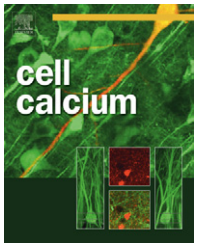
Cell Calcium (2008) 44, 567–579

journal homepage: www.elsevier.com/locate/ceca



Purinergic-mediated Ca^{2+} influx in *Dictyostelium discoideum*

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Received 13 February 2008; received in revised form 31 March 2008; accepted 6 April 2008
Available online 16 May 2008

KEYWORDS
Purinergic;
P2X;
P2Y;
Dictyostelium;
ATP;
ADP;
Aequorin;
Calcium

Summary The presence of five P2X-like genes (*p2xA–E*) in *Dictyostelium* suggests that nucleotides other than cAMP may act as extracellular signalling molecules in this model eukaryote. However, p2xA was found to have an exclusively intracellular localisation making it unclear whether *Dictyostelium* utilise P2 receptors in a manner analogous to vertebrates. Using an apoaequorin expressing strain we show here that *Dictyostelium* do possess cell surface P2 receptors that facilitate Ca^{2+} influx in response to extracellular ATP and ADP (EC_{50} = 7.5 μM and 6.1 μM, respectively). Indicative of P2X receptor activation, responses were rapid reaching peak within 2.91 ± 0.04 s, required extracellular Ca^{2+}, were inhibited by Gd^{3+}, modified by extracellular pH and were not affected by deletion of either the single *Gβ* or *iplA* genes. Responses also remained unaffected by disruption of *p2xA* or *p2xE* showing that these genes are not involved. Cu^{2+} and Zn^{2+} inhibited purine-evoked Ca^{2+} influx with IC_{50} values of 0.9 and 6.3 μM, respectively. 300 μM Zn^{2+} completely abolished the initial large rapid rise in intracellular Ca^{2+} revealing the presence of an additional smaller, slower P2Y-like response. The existence of P2 receptors in *Dictyostelium* makes this organism a valuable model to explore fundamental aspects of purinergic signalling.

Introduction

Sequencing of the *Dictyostelium* genome [1] has revealed a diverse complement of predicted cell surface receptors and ion channels, many with mammalian homologues, thereby increasing the interest in this amoeboid protozoan as a model eukaryotic organism. In addition to the seven well-characterised cAMP and cAMP-like receptors involved in chemotaxis during aggregation, 48 additional putative seven transmembrane receptors (7TM) are present [1,2] including receptors for glutamate [3] and GABA [4]. In terms of the complement of ion channels, an intriguing finding is the presence of a family of five *Dictyostelium* genes predicted to code for proteins with weak homology to vertebrate P2X

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doi:10.1016/j.ceca.2008.04.001

receptors (Dictybase [5] genes: *p2xA*, *p2xB*, *p2xC*, *p2xD* and *p2xE*).

Vertebrate P2X receptors are cell surface cation selective ion channels gated by extracellular ATP and consist of seven distinct subtypes ($P2X_{1-7}$), which assemble as functional homo- or hetero-trimeric channels. Vertebrate P2X receptors play a fundamental role in a wide array of physiological processes including neurotransmission, smooth muscle contraction, immune cell function, bone formation and platelet aggregation [6,7].

Recently *Dictyostelium p2xA* has been shown to produce a cell surface ATP-gated ion channel when exogenously expressed in the human embryonic kidney cell line HEK293 [8]. The native P2XA protein in *Dictyostelium* however was found to have an exclusively intracellular localisation being present on the contractile vacuole membrane where it plays a role in osmoregulation [8]. It is not clear therefore whether *Dictyostelium* utilises P2 receptors in the cytoplasmic membrane to sense extracellular nucleotides in a manner analogous to purinergic signalling in vertebrates.

In addition to ionotropic P2X receptors, vertebrate purinergic signalling is also mediated by metabotropic P2Y receptors. Unlike the P2X channels, P2Y receptors are 7TM G-protein coupled receptors and have a wider agonist profile responding to purines, pyrimidines and UDP—glucose [9]. Eight P2Y receptors have been characterised in mammals ($P2Y_{1,2,4,6,11-14}$) [9—12] and these couple via G_q/G_{11} to phospholipase C-β ($P2Y_{1,2,4,6}$ and $_{11}$), via G_i/G_o to inhibit adenyl cyclase ($P2Y_{12,13}$ and $_{14}$) and to G_s to stimulate adenyl cyclase ($P2Y_{11}$). Whilst the literature has tended not to classify the *Dictyostelium* cAMP receptors as P2 receptors, it could be argued that these correspond to *Dictyostelium* P2Y receptors since they are 7TM receptors activated by an extracellular purine ligand (cAMP). It is therefore possible that some of the orphan *Dictyostelium* 7TM receptors are activated by extracellular nucleotides other than cAMP.

Early studies performed prior to the discovery of P2 receptors demonstrated that extracellular ATP could enhance chemoattractant-mediated cell aggregation in *Dictyostelium* [13,14] and also increase basal uptake of $^{45}Ca^{2+}$ [15]. However, these studies were performed on the basis of ATP being a substrate for extracellular kinases rather than a signalling molecule *per se* making interpretation of the results with respect to P2 receptor function unclear.

