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P2 receptor-mediated signal transduction in Ehrlich ascites tumor cells

Stine Falsig Pedersen *, Susanne Pedersen, Ian Henry Lambert, Else Kay Hoffmann

August Krogh Institute, Department of Biochemistry, Universitetsparken 13, University of Copenhagen, DK-2100 Copenhagen Ø, Denmark

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Abstract

The mechanisms, by which the P2 receptor agonists adenosine 5′-triphosphate (ATP) and uridine 5′-triphosphate (UTP) evoke an increase in the free cytosolic calcium concentration ($[Ca^{2+}]_i$) and in intracellular pH (pH_i), have been investigated in Ehrlich ascites tumor cells. The increase in $[Ca^{2+}]_i$ evoked by ATP or UTP is abolished after depletion of intracellular Ca^{2+} stores with thapsigargin in Ca^{2+}-free medium, and is inhibited by U73122, an inhibitor of phospholipase C (PLC), indicating that the increase in $[Ca^{2+}]_i$ is primarily due to release from intracellular, Ins(1,4,5)P_3-sensitive Ca^{2+} stores. ATP also activates a capacitative Ca^{2+}-entry pathway. ATP as well as UTP evokes a biphasic change in pH_i, consisting of an initial acidification followed by alkalinization. Suramin and 4,4′-diisothiocyano-2,2′-stilbene-disulfonic acid (DIDS) inhibit the biphasic change in pH_i, apparently by acting as antagonists at P2 receptors. The alkalinization evoked by the P2 receptor agonists is found to be due to activation of a 5′-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA)-sensitive Na^+/H^+ exchanger. ATP and UTP elicit rapid cell shrinkage, presumably due to activation of Ca^{2+} sensitive K^+ and Cl^- efflux pathways. Preventing cell shrinkage, either by incubating the cells at high extracellular K^+ concentration, or by adding the K^+-channel blocker, charybdotoxin, does not affect the increase in $[Ca^{2+}]_i$, but abolishes the activation of the Na^+/H^+ exchanger, indicating that activation of the Na^+/H^+ exchanger is secondary to the Ca^{2+}-induced cell shrinkage. 0005-2736/98/$ – see front matter © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Adenosine 5′-triphosphate; Uridine 5′-triphosphate; $[Ca^{2+}]_i$; Phospholipase C; U73122; pH_i regulation; Na^+/H^+ exchanger

1. Introduction

Extracellular adenosine 5′-triphosphate (ATP) is involved in the regulation of numerous biological processes, including neurotransmission, transepithelial chloride secretion, cell proliferation and apoptosis [1–5]. Furthermore, recent reports have assigned important roles for both ATP and uridine 5′-triphosphate (UTP) as autocrine messengers in a diverse range of cell types under various conditions of stress, including mechanical and osmotic stresses [6–10]. Extracellular ATP exerts its effects via two major receptor families, the P2X receptors, which are ligand gated ion channels, and the P2Y receptors, which are G protein-coupled [2,11]. It is well established that in most tissues studied, P2Y receptor stimulation leads to the activation of an inositol phospholipid-specific phospholipase C (PLC), leading to the formation of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) [1,2,11]. Ins(1,4,5)P_3 elicits release of Ca^{2+} from Ins(1,4,5)P_3-sensitive stores, which is in many cells accompanied by Ca^{2+} influx ascribed to the capacitative Ca^{2+} entry (CCE) pathway [1,2,13–16]. Moreover, P2 receptor

* Corresponding author. Fax: +45-35-32-15-67;
E-mail: sfpedersen@aki.ku.dk

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stimulation has been shown to activate multiple protein kinases, including protein kinase C (PKC), and the mitogen activated protein (MAP) kinase cascades [1,10,17], as well as several different phospholipases [1,12,18,19].

In Ehrlich ascites tumor cells, micromolar concentrations of ATP cause PtdIns(4,5)P_2 hydrolysis, increase cellular levels of Ins(1,4,5)P_3 and phosphatidic acid (PA), and elicit an increase in $[Ca^{2+}]_i$ [21,22]. We have recently reported that the Ehrlich cells possess $P2Y_1$- and $P2Y_2$ receptors, but probably not P2X receptors [6]. ATP acting via P2Y receptors has been found to activate several types of ion channels, including Ca^{2+}-dependent K^+ channels in Ehrlich cells [6,23] and in many other cell types [13,24,25], as well as both Ca^{2+}-dependent [24,25] and -independent [26] Cl^- channels. Although simultaneous activation of K^+ and Cl^- channels is expected to lead to cell shrinkage, the effect of ATP on cell volume in Ehrlich cells is unclear, i.e., no effect [21] or cell shrinkage [22] has been reported previously. It may also be noted that acceleration of the regulatory volume decrease (RVD) response after addition of ATP has recently been described in Ehrlich cells [23].

In several cell types including Ehrlich cells, ATP has been shown to exert a biphasic effect on pH_i, consisting of an initial acidification, followed by alkalinization to above the initial steady state pH_i [27–30]. This is not universally observed, thus, in various other cells, only acidification [31], only alkalinization [32], or no effect on pH_i [33] is seen upon addition of ATP. In rat cardiac myocytes [29,34] and rabbit osteoclasts [31], ATP-induced acidification is suggested to reflect activation of a Cl^-/HCO_3^- exchanger. In Ehrlich cells, the nature of the initial acidification has not been studied, whereas the subsequent alkalinization has previously been shown to reflect the activation of a Na^+/H^+ exchanger [27]. The signal transduction pathway involved was not elucidated, although a partial dependence on PKC was suggested.

In some cell types, activation of a Na^+/H^+ exchanger by other Ca^{2+} mobilizing agonists has been suggested to be a consequence of Ca^{2+}-induced cell shrinkage [35–38]. However, for the ATP-induced activation of the Na^+/H^+ exchanger, this possibility has not previously been investigated.

The homeostasis of $[Ca^{2+}]_i$, pH_i, and cell volume is tightly interrelated, and minor modulations of these parameters may be part of the signaling cascades triggering such diverse events as apoptosis or proliferation (see e.g. Ref. [39]). In the present study, we investigate the consequences of P2 receptor stimulation in Ehrlich cells with respect to $[Ca^{2+}]_i$, pH_i, and cell volume. The aim of the study was to elucidate the mechanisms of, and causal relationship between, these changes, in order to gain new insight into how P2 receptor agonists may exert their diverse cellular functions.

A part of this investigation has previously been presented in abstract form [40].

