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Mechanical stress induces release of ATP from Ehrlich ascites tumor cells

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Abstract

The supernatant from a suspension of Ehrlich cells exposed to centrifugation at $700 \times g$ for 45 s induced a transient increase in the intracellular concentration of free, cytosolic Ca^{2+}, $[Ca^{2+}]_i$, as well as activation of an outwardly rectifying whole-cell current when added to a suspension of non-stimulated cells. These effects were inhibited by suramin, a non-specific P2 receptor antagonist, and mimicked by ATP. Reversed phase HPLC analysis revealed that the supernatant from Ehrlich cells exposed to centrifugation contained 2.6 ± 0.2 μM ATP, and that the mechanical stress-induced release of ATP was inhibited by glibenclamide and verapamil, non-specific inhibitors of the cystic fibrosis transmembrane conductance regulator and P-glycoprotein, respectively. After trypan blue staining, less than 0.5% of the cells were unable to extrude the dye. Addition of extracellular ATP induced a suramin-sensitive, transient, concentration-dependent increase in $[Ca^{2+}]_i$, activation of an outwardly rectifying whole-cell current and a hyperpolarization of the plasma membrane. The ATP-induced hyperpolarization of the plasma membrane was strongly inhibited in the presence of charybdotoxin (ChTX), an inhibitor of several Ca^{2+}-activated K^+ channels, suggesting that stimulation of P2 receptors in Ehrlich cells evokes a Ca^{2+}-activated K^+ current. The relative potencies of several nucleotides (ATP, UTP, ADP, 2-MeSATP, α,β-MeATP, bzATP) in eliciting an increase in $[Ca^{2+}]_i$, as well as the effect of repetitive addition of nucleotides were investigated. The results lead us to conclude that mechanical stimulation of Ehrlich cells leads to release of ATP, which in turn stimulates both $P2Y_1$ and $P2Y_2$ receptors, resulting in Ca^{2+} influx as well as release and activation of an outwardly rectifying whole-cell current.

Keywords: Mechanical stress; ATP release; $[Ca^{2+}]_i$; Whole-cell patch clamp; P2Y receptor

Abbreviations: α,β-MeATP, α,β-methyleneadenosine 5′-triphosphate; ADO, adenosine; ADP, adenosine 5′-diphosphate; AM, acetoxymethyl ester; AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; BSA, bovine serum albumin; bzATP, 2′- and 3′-*O*-(4-benzoylbenzoyl)adenosine 5′-triphosphate; cAMP, cyclic adenosine 5′-monophosphate; ChTX, charybdotoxin; DIDS, 4,4′-diisothiocyano-2,2′-stilbene disulfonic acid; DMSO, dimethylsulfoxide; EGTA, ethylene-glycol-bis-β-amino-ethyl-(ether)*N*,*N*,*N*′,*N*′-tetraacetic acid; GTP, guanosine 5′-triphosphate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid; $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; MAP, mitogen-activated protein; 2-MeSATP, 2-methylthioadenosine 5′-triphosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PI-PLC, phospholipase C; PKC, protein kinase C; TES, *N*-tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid; UTP, uridine 5′-triphosphate

