the presence of apyrase or Gd^{3+}. These data strongly suggest that NO production lies downstream of ATP sensing via a Gd^{3+}-sensitivity receptor. In mammalian cells such as red blood cells and vascular endothelium, NO is produced in response to mechanical stress [40-41]. There is also evidence that NO is produced by mammalian cells during osmotic and trauma-induced swelling [40-41]. Activation of P2 receptor also stimulates NO production in various mammalian cells types [42-46]. There are also a number of studies linking extracellular ATP to NO production in plant cells [47-49]. In mammalian cells, NO is produced by nitric oxide synthase (NOS), though in plants the identification of a mammalian-like NOS homologue mains elusive. This is despite the identification of NO associated proteins [48]. Nitrate reductases have been identified as enzymes responsible for NO production in plants [49]. Evidence is also lacking for a homologue of mammalian NOS in *Dictyostelium*. The genome however does predict the existence of a homologue of NOS-interacting protein [48]. In addition to the requirement of NO for cell volume regulation shown in this study, NO is known to control cellular aggregation and differentiation during multicellular development *Dictyostelium* [50-52].

In summary, we demonstrate that ATP is secreted during osmotic swelling in *Dictyostelium* via a NEM-sensitive mechanism. Based on the pharmacology of the cellular response, we suggest that

extracellular ATP activates a Gd^{3+}-sensitive receptor to increase intracellular NO which in-turn initiates cell volume recovery.

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Figure Legends

Figure 1 Extracellular ATP released during cell swelling is required for regulatory cell volume decrease. (A) Representative light-scattering (LS) experiment showing cell size changes in suspension of *Dictyostelium* amoeba immediately after exposure to isotonic conditions or hypotonic challenge. (B) Representative plot showing extracellular ATP concentration with time. (C) Mean peak extracellular ATP concentrations measured under isotonic and hypotonic conditions (*N*=5). (D) Representative trace showing effect of apyrase (2U/mL) on regulatory cell volume decrease during hypotonic challenge, and rescue of control response by βγ-imidoATP (100μM). (E) Lack of effect of apyrase (2U/mL) on cell size during isotonic conditions. * denotes *P*<0.05 throughout.

Figure 2 Role of vesicular fusion in swelling-induced ATP release. (A) Representative light-scattering (LS) experiment showing cell size changes in suspensions of wild-type *Dictyostelium* amoeba immediately after exposure to hypotonic challenge. Cells are pre-treated with *N*-ethylmaleimide (NEM; 1mM, 15mins) or not (control). (B) Representative plot showing extracellular ATP concentration with time. (C) Mean peak extracellular ATP concentrations measured under hypotonic conditions with (NEM) and without (control) NEM pre-treatment for wild-type cells (*N*=6). (D) Representative LS experiments showing cell size changes in suspensions of wild-type *Dictyostelium* amoeba and temperature-sensitive *N*-ethyl maleimide-sensitive factor (NSF) mutant cells. Arrows indicate sample points for extracellular ATP measurements as shown representative plot (E). (F) Mean peak extracellular ATP concentrations measured under hypotonic conditions for wildtype and NSF mutant amoeba (*N*=6). * denotes *P*<0.05 throughout.

Figure 3 Role of contractile vacuole voiding in swelling-induced ATP release. (A) Representative light-scattering (LS) experiment showing cell size changes in suspensions of wild-type *Dictyostelium* amoeba and drainin knock-out (KO) amoeba immediately after exposure to hypotonic

challenge. (B) Representative plot showing extracellular ATP concentration with time. (C) Mean peak extracellular ATP concentrations measured under hypotonic conditions for wildtype and drainin KO amoeba (N=5). * denotes P<0.05 throughout.

Figure 4 Gd^{3+} inhibits cell volume recovery from swelling and rescue by βγ-imidoATP during apyrase-induced swelling. (A) Representative light-scattering (LS) experiment showing cell size changes in suspensions of wild-type *Dictyostelium* amoeba immediately after exposure to hypotonic challenge in the presence (Gd^{3+}) or absence (control) of Gd^{3+} (100μM). (B) Representative plot showing extracellular ATP concentration with time. (C) Mean peak extracellular ATP concentrations measured under hypotonic conditions in the absence or presence of Gd^{3+} (100μM) (N=5). (D) Representative trace and mean peak cell size (D) showing cell changes under hypotonic conditions. Cells are exposed to apyrase (2U/mL) and βγ-imidoATP (100μM), and with Gd^{3+}(100μM) where indicated. Control values are from cells exposed to treatment (N=5). * denotes P<0.05 throughout.