2. Materials and methods

2.1. Cell suspensions and incubation media

The Ehrlich ascites tumor cells (hyperdiploid strain) were maintained in NMRI mice by weekly intraperitoneal transplantation. The cells in the ascites fluid were harvested in standard incubation medium containing heparin (2.5 IU/ml), washed 2–3 times by centrifugation ($700 \times g$, 45 s) in standard medium without heparin, resuspended at a cytocrit of 4% and preincubated for 15–30 min before initiation of experiments (for details, see Ref. [41]). The standard incubation medium was of the following composition (mM): 150 Na^+, 5 K^+, 154 Cl^-, 1 Mg^{2+}, 1 Ca^{2+}, 1 SO_4^{2-}, 1 PO_4^{3-}, 3,3,3-(*N*-morpholino)propanesulfonic acid (MOPS), 3,3-*N*-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES); 3,3-*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes); pH was adjusted to 7.4 with NaOH. In Ca^{2+}-free medium, addition of Ca^{2+} was omitted and ethylene glycol-bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA, 2 mM) was added. In 'high $[K^+]$'-media, 48 mM KCl was substituted for 48 mM NaCl, to yield a total $[K^+]$ of 53 mM. In KCl media, NaCl was substituted by KCl in equimolar amounts. In these media, pH_o was adjusted with KOH. In HCO_3^- media, 25 mM $NaHCO_3$ was substituted for 25 mM NaCl. In experiments performed at pH_o 8.2, MOPS, TES and Hepes were replaced by 5 mM *N,N*-bis(2-hydroxymethyl)glycine (Bicine) and 5 mM *N*-tris(hy-

droxymethyl)methylglycine (Tricine). Unless otherwise indicated, experiments were carried out at 37°C.

2.2. Measurement of pH_i

Loading of the Ehrlich cells with 2′,7′-bis-(2-carboxyethyl)-5,6-carboxyfluorescein, tetraacetoxymethyl ester (BCECF-AM) and measurement of pH_i was performed essentially as previously described [42]. Briefly, cell suspensions were incubated with BCECF-AM (3.6 μM) at 37°C for 30 min, followed by two washes in standard medium containing bovine serum albumin, fraction V (BSA) (1 mg/ml) and resuspension in this medium at a cytocrit of 8%. Following dilution of the BCECF-loaded cell suspension to a cytocrit of 0.3%, pH_i was estimated using a Perkin–Elmer LS-5 luminescence spectrophotometer. Emission was measured at 525 nm after excitation at 445 nm and 495 nm (band width 5 nm). Extracellular fluorescence (comprising 10–20% of total fluorescence) was subtracted prior to calculation of the 445 nm/495 nm ratio. Linear in situ calibration of the BCECF fluorescence signal to pH_i was performed after each experiment, according to the nigericin method [43]. Three to four different values of pH_o were used to construct a linear calibration curve ($r > 0.98$). The compounds used (EIPA, thapsigargin, DIDS, suramin) were found to have no, or only insignificant, intrinsic fluorescence at the settings used.

2.3. Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was assessed in cell suspensions using the Ca^{2+}-sensitive fluorescent probe fura-2, according to [44]. Briefly, cell suspensions were incubated at 37°C for 20 min with fura-2-AM (2 μM), washed once in standard medium containing 0.2% BSA, and once in the experimental medium, and resuspended in this medium at a cytocrit of 5%. Following dilution of the fura-2 loaded cell suspensions to a cytocrit of 0.5%, $[Ca^{2+}]_i$ was estimated using a Perkin–Elmer LS-5 luminescence spectrophotometer. The excitation wavelengths were 340 nm and 380 nm, emission was measured at 510 nm (band width 5 nm). Extracellular fluorescence (15–25% of total fluorescence) was subtracted from the intensities measured after excitation at each wavelength before calculation of the 340 nm/380 nm ratio. The fura-2 signal was calibrated in vitro according to Ref. [45], as previously described for Ehrlich cells in Ref. [44]. A new calibration was performed after any alterations in the experimental setup.

2.4. Measurement of cell volume

Cell volume was measured by electronic cell sizing using a Coulter Multisizer II (Coulter, Luton, Beds, UK), after dilution of the cells to a cytocrit of 0.016% in the standard medium. The tube orifice was 100 μm. Mean cell volume was calculated as the median of the cell volume distribution curves after calibration with latex beads (diameter 14.1 μm, Coulter). Media used for volume measurements were filtered (Millipore filters, 0.45 μm) prior to use.

2.5. Reagents

Unless otherwise indicated, all reagents were analytical grade and obtained from Sigma (St. Louis, MO, USA). 5′-(*N*-ethyl-*N*-isopropyl)amiloride, hydrochloride (EIPA), BCECF-AM, fura-2-AM and fura-2-P (pentapotassium salt) were purchased from Molecular Probes (Leiden, Netherlands). Thapsigargin and charybdotoxin were from Alamone Labs, Jerusalem, Israel. Heparin was from Løvens Kemiske (Ballerup, Denmark). 1-(6-(17b-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1*H*-pyrrole-2,5-dione (U73122) and 1-(6-(17b-3-metoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-pyrrolidine-dione (U73343) were from Biomol Research Laboratories (Plymouth, PA, USA), and were dissolved at 10 mM in chloroform. Suramin was from Research Biochemicals (MA, USA), and was dissolved at 100 mM in double distilled water and kept refrigerated until use. Adenosine, ATP and UTP were prepared as 10.7 mM stock solutions in standard medium containing 0.1 mM EGTA (free concentration of nucleotides calculated using EQCal (Biotool, 1988) and assuming the same affinity for Ca^{2+} and Mg^{2+} for all nucleotides. Charybdotoxin (5 μM) was dissolved in standard medium containing 0.1% BSA. 4,4′-Diisothiocyano-2,2′-stilbene-disulfonic acid (DIDS) was dissolved at 10.7 mM in double distilled water. Nigericin (1 mg/ml) and thapsigargin (1 mM) were dissolved in 96% ethanol. BCECF-AM (1.2 mM) and fura-2-AM (1 mM) were dissolved in anhydrous dimethylsulfox-

ide (DMSO). Unless otherwise noted, all these reagents were kept at −20°C until use. The maximal final vehicle concentrations were: DMSO 0.5%, chloroform 0.1%, ethanol 0.2%. At these concentrations, the experiments were unaffected by these compounds (data not shown).