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1. Introduction

Extracellular ATP is involved in many physiological processes (see [1]). The role of ATP as an autocrine messenger upon exposure of endothelial cells to shear stress, e.g. by an increase in blood flow, is well known [2]. Release of ATP after exposure to various physiological and pathological stimuli has also been reported in other cell types, including an epithelial cell line [3] and rat hepatoma cells [4]. In addition, it has recently been demonstrated that human astrocytoma cells release UTP upon mechanical stimulation [5]. Accordingly, ATP and UTP are proposed to act as autocrine messengers (see [1,5]). The effects of extracellular nucleotides are mediated via specific cell surface receptors, the P2 receptors. The P2 receptors, which are highly responsive to adenosine 5′-triphosphate (ATP) as well as to other nucleotides, are subdivided into P2X and P2Y receptors based on their structure. P2X receptors are ligand-gated ion channels, whereas the P2Y receptors are G-protein coupled receptors containing seven transmembrane domains (see [6,7]). The family of P2 receptors is rapidly expanding, and at present seven different P2X receptor subtypes and seven, possibly eight, different subtypes of the P2Y receptor, have been identified and cloned [7]. Generally, $P2X_{1-6}$ receptors are characterized by their high affinity for α,β-methyleneATP (α,β-MeATP), whereas the pore-forming $P2X_7$ (P2Z) receptor is characterized by its sensitivity to extracellular benzoylATP (bzATP). The $P2Y_1$ receptors are, in most cell types, found to have a relatively high affinity for 2-methylthioATP (2-MeSATP) and adenosine 5′-diphosphate (ADP), whereas they are insensitive to uridine 5′-triphosphate (UTP). On the other hand, the $P2Y_2$ receptors have a much higher affinity for UTP than for 2-MeSATP and are, normally, not responsive to ADP [6,8].

In Ehrlich ascites tumor cells, it has previously been demonstrated that addition of extracellular ATP leads to a stimulation of the phospholipase C (PLC)/inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) signaling pathway, resulting in an increase in $[Ca^{2+}]_i$ [9–11] and cell shrinkage due to activation of Ca^{2+}-activated K^+ and Cl^- conductance pathways [11]. However, Dubyak and De Young [9] found no detectable effect on cell volume after addition of ATP. In the present study, we demonstrate that Ehrlich cells release ATP as an autocrine messenger during mechanical stimulation, and we furthermore characterize the P2 receptors involved in mediating the subsequent cellular responses.

A part of this investigation has been presented in an abstract form at the joint meeting of the Scandinavian and German Physiological Societies in Hamburg, 1998 [12].

2. Materials and methods

2.1. Cell suspension and incubation media

The Ehrlich ascites tumor cells (hyperdiploid strain) were maintained by weekly intraperitoneal transplantation in white NMRI mice (25–30 g). The cells were harvested from the mice one week after inoculation, as described in [13]. For the electrophysiological experiments and for measurements of $[Ca^{2+}]_i$ in single cells, the cells were suspended at a cytocrit of 0.2–0.5% in standard Krebs medium (in mM: 150 NaCl, 6 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, pH adjusted to 7.4 with NaOH), and for estimation of $[Ca^{2+}]_i$ in cell suspensions, the cells were suspended at a cytocrit of 4% in standard Ringer (in mM: 143 NaCl, 5 KCl, 1 $MgSO_4$, 1 Na_2HPO_4, 1 $CaCl_2$, 3.3 MOPS, 3.3 TES, 5 HEPES, pH adjusted to 7.4 with NaOH). In Ca^{2+}-free media, Ca^{2+} was omitted and 2 mM EGTA added. The internal pipette solution used for electrophysiological recordings in the voltage clamp mode was (in mM): 40 KCl, 100 K-aspartate, 2 $MgCl_2$, 4 Na_2ATP, 0.1 EGTA, 10 HEPES, pH adjusted to 7.2 with KOH, and in the current clamp mode (in mM): 40 KCl, 100 K-aspartate, 1 $MgCl_2$, 0.1 EGTA, 10 HEPES, pH adjusted to 7.2 with KOH. The electrophysiological experiments were executed at room temperature, whereas the measurements of $[Ca^{2+}]_i$ in cell suspensions were performed at 37°C.