Figure 5 Nitric oxide production during hypotonic swelling is blocked by apyrase and Gd^{3+}. (A) Relative quantification of cellular nitric oxide production in suspensions of *Dictyostelium* amoeba under hypotonic or isotonic conditions (N=5). (B) Representative trace showing inhibition of cell volume recovery in cells treated with nitric oxide scavenging agent (PTIO) during hypotonic challenge. (C) Effect of apyrase (2U/mL) or Gd^{3+} (100μM) on nitric oxide production in cell under hypotonic stress (N=5). Representative traces showing effect of nitric oxide donor (SNP, 500μM) on swelling induced by apyrase (2U/mL) and Gd^{3+}(100μM).

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Eukaryotic Cell

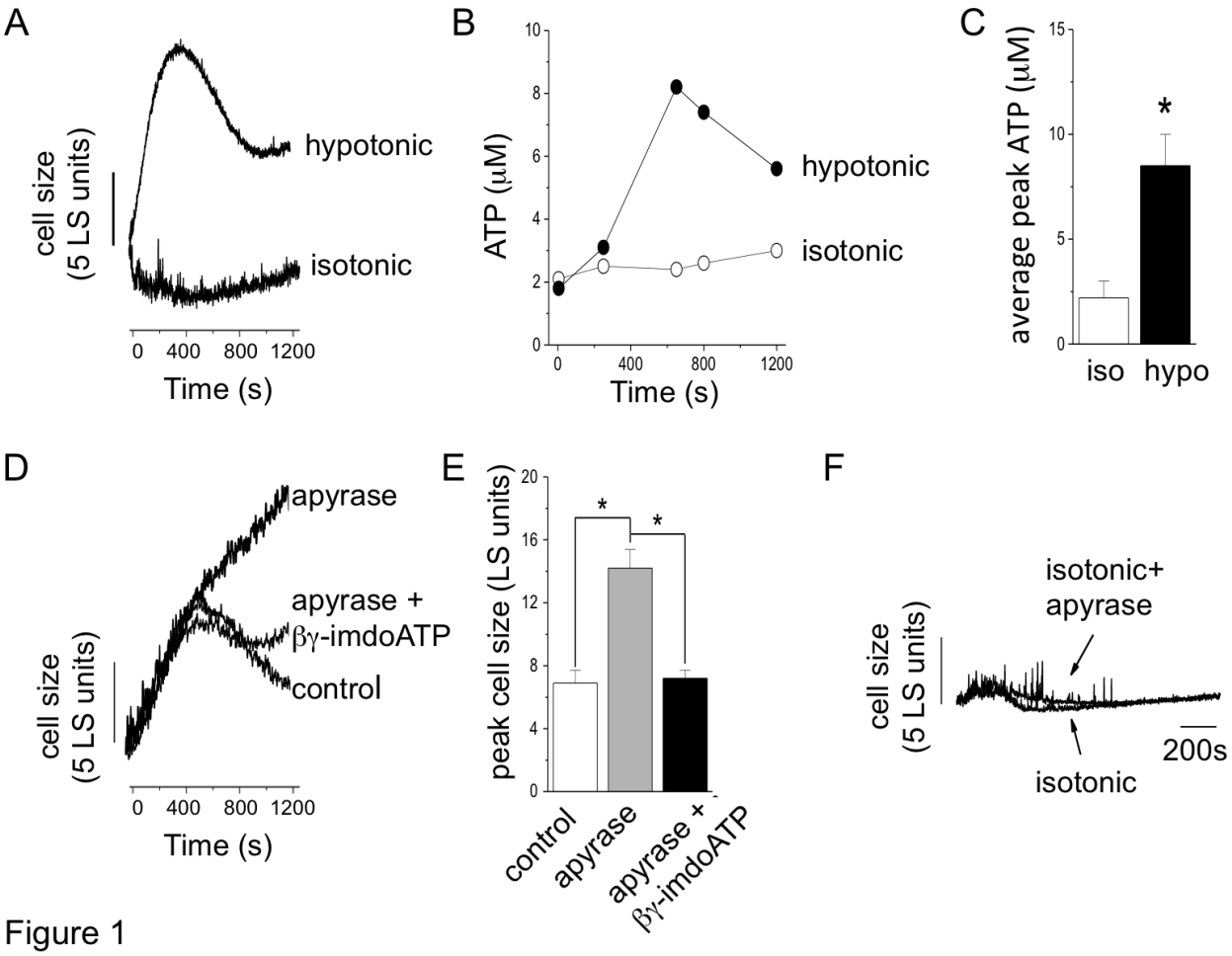


Figure 1

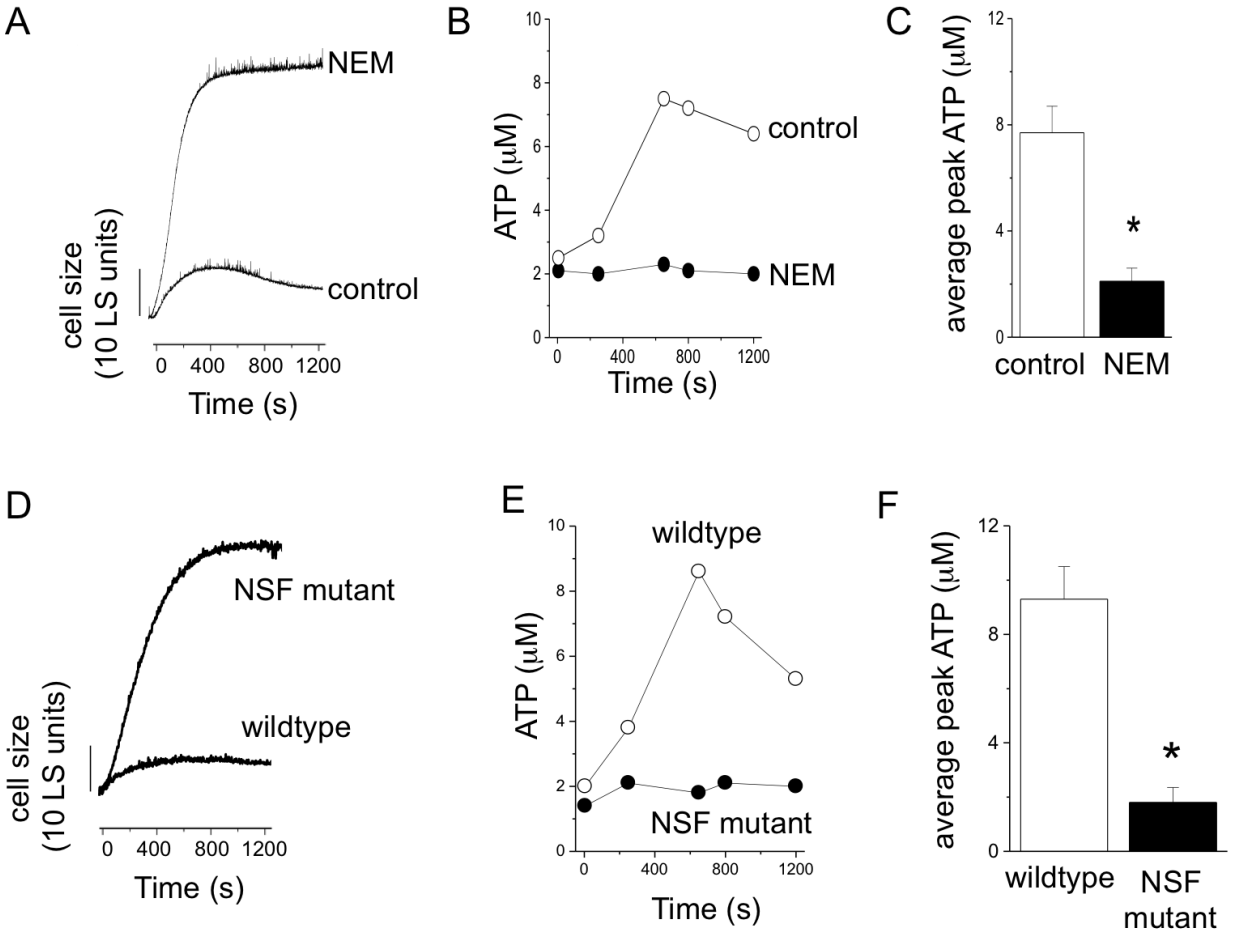
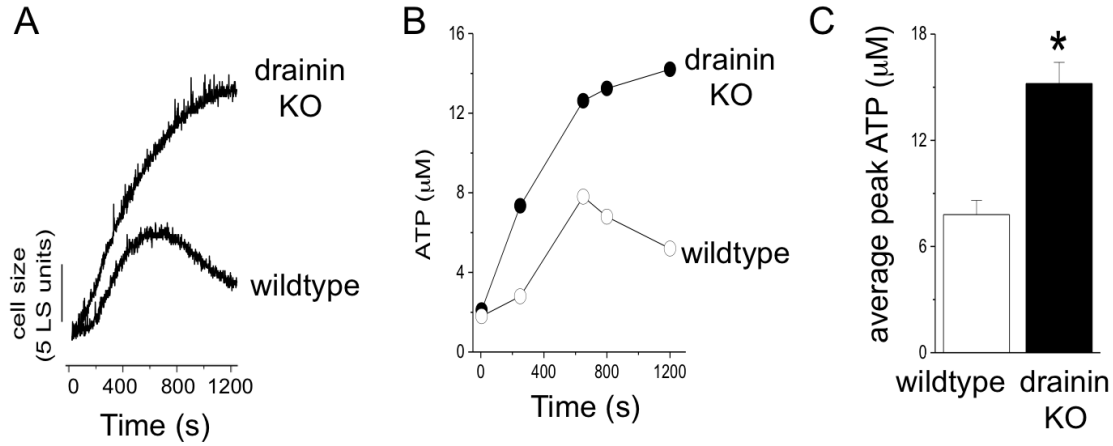