2.6. Data analysis and statistics

The rate of change in pH_i was calculated as the slope of the linear fit of the measurements obtained 0–60 s after the maximal acidification, during which time the change in pH_i can be considered linear ($r > 0.98$). EC_{50} values for the effects of ATP and UTP on pH_i were obtained from fits to the Michaelis–Menten equation. EC_{50} values for inhibition of the ATP- and UTP-induced changes in pH_i by DIDS (Fig. 4) were calculated using the equation $y = y_{max} \cdot (1 - 1/(1 + EC_{50}/[DIDS]))$, where y_{max} is the initial acidification, or the rate of alkalinization, respectively, and y is the corresponding value at any given concentration of DIDS ([DIDS]).

Results are presented as means ± S.E.M. with the number of independent experiments in parentheses. Single experiments shown are representative of at least three individual experiments. Significance was tested using a Student's t-test (level of significance $P < 0.05$).

3. Results

3.1. Sources of the increase in $[Ca^{2+}]_i$ induced by the P2 receptor agonists

As seen in Fig. 1, stimulation of Ehrlich ascites tumor cells suspended in standard experimental medium (1 mM Ca^{2+}) with ATP (15 μM) elicited a rapid increase in the free cytosolic calcium concentration ($[Ca^{2+}]_i$), followed by a lag phase of elevated $[Ca^{2+}]_i$. The mean increase in $[Ca^{2+}]_i$ was 202 ± 52 nM ($n = 12$). When ATP (15 μM) was added to cells preincubated in Ca^{2+}-free medium (2 mM EGTA), the increase in $[Ca^{2+}]_i$ was significantly reduced, to 123 ± 56 nM ($P < 0.02$, $n = 13$). As seen, UTP (15 μM) also elicited a transient increase in $[Ca^{2+}]_i$. The mean UTP-induced increase in $[Ca^{2+}]_i$ was 207 ± 21 nM ($n = 4$) in standard experimental medium, and 143 ± 44 nM ($n = 3$) in Ca^{2+} free medium, not significantly lower than that seen in standard medium.

The role of phospholipase C (PLC) in the signaling events leading to the increase in $[Ca^{2+}]_i$ induced by P2 receptor stimulation was studied using the PLC inhibitor U73122, and the inactive analog, U73343 [46,47]. After preincubation of the cells in standard medium with the inactive analog, U73343 (10 μM), the increase in $[Ca^{2+}]_i$ induced by 15 μM ATP was 242 ± 19 nM ($n = 4$), not significantly different from the increase seen after addition of 15 μM ATP to

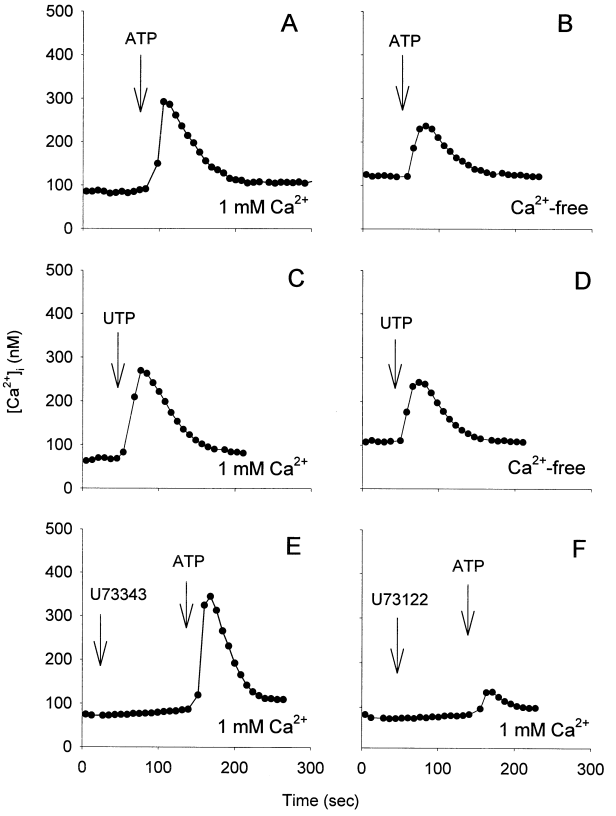

Fig. 1. Contributions of Ca^{2+} release and Ca^{2+} influx to the increase in $[Ca^{2+}]_i$ elicited by ATP and UTP. $[Ca^{2+}]_i$ was measured spectrophotometrically in fura-2-loaded Ehrlich cells suspended at a cytocrit of 0.5% in standard medium (A, C, E, F) or in Ca^{2+}-free medium containing 2 mM EGTA (B and D). (A, B) ATP (15 μM) was added at the time indicated by arrow ($n = 12$ in standard medium, $n = 13$ in Ca^{2+}-free medium). (C, D) UTP (15 μM) was added at the time indicated by arrow ($n = 4$ in standard medium, $n = 3$ in Ca^{2+}-free medium). (E, F) The PLC-inhibitor, U73122 (10 μM, $n = 4$, $P < 0.001$), or the inactive analogue, U73343 (10 μM, $n = 4$), was added 2 min prior to stimulation of the cells with ATP. Similar results were obtained with UTP ($n = 2$–3, data not shown).

control cells. In contrast, after preincubation with U73122 (10 μM), the increase in $[Ca^{2+}]_i$ induced by 15 μM ATP was reduced by 82%, to 43 ± 19 nM ($n = 4$), which was significantly lower than that observed in cells preincubated with U73343 ($P < 0.001$) and that observed in control cells ($P < 0.001$) (Fig. 1E,F). A similar pattern was observed following stimulation with 15 μM UTP ($n = 3$, data not shown).

Thapsigargin (TG) is a sesquiterpene lactone well known to inhibit the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) responsible for the reuptake of Ca^{2+} into the $Ins(1,4,5)P_3$ sensitive stores. Thapsigargin application therefore results in Ca^{2+} depletion of these stores [48]. As seen in Fig. 2A, in Ca^{2+}-free medium (2 mM EGTA), addition of TG (500 nM) leads to a slow increase in $[Ca^{2+}]_i$, representing discharge of $Ins(1,4,5)P_3$-sensitive stores, followed by a decrease, which is due to the subsequent extrusion of Ca^{2+} across the plasma membrane. Upon a subsequent addition of ATP, the increase in $[Ca^{2+}]_i$ was essentially abolished, reinforcing the notion that ATP-induced Ca^{2+} release occurs primarily from $Ins(1,4,5)P_3$-sensitive stores.