In light of these early studies and the more recent finding that the *Dictyostelium p2xA* gene codes for an intracellular protein [8] we sought to establish whether *Dictyostelium* possess cell surface P2 receptors for extracellular nucleotides. Vertebrate P2X receptors are permeable to Ca^{2+} [16] as are *Schistosoma mansoni* P2X [17] and *Dictyostelium p2xA* [8]. Activation of $P2Y_{1,2,4,6\text{ and }11}$ receptors also result in an increase in intracellular Ca^{2+} via activation of inositol 1,3,5-trisphosphate receptors leading to release of Ca^{2+} from intracellular stores. We therefore assessed P2 receptor function in *Dictyostelium* by utilising an apoaequorin expressing strain [18] to detect extracellular nucleotide-evoked increase in intracellular Ca^{2+} levels. Using this technique, we clearly demonstrate the presence of cell surface P2 receptors for extracellular ATP and ADP in *Dictyostelium*.

Materials and methods

Materials

Benzyl coelenterazine (h-CTZ) was from Lux Biotechnology. Adenosine 5′-triphosphate (ATP) magnesium salt, adenosine 5′-diphosphate (ADP), adenosine monophosphate (AMP), uridine 5′-triphosphate (UTP), adenosine, α,β-methyleneadenosine 5′-triphoshate (αβ-MeATP), 2′(3′)-*O*-(4-benzoylbenzoyl)adenosine 5′-triphosphate (BzATP), 2-(methylthio)adenosine 5′-triphosphate (2-MeSATP), suramin, pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) (PPADS), apyrase and ATP bioluminescent assay kit were from Sigma. Pluronic F-127 was from Invitrogen. α-[^{32}P]-dCTP was from Amersham.

Dictyostelium transformation

Cells were harvested from axenic medium, washed twice in ice-cold H_{50} buffer (20 mM HEPES, 50 mM KCl, 10 mM NaCl, 1 mM $MgSO_4$, 5 mM $NaHCO_3$, 1 mM $NaH_2PO_4{\cdot}2H_2O$, pH 7.0), resuspended at 5×10^7 cells/ml in H_{50} buffer and 105 μl electroporated (two pulses of 0.75 kV at 25 μF, Bio-Rad Genepulser) with 15 μg of pPROF120 plasmid [18] in a 1 mm cuvette. Transformant selection at 30 μg/ml G418 commenced after 24 h.

p2xA and *p2xE* gene disruption

The *p2xA*$^-$ and *p2xE*$^-$ strains were produced in Ax2 by homologous recombination using disruption vectors based on pLPBLP [19,20] (pDT23 and p2XEKO, respectively). Flanking homology regions were generated by polymerase chain reaction (PCR) with the following primer pairs: *p2xA* 5′-flanking region (1846 bp) forward primer: 5′-TAAAGGGCC CAACTTCATTGTCTCATTCTAGTCATAGTAATCCAATTG-3′; reverse primer: 5′-TGAAGTCGACCTAAACGTCTGTCTCTAATTCTTACAATCTTTACTGTAG-3′. *p2xA* 3′-flanking region (1678 bp) forward primer: 5′-ATGAACTAGTGAATAACAACTG ATGAAGGTGAAGATGTTTTATATAC-3′; reverse primer: 5′-GGGGCGGCCGCTACAAAATAAAATCAAAAATGACAATAACAACTATTTAAC-3′. *p2xE* 5′-flanking region (990 bp) forward primer: 5′-ACCTGGGCCCAAAGACTCATTATTTTCC-3′; reverse primer: 5′-ACCTAAGCTTTCTATTTTTGCAATTAATAATTAC-3′. *p2xE* 3′-flanking region (1526 bp) forward primer: 5′-AGTATGCGGCCGCTATGCCCCAAAGAAGTAG-3′ reverse primer: 5′-TCACCCCGCGGTCACCATTAACGAAACC-3′). Flanking homology regions were cloned on either side of the blasticidin S resistance cassette via the exogenous restriction enodnuclease sites introduced by the primers during amplification (*p2xA*: 5′ApaI—SalI and 3′ SpeI—NotI, *p2xE*: 5′ ApaI—HindIII and 3′ NotI—SacII). Disruption cassettes were liberated as ApaI/NotI and ApaI/SacII fragments, for *p2xA*$^-$ and *p2xE*$^-$, respectively, and 15 μg used to transform Ax2 amoebae. Two independent null strains were isolated from 96-well tissue culture plates in axenic medium [21], supplemented with vitamins (0.1 mg/l B_{12}, 0.02 mg/l biotin and 0.2 mg/l riboflavin) plus 10 μg/ml blasticidin S as the selective agent. *p2xA* and *p2xE* gene disruptants among the many blasticidin resistant clones were identified by PCR, using oligonucleotides located outside the disruption cassette and within the blasticidin resistance cassette, with South-