Figure 2



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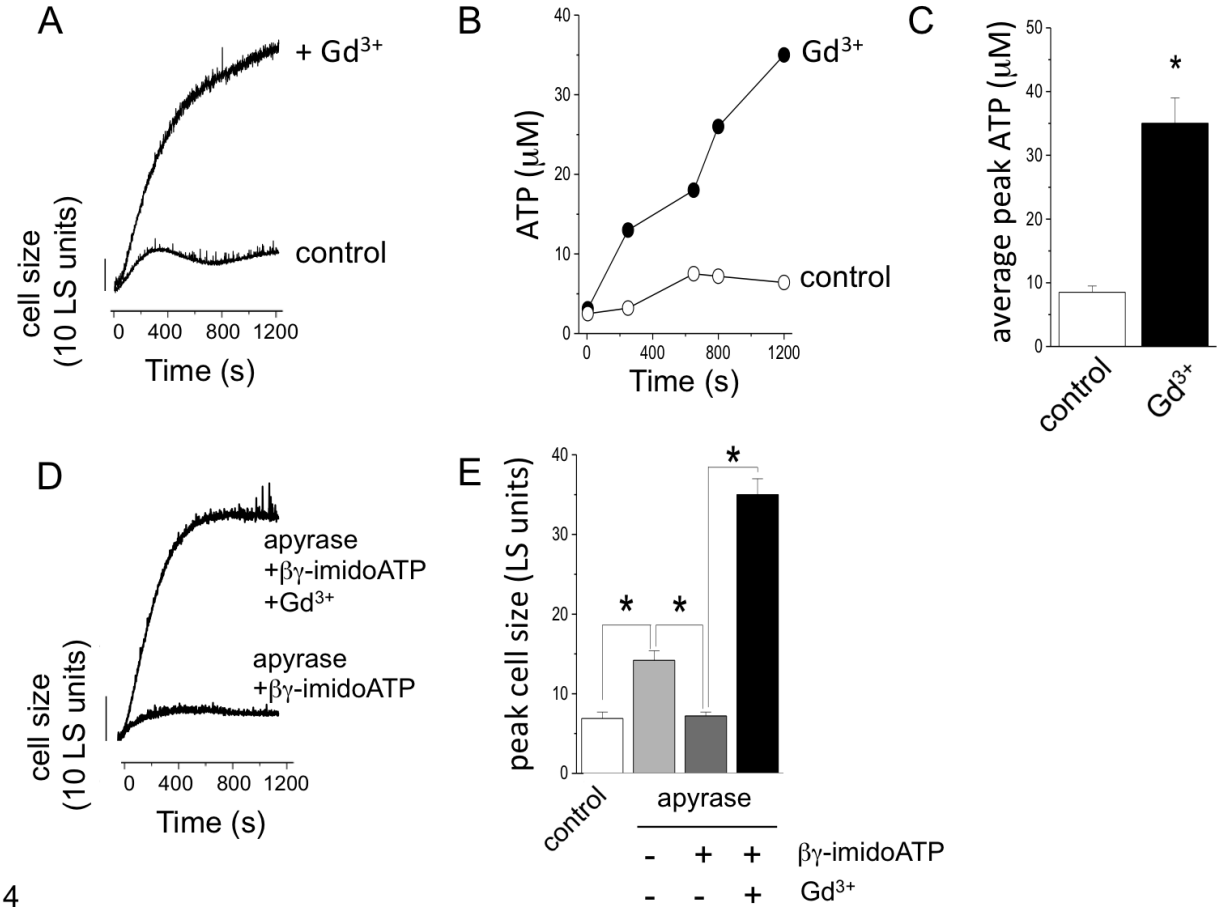