In contrast to the transient increase in $[Ca^{2+}]_i$ seen in Ca^{2+}-free medium (2 mM EGTA), treatment of cells suspended in standard medium (1 mM Ca^{2+}) with TG (500 nM) resulted in a sustained elevation of $[Ca^{2+}]_i$ (Fig. 2B,C). This has been described in many cell types, including Ehrlich cells, and reflects activation of Ca^{2+} influx from the extracellular medium as a consequence of the depletion of $Ins(1,4,5)P_3$-sensitive stores by TG [49,50]. The effect of a subsequent stimulation with ATP (1 μM) was found to be dependent on the time elapsed between TG treatment and stimulation. Thus, if ATP was added shortly (3–4 min, Fig. 2B) after TG treatment, a transient increase in $[Ca^{2+}]_i$ was seen, which was possibly due to release of Ca^{2+} from stores which had not yet been depleted, and which was followed by a rapid decrease below the level prior to addition of ATP (Fig. 2B). In contrast, if a longer time (6 min and above) had elapsed after TG treatment, presumably resulting in effective depletion of the $Ins(1,4,5)P_3$-sensitive Ca^{2+} stores, a subsequent addition of ATP lead to a transient decrease in $[Ca^{2+}]_i$ (Fig. 2C). The mechanisms involved in the ATP-evoked decrease in $[Ca^{2+}]_i$ were not further investigated here, but several possibilities can be envisaged (see Section 4).

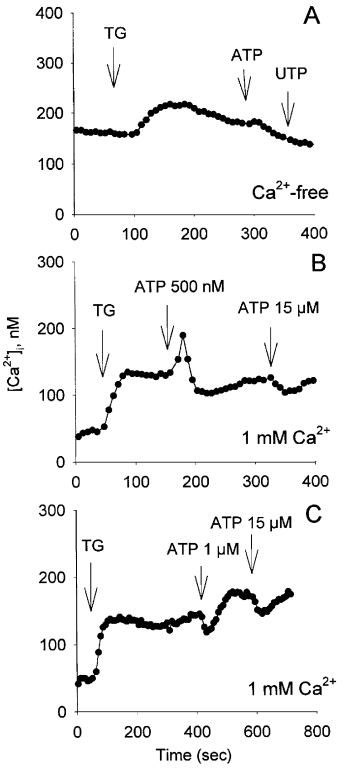

Fig. 2. Effects of ATP on $[Ca^{2+}]_i$ in thapsigargin-treated cells. $[Ca^{2+}]_i$ was measured spectrophotometrically in fura-2-loaded Ehrlich cells suspended at a cytocrit of 0.5% in Ca^{2+} free medium containing 2 mM EGTA (A) or in standard experimental medium (B and C). (A) Thapsigargin (TG, 500 nM) was added as indicated by the arrow, followed by addition of 15 μM ATP and 15 μM UTP, as indicated. The experiment is representative of three independent experiments. (B, C) TG (500 nM) was added as indicated by the arrow, followed by addition of ATP at the concentrations indicated. The experiments shown are representative of seven (B) and three (C) independent experiments.

3.2. Effect of ATP and UTP on pH_i

Addition of ATP (15 μM) elicited a transient intracellular acidification, followed by alkalinization. The initial ATP-induced acidification and the subsequent alkalinization were inhibited by 68% and 73%, respectively, in the presence of suramin (100 μM), a non-specific inhibitor of P2-purinoceptors [51] (Fig. 3, Table 1). As seen in Table 1, the changes in pH_i induced by UTP were of similar magnitudes as those induced by ATP, but were more potently inhibited by suramin. Thus, suramin inhibited the initial UTP-

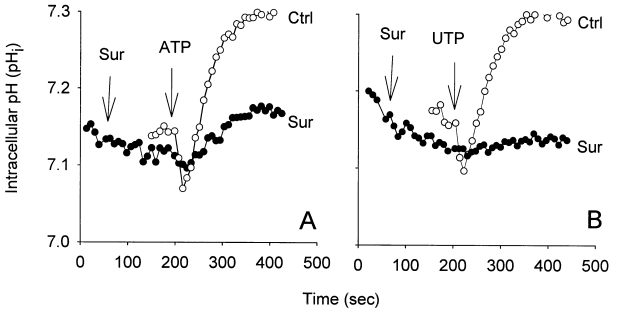

Fig. 3. ATP and UTP elicit a suramin-sensitive, biphasic change in pH_i. pH_i was measured spectrophotometrically in BCECF-loaded Ehrlich cells suspended at a cytocrit of 0.3% in the standard experimental medium. 15 μM ATP (A) or UTP (B) was added at the time indicated by arrow, in control cells (open circles) or in the presence of suramin (100 μM), added 2 min prior to addition of the agonists. The traces shown are representative of 3–5 experiments for each condition, and are summarized in Table 1.

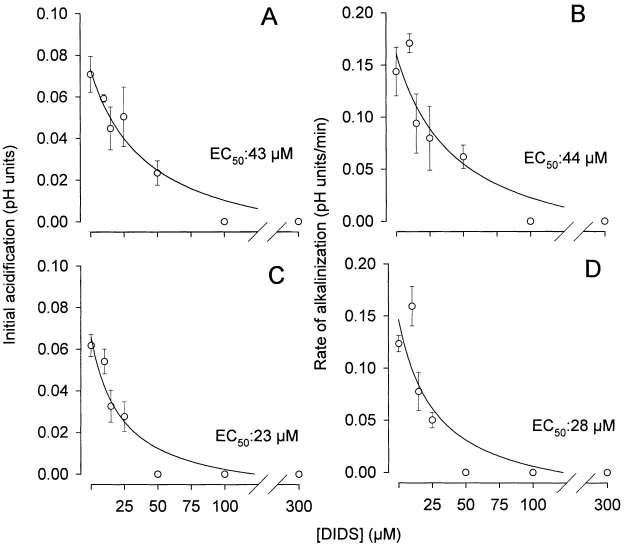

Fig. 4. Inhibition of ATP- and UTP-induced changes in pH_i by DIDS. pH_i was measured spectrophotometrically in BCECF-loaded Ehrlich cells suspended at a cytocrit of 0.3% in the standard experimental medium. DIDS (10–300 μM) was added 1 min prior to addition of 15 μM ATP (A and B) or UTP (C and D). EC_{50} values are calculated using the equation $y = y_{max} \cdot (1 - 1/(1 + EC_{50}/[DIDS]))$, where y_{max} is the initial acidification (A and C) or the rate of alkalinization (B and D), respectively, and y is the corresponding value at any given concentration of DIDS ([DIDS]). The figures shown are based on 3–5 independent experiments at each concentration.

induced acidification by 84%, and the subsequent alkalinization by 94%, respectively. In contrast, the changes in pH_i induced by a higher dose of ATP (100 μM) were unaffected by suramin (Table 1), indicating that suramin is a competitive inhibitor of P2Y receptors in Ehrlich cells. Furthermore, although other mechanisms cannot be excluded, these results suggest that the overall affinity for ATP at P2Y receptors in Ehrlich cells is higher than for UTP.

In some cells, the initial, ATP-induced acidification has been suggested to reflect the activation of a Cl^-/HCO_3^- exchanger [29]. In Ehrlich cells, the ATP-induced acidification was transient and of similar magnitude in control cells and in the presence of EIPA to inhibit the Na^+/H^+ exchanger ($n = 4$, data not shown). Moreover, this acidification was not augmented under conditions at which the activity of the Cl^-/HCO_3^- exchanger is known to be readily detectable in these cells (pH_o 8.2, 25 mM HCO_3^-, see Ref. [54]) ($n = 2$, data not shown). These findings argue against the notion that P2 receptor agonists

Table 1
Effect of suramin on the ATP- and UTP-evoked changes in pH_i

	ATP, 15 μM		ATP, 100 μM		UTP, 15 μM	
	Initial pH_i decrease[a]	Rate of pH_i increase[b]	Initial pH_i decrease	Rate of pH_i increase	Initial pH_i decrease	Rate of pH_i increase
Control	0.056 ± 0.005 (4)	0.14 ± 0.017 (4)	0.070 ± 0.005 (4)	0.10 ± 0.013 (4)	0.056 ± 0.0045 (5)	0.130 ± 0.005 (5)
Suramin, 100 μM	0.018 ± 0.0091 (3)	0.04 ± 0.017 (3)	0.070 ± 0.019 (3)	0.10 ± 0.026 (3)	0.009 ± 0.0002 (3)	0.008 ± 0.0037 (3)
Percent inhibition	68	73	0	0	84	94

The experimental protocol was as described in the legend to Fig. 3. The ATP- and UTP-induced acidification was calculated as the total decrease in pH_i following addition of the agonist. The ATP- and UTP-induced alkalinization was calculated as the slope of the linear fit of the measurements obtained 0–60 s after the maximal acidification, during which time the change in pH_i can be considered linear ($r > 0.98$). The number of experiments is given in parenthesis.

[a] In pH units.

[b] In pH units min^{-1}.

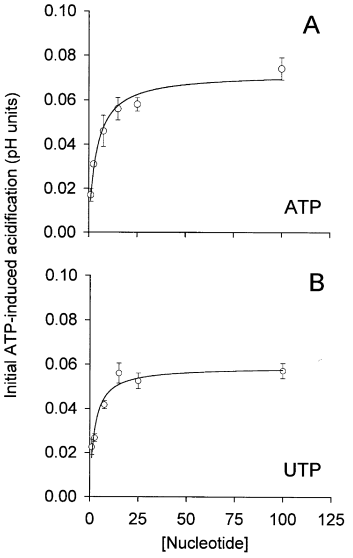

Fig. 5. Concentration-dependency of the ATP- and UTP-induced acidification. pH_i was measured spectrophotometrically in BCECF-loaded Ehrlich cells suspended at a cytocrit of 0.3% in the standard experimental medium. The graphs show the total intracellular acidification induced by various concentrations (1–100 μM) of ATP (A) or UTP (B). The acidification was calculated as the difference between the steady state pH_i prior to, and the minimum pH_i reached after addition of ATP or UTP (see Fig. 3). The EC_{50} value and the maximal acidification induced were estimated by fitting the data to the Michaelis–Menten equation. The following values were obtained. ATP: EC_{50} 3.8 μM, maximal acidification 0.07 pH units; UTP: EC_{50} 2.6 μM, maximal acidification 0.06 pH units. The graphs shown are based on data from 3–5 independent experiments for each condition.

activate a Cl^-/HCO_3^- exchanger in these cells, and indicate that other mechanisms are involved in the ATP-induced acidification (see Section 4).

The stilbene derivative 4,4′-diisothiocyano-2,2′-stilbene-disulfonic acid (DIDS) is commonly used as an inhibitor of Cl^-/HCO_3^- exchangers [52]. However, DIDS has been reported to be a competitive antagonist at P2 receptors [53], and hence unfit for evaluating possible effects of P2 agonists on Cl^-/HCO_3^- exchange. As seen in Fig. 4, DIDS inhibited the initial acidification following addition of ATP or UTP (15 μM), as well as the subsequent alkalinization, in a concentration-dependent manner. The EC_{50} values for the DIDS-induced inhibition of ATP-induced acidification and alkalinization were estimated at 43 and 44 μM, respectively (Fig. 4A,B), while the corresponding values for the UTP-induced responses were 23 and 28 μM, respectively (Fig. 4C,D). These latter results cannot be explained by an inhibitory effect of DIDS on a Cl^-/HCO_3^- exchanger, but are a strong indication that this compound is an inhibitor of P2Y receptors, or at least of an early step in the signal transduction cascade. Furthermore, they confirm the above observation that UTP has a lower overall affinity at P2 receptors in these cells than does ATP. The concentration–response relationships of the ATP- and UTP-induced initial acidification are shown in Fig. 5. As seen, the acidification induced by both P2 receptor agonists is well fitted by simple Michaelis–Menten kinetics, and the responses were similar, although, at any given concentration, the acidification induced by UTP appears to be slightly lower than that induced by ATP.

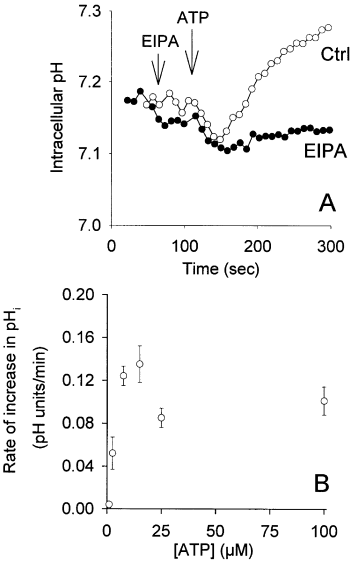

Fig. 6. Activation of an EIPA-sensitive Na^+/H^+ exchanger by ATP. pH_i was measured spectrophotometrically in BCECF-loaded Ehrlich cells suspended at a cytocrit of 0.3% in the standard experimental medium (see Section 2). (A) ATP (15 μM) was added at the time indicated by the arrow, in control experiments (open circles), or in the presence of EIPA (10 μM, closed circles). The experiment shown is representative of four (control) and three (EIPA) experiments, respectively. (B) Concentration-dependency of the ATP-induced activation of the Na^+/H^+ exchanger. The experimental protocol was as in A. The ATP concentration required for half-maximal effect was estimated at about 3 μM ($n=3$–4 at each concentration). Similar experiments with UTP resulted in an estimated half-maximal concentration of about 5.5 μM ($n=3$–5 at each concentration).