Figure 4

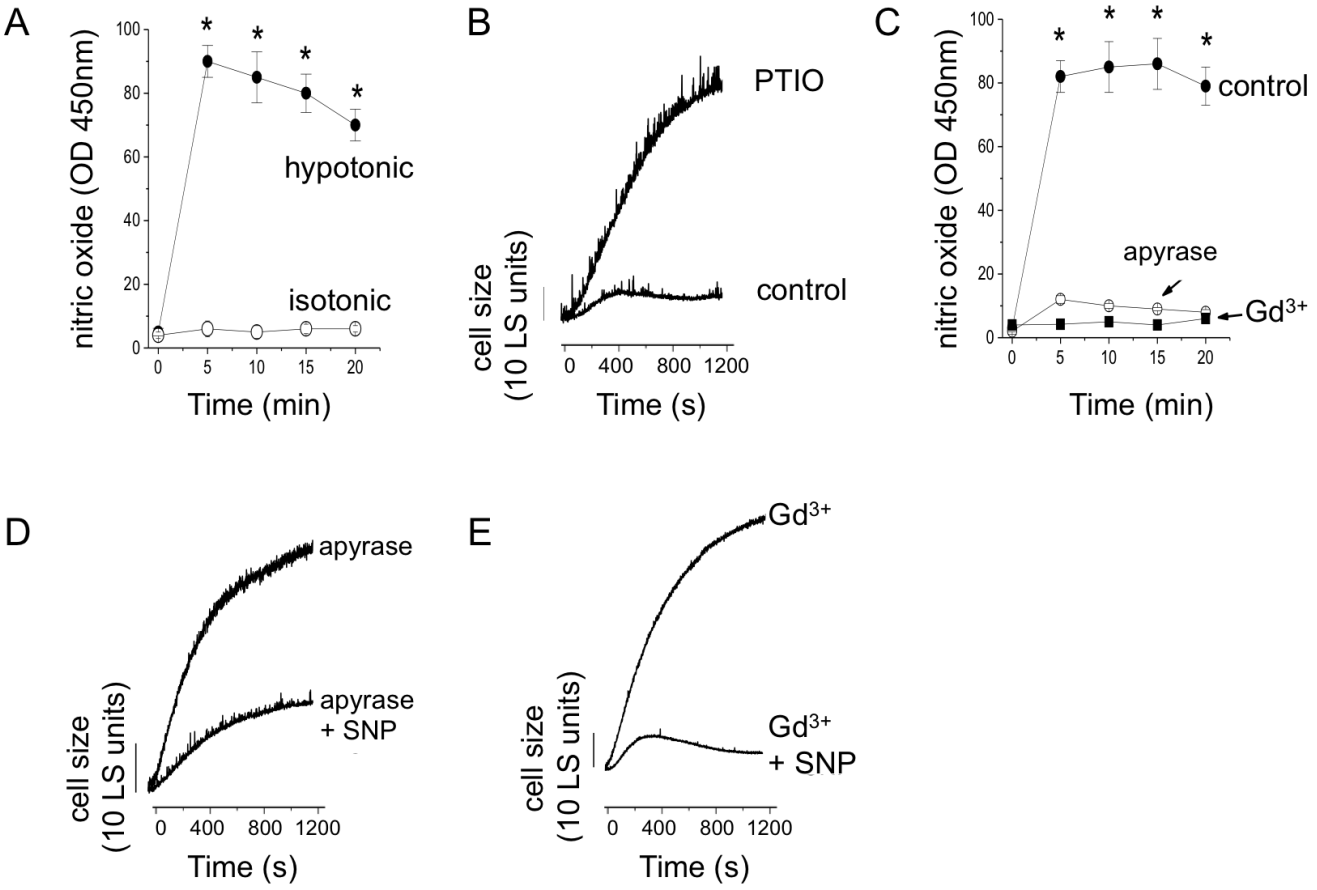

Figure 5