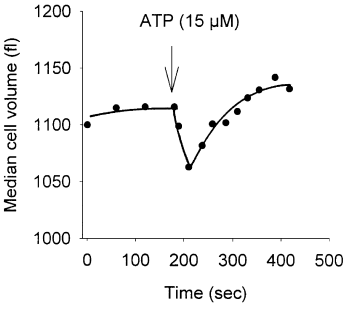

Fig. 7. Effect of ATP on cell volume. Cell volume was measured by electronic cell sizing using a Coulter Multisizer II after dilution of the cells to a cytocrit of 0.016% in the standard medium. ATP (15 μM) was added at the time indicated by the arrow. The median cell volume was calculated as the median of the cell volume distribution curves after calibration with latex beads. The ATP-induced cell shrinkage was 10 ± 3% ($n = 5$) of the normal cell volume (measured at the time of maximal cell shrinkage, approximately 30 s after stimulation). Similar results were obtained with UTP (15 μM, $n = 2$).

3.3. ATP and UTP activate an EIPA-sensitive Na^+/H^+ exchanger

Fig. 6A shows that the P2 receptor-mediated intracellular alkalinization was abolished in the presence of EIPA (10 μM), suggesting that it represents activation of the NHE-1 or NHE-2 isoform of Na^+/H^+ exchangers (see Ref. [35]). This extends the previous observation [27] of activation of amiloride-sensitive Na^+/H^+ exchange by ATP in these cells. Fig. 6B shows the rate of intracellular alkalinization, reflecting the activation of the Na^+/H^+ exchanger, as a function of the concentration of ATP. As seen, the rate of intracellular alkalinization increased with increasing ATP concentration up to about 15 μM ATP, with a half-maximal effect estimated at about 3 μM. At ATP concentrations above 15 μM, a slight decrease in the rate of alkalinization was seen, which was not further investigated in the present study. Similar results were found for UTP; however, the UTP concentration required for half-maximal effect was somewhat higher, estimated at about 5.5 μM ($n = 3$–4 at each concentration, data not shown).

3.4. Role of cell shrinkage in the P2 receptor-mediated activation of Na^+/H^+ exchange

In Ca^{2+}-free medium (2 mM EGTA) and in the presence of TG, the ATP-induced, biphasic change in pH_i was completely abolished ($n = 3$, data not shown). This indicates that the increase in $[Ca^{2+}]_i$, which is also abolished under these conditions (Fig. 2A), is required at some step in the signal transduction mechanism leading to activation of the Na^+/H^+ exchanger. Activation of K^+ and Cl^- efflux by other Ca^{2+} mobilizing agonists has previously been shown to elicit cell shrinkage in these cells [55]. As seen in Fig. 7, stimulation of Ehrlich cells in standard medium with ATP (15 μM) induced a rapid cell shrinkage (10 ± 3% of the original cell volume within 30 s ($n = 5$)), followed by rapid volume recovery. Similar results were obtained with UTP (15 μM, $n = 2$, data not shown).

Cell shrinkage is a potent stimulus for activation of the Na^+/H^+ exchanger in Ehrlich cells [38,42] as well as in many other cell types [35]. We therefore

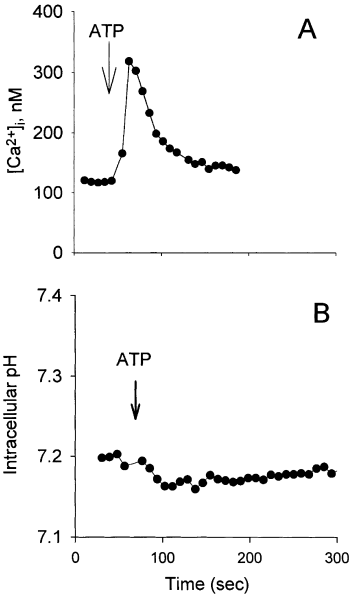

Fig. 8. Effect of preventing cell shrinkage on the ATP-evoked changes in $[Ca^{2+}]_i$ and pH_i. (A) $[Ca^{2+}]_i$ was measured spectrophotometrically in fura-2-loaded Ehrlich cells suspended at a cytocrit of 0.5% in 'high K^+' medium (53 mM K^+, see Section 2). ATP (15 μM) was added as indicated by the arrow. The increase in $[Ca^{2+}]_i$ is not significantly inhibited in cells suspended in 'high K^+' medium as compared to cells suspended in standard experimental medium ($n = 3$ and 12, respectively) (B) Cellular pH (pH_i) was measured spectrophotometrically in BCECF-loaded Ehrlich cells suspended at a cytocrit of 0.3% in the standard experimental medium. ATP (15 μM) was added as indicated by the arrow ($n = 3$). It has previously been shown that cell shrinkage induced by Ca^{2+} mobilizing agonists is prevented in the 'high K^+' medium [56].

investigated the role of cell shrinkage in the P2 receptor-mediated activation of the exchanger, taking advantage of the fact that K^+ loss, and concomitant cell shrinkage have previously been shown to be exactly prevented at 53 mM extracellular K^+ in these cells [56]. As seen in Fig. 8A, when the Ehrlich cells were incubated at this $[K^+]_o$, the increase in $[Ca^{2+}]_i$ induced by stimulation with ATP (15 μM) was 231 ± 10 nM ($n = 3$), which is not significantly different from that seen in standard medium (compare Fig. 1A, 202 ± 52 nM, $n = 12$). Similar results were obtained with UTP ($n = 3$, data not shown). The lack of effect of high $[K^+]_o$ on the ATP-induced increase in $[Ca^{2+}]_i$ was to be expected, since the increase in $[Ca^{2+}]_i$ was primarily due to release of Ca^{2+} from intracellular stores, and hence was little affected by the altered driving force for Ca^{2+} influx (Figs. 1 and 2). In contrast, under these conditions, stimulation with ATP (15 μM) did not activate the Na^+/H^+ exchanger. In the 'high $[K^+]$' medium, it can be calculated that the driving force for the Na^+/H^+ exchanger is reduced by about 16%. In order to rule out the possibility that the inhibition of the exchanger under these conditions reflects the reduction in driving force, an alternative approach was employed. In Ehrlich cells, the blocker of Ca^{2+}-activated K^+ channels, charybdotoxin (ChTX, 100 nM), completely inhibits ATP-induced K^+ efflux [23], and furthermore inhibits K^+ loss (and hence, cell shrinkage) induced by Ca^{2+} mobilizing agonists by about 70% [57]. The ATP-induced increase in $[Ca^{2+}]_i$ was not inhibited by ChTX [6]; however, in the presence of ChTX (100 nM), the ATP-induced activation of the Na^+/H^+ exchanger was strongly attenuated (data not shown). Taken together, these observations are in agreement with the hypothesis that the activation of the Na^+/H^+ exchanger by P2 receptor agonists is secondary to the cell shrinkage induced by an increase in $[Ca^{2+}]_i$.

Finally, it should be noted that pH_i is unaffected by adenosine and isoproterenol, indicating that activation of the exchanger does not reflect extracellular breakdown of ATP and stimulation of P1 receptors (data not shown). This is supported by the finding that activation of the exchanger is inhibited by suramin, which is not an inhibitor at P1 receptors (see e.g. Ref. [22]), and is a particularly important point in transformed cells such as Ehrlich cells, which exhibit unusually high ecto-ATPase and ectonucleotidase activity [1,20].

4. Discussion

4.1. Mechanisms of the P2 receptor mediated increase in $[Ca^{2+}]_i$

The present study investigates the cellular events initiated via P2 receptor activation in Ehrlich ascites tumor cells. Stimulation with ATP (15 μM) or UTP (15 μM) elicits an increase in $[Ca^{2+}]_i$, of which only the ATP-induced increase is significantly reduced in Ca^{2+}-free medium. The increase in $[Ca^{2+}]_i$ induced by both P2Y receptor agonists is mainly mediated via activation of a PLC, followed by release of Ca^{2+} from $Ins(1,4,5)P_3$-sensitive stores. Similar results have been reported in many other cell types [1,25,59]. The activation of PLC by P2Y receptor activation has been reported to be either pertussis toxin-insensitive (G_q mediated), or at least partly pertussis toxin-sensitive (mediated via βγ subunits from G_i proteins) (see Refs. [11,58]). In contrast, in rat cardiac myocytes the increase in $[Ca^{2+}]_i$ induced by ATP is suggested to be secondary to intracellular acidification [60]. This can be excluded in the present study, since the ATP-induced acidification is abolished when the increase in $[Ca^{2+}]_i$ is prevented (Fig. 2A).

Regarding the nature of the ATP-activated Ca^{2+} influx pathway, it is interesting to note that while about 40% of the ATP-induced increase in $[Ca^{2+}]_i$ under control conditions is due to Ca^{2+} influx (compare Fig. 1A and B), the residual increase in $[Ca^{2+}]_i$ in cells treated with the PLC inhibitor, U73122, is less than 20% of that observed under control conditions. Thus, most of the Ca^{2+} influx appears to be dependent on an increase in $Ins(1,4,5)P_3$, suggesting that depletion of $Ins(1,4,5)P_3$-sensitive Ca^{2+} stores by ATP stimulates capacitative Ca^{2+} entry (CCE) in these cells, in agreement with findings from many other cell types [12–15]. The nature of the CCE pathway(s) is essentially obscure, although this phenomenon is usually observed following stimulation with TG [49,62,63,66].

The origin of the residual increase in $[Ca^{2+}]_i$ seen in the presence of U73122 was not further investi-

gated. However, it may be noted that this could be a nonspecific effect of this U73122, because it has been reported that this compound may in itself cause an increase in $[Ca^{2+}]_i$, via effects not related to inhibition of PLC [61].

When the Ehrlich cells are preincubated with TG long enough (about 6 min) to presumably completely deplete Ins(1,4,5)P_3-sensitive Ca^{2+} stores and fully activate CCE, ATP does not elicit a further increase in $[Ca^{2+}]_i$, even in the presence of extracellular Ca^{2+} (Fig. 2C). This supports the notion that the main Ca^{2+} entry pathway is CCE, which is already maximally activated under these conditions, and is also in congruence with our recent finding that P2X receptors are not present in these cells [6].

A notable finding is the decrease in $[Ca^{2+}]_i$ in TG-treated cells upon a subsequent stimulation with ATP (Fig. 2). This is only seen in the presence of extracellular Ca^{2+}, indicating that in these cells, ATP may inhibit the Ca^{2+} influx already activated by TG. In CFPAC-1 epithelial cells, stimulation with ATP also induced a decrease in $[Ca^{2+}]_i$; however, in those cells, this effect was shown to be due to stimulation of Ca^{2+} efflux [64]. The mechanism involved in the apparent inhibition of CCE by ATP under these conditions awaits further investigation, but several putative schemes can be proposed. It could be a consequence of ATP-induced cell shrinkage, since cell shrinkage has been found to inhibit Ca^{2+} entry in Ehrlich cells [38]. This would require that K^+ and/or Cl^- currents are still activated by ATP in the absence of a bulk increase in $[Ca^{2+}]_i$. In this regard it may be noted that Ca^{2+}-independent activation of Cl^- currents by ATP has been reported [26]. The apparent inhibition of Ca^{2+} entry by ATP could also reflect phosphorylation-dependent events, since CCE is found to be inactivated by PKC in many cell types [15,63,65]. TG does not elicit a measurable increase in the cellular level of Ins(1,4,5)P_3 (see Ref. [49]), whereas ATP is known to increase Ins(1,4,5)P_3 levels in Ehrlich cells, and thus is expected to activate a PKC [19]. Consequently, it may be speculated that in TG treated cells stimulation with ATP, in concert with the already elevated $[Ca^{2+}]_i$, leads to a substantial activation of PKC, resulting in inhibition of CCE. Whatever the mechanism, inhibition of CCE by ATP could provide an important feedback regulation of $[Ca^{2+}]_i$ following stimulation with this agonist.

4.2. ATP and UTP elicit a biphasic change in pH_i, which is inhibited by suramin and DIDS

As reported previously, ATP and UTP both elicit a biphasic change in pH_i in Ehrlich cells [27]. Suramin is the most widely used P2 receptor antagonist, although it has a range of other biological activities [see [22,67]]. This drug inhibits the biphasic effect on pH_i elicited by a moderate (15 μM), but not by a higher (100 μM), concentration of ATP, suggesting that suramin is a competitive inhibitor of P2Y receptors in Ehrlich cells. Changes in pH_i induced by UTP are inhibited to a greater extent than those induced by ATP (Fig. 3, Table 1). Similarly, the increase in $[Ca^{2+}]_i$ elicited by a low concentration (10 μM) of ATP in these cells is abolished by suramin [6]. Our results are in agreement with a recent study in which suramin is reported to be a predominantly competitive inhibitor at P2X and P2Y receptors [51]. DIDS, a stilbene derivative commonly used as an inhibitor of anion transport [52], was also found to inhibit the ATP- and UTP-induced changes in pH_i. This is in agreement with findings in renal pigment epithelial cell [68], as well as with a recent report indicating that DIDS is a competitive inhibitor at P2Y receptors in the rat vas deferens [53].

Similar to suramin, DIDS is a more potent inhibitor of UTP-mediated (EC_{50} about 25 μM) than ATP-mediated (EC_{50} about 44 μM) effects on pH_i. This is in good agreement with the higher EC_{50} value for the UTP-evoked, compared to the ATP-evoked, increase in $[Ca^{2+}]_i$, and is also consistent with our recent report of two main receptor populations in Ehrlich cells: a $P2Y_1$ receptor activated preferentially by ATP and 2-methylthioadenosine 5′-triphosphate (2-MeSATP), and a $P2Y_2$ receptor activated preferentially by UTP and ATP [6].

As discussed in Section 3.2, the initial acidification induced by P2 receptor stimulation is unlikely to be due to activation of a Cl^-/HCO_3^- exchanger. Other possible mechanisms which could account for a cytoplasmic acidification following stimulation with Ca^{2+} mobilizing agonists such as ATP and UTP include: (i) competition for intracellular buffers by H^+ and Ca^{2+}, (ii) H^+ countertransport by mitochondrial-, endoplasmic reticulum- and plasma membrane Ca^{2+}-ATPases [69], (iii) a Ca^{2+}-mediated increase in metabolic acid production, and (iv) hyperpolariza-

tion-induced H^+ influx across the plasma membrane [70]. The latter possibility is in agreement with our finding that the activation of K^+ channels by ATP results in a significant hyperpolarization in these cells [6].

4.3. Activation of a Na^+/H^+ exchanger by ATP is secondary to Ca^{2+}-induced cell shrinkage

In the present study, P2 receptor activation is found to elicit transient cell shrinkage under isotonic conditions, at least partly due to the activation of a ChTX-sensitive, Ca^{2+} dependent K^+ current by ATP [6,23]. The nature of the Cl^- current which must also be activated by ATP in order to elicit net loss of KCl and concomitant cell shrinkage has not been directly demonstrated (for a discussion, see Ref. [6]). However, substantial whole cell Cl^- currents are known to be activated after small increases in $[Ca^{2+}]_i$ in these cells [71].

Activation of the ubiquitous NHE-1 isoform of the Na^+/H^+ exchangers by osmotic cell shrinkage and by growth factors are known to share several similarities, although both pathways are incompletely understood [35]. The present study demonstrates that P2 receptor-mediated activation of the exchanger can be inhibited in the presence of a maximal increase in $[Ca^{2+}]_i$, by preventing the agonist-induced cell shrinkage (Fig. 8). Thus, it is suggested that activation of the Na^+/H^+ exchanger is preferentially mediated by the ATP-induced cell shrinkage, and that the role of Ca^{2+} in the activation process is to elicit cell shrinkage. Similar results were obtained after stimulation with bradykinin in transformed NIH-3T3 fibroblasts [37], with carbachol in rat acinar cells [36], and with thrombin and bradykinin in Ehrlich cells [38], suggesting that it may be a general mechanism involved in the activation of the exchanger by many types of growth factors, mitogens and neurotransmitters. It may be noted that the present findings are compatible with the previously proposed partial role for PKC in the ATP-induced activation of the exchanger in these cells [27]. Thus, in Ehrlich cells, PKC is activated within 1 min of osmotic cell shrinkage [72], and shrinkage-induced activation of the exchanger appears to be at least partly dependent on a PKC isoform [42]. In other cell types, the activation of a Na^+/H^+ exchanger by ATP has been reported to be either Ca^{2+}- and PKC-dependent, and pertussis toxin-insensitive [28]; PKC dependent [30]; Ca^{2+}- and PKC-independent [34]; or PKC-independent, partially Ca^{2+}-dependent, and pertussis toxin-sensitive [32].

4.4. Functional significance of P2 receptors in Ehrlich cells

We have recently reported that in Ehrlich cells, ATP is released by mechanical stress [6], and accelerates the RVD process [23], suggesting that nucleotides may play a role as autocrine messengers during RVD in these cells.

The present study suggests an important role for cell volume in the signal transduction events between ATP-induced changes in $[Ca^{2+}]_i$ and activation of a Na^+/H^+ exchanger and concomitant elevation of pH_i. Cell shrinkage is known to inhibit Ca^{2+} entry in Ehrlich cells [38], and in HT_{29} cells [73]. Thus, it may be speculated that the ATP-evoked cell shrinkage and intracellular alkalinization could play important roles in the feedback regulation of Ca^{2+} signaling events. Moreover, in rat pancreatic acinar cells [74], alkaline pH_i reduces agonist-induced release of $[Ca^{2+}]_i$. Finally, given the numerous cellular processes known to be regulated by pH_i (see e.g. Ref. [75]), the ATP-evoked intracellular alkalinization is likely to be important in mediating cellular effects evoked following stimulation of P2 receptors.

In conclusion, in Ehrlich cells, ATP and UTP activate release of Ca^{2+} from $Ins(1,4,5)P_3$ sensitive stores, and ATP also activates Ca^{2+} entry from the extracellular medium, by a mechanism which is mainly dependent on PLC. In contrast, in TG-treated cells in Ca^{2+}-containing medium, ATP evokes a transient decrease in $[Ca^{2+}]_i$, possibly due to inhibition of CCE. ATP and UTP elicit an initial decrease in pH_i, followed by an increase, both of which are inhibited by suramin and by DIDS. The P2 receptor-mediated increase in pH_i is due to activation of a Na^+/H^+ exchanger, by a mechanism which appears to be mainly secondary to the ATP-induced cell shrinkage elicited by activation of Ca^{2+}-dependent K^+ and Cl^- channels.

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