2.2. Reagents and stock solutions

Unless otherwise indicated, all chemicals were purchased from Sigma (St. Louis, MO) and stored at −20°C. Fura-2-AM was obtained from Molecular

Probes (Leiden, The Netherlands) or from TEFLABS (Austin, TX) and prepared as 1 mM stock solution in dry DMSO. ADO, ATP, ADP, UTP, GTP, α,β-MeATP, 2-MeSATP and bzATP were prepared as 10.7 mM stock solutions in standard Ringer containing 0.1 mM EGTA. The free concentration of the nucleotides was estimated at 10.7 mM using EQCAL software (BioTool, 1988), assuming that the nucleotides bind Mg^{2+} and Ca^{2+} with the same potency. 2-MeSATP and suramin were purchased from Research Biochemicals International (MA, USA) and suramin was prepared as an aqueous stock solution at 100 mM and stored at 4°C. Charybdotoxin was prepared as a 50 μM aqueous stock solution. Nystatin was prepared as a stock solution (100 mg/ml) in DMSO. Isoproterenol was prepared as an aqueous stock solution at 10 mM.

2.3. Estimation of $[Ca^{2+}]_i$ using fura-2

2.3.1. In single cells

Loading of the Ehrlich cells with fura-2-AM and measurement of $[Ca^{2+}]_i$ in single cells during the electrophysiological experiments were performed as described in [14]. The ratio of the fluorescence emitted at 510 nm after excitation at 360 and 390 nm (ratio 360/390), respectively, was corrected for extracellular fluorescence and used as an estimate of $[Ca^{2+}]_i$.

The fura-2-loaded cells were diluted in standard Krebs medium on a poly-L-lysine coated cover glass, mounted on the stage of an inverted Zeiss Axiovert 100 microscope, superfused with standard Krebs medium to wash away extracellular fura-2-AM, and left for 2 min to allow the cells to settle. $[Ca^{2+}]_i$ was followed over time using a photomultiplier-based system, in which a filter wheel, amplifier and controller (Luigs and Neumann, Ratingen, Germany) and a photomultiplier tube (Hamamatsu, Tokyo, Japan) were connected to the inverted microscope.

2.3.2. In cell suspensions

Loading of the cells with fura-2-AM and measurement of $[Ca^{2+}]_i$ was conducted according to [15]. The fura-2 loaded cells were diluted to a cytocrit of 0.5% in the cuvette, and the fluorescence was measured using a Perkin-Elmer LS-5 Luminescence Spectrometer. The fluorescence emission, collected at 510 nm after excitation at 340 and 380 nm, respectively, and corrected for extracellular fluorescence, was calibrated in vitro as previously described [15], and $[Ca^{2+}]_i$ was calculated as described in [16].

2.4. Mechanical stimulation

Ehrlich cells at a cytocrit of 2% were centrifuged at $700 \times g$ for 45 s. Subsequently, the supernatant was transferred to a vial and used directly (within a few minutes).

2.5. Electrophysiological experiments

The whole-cell membrane current was measured according to the protocol described in [14]. The pipette resistance was 3–5 MΩ. The currents were monitored using an EPC-7 or an EPC-9 patch clamp amplifier (List Electronic, Germany) and sampled at 2 ms intervals. In voltage clamp experiments, linear voltage ramps from −100 to +100 mV were applied every 15 s. The whole-cell membrane potential (V_m) was measured applying the current clamp technique to patches perforated with nystatin (0.4 mg/ml). The liquid junction potential between the pipette and bath solutions was calculated at −12.5 and −11.9 mV for the voltage clamp and current clamp experiments, respectively (see [17]), using Axopatch software, and the recorded potentials were corrected accordingly.

2.6. Combined measurements of $[Ca^{2+}]_i$ and V_m or whole-cell current

In the experiments shown in Figs. 1–3, the measurements of $[Ca^{2+}]_i$ and the whole-cell recordings were performed simultaneously on the same cell.

2.7. Estimation of nucleotide content

2.7.1. ATP

Samples were deproteinized with 0.4 N perchloric acid and centrifuged ($15\,000 \times g$, 5 min) before neutralisation with 0.4 N KOH, and the ATP concentration was subsequently estimated by reversed phase high-pressure liquid chromatography (HPLC). Separation of nucleotides was performed on a Nucleosil column (C18, 4×250 mm, 5 μm particles), using a 100 mM KH_2PO_4 buffer (pH = 7.0) containing 9.5%

(v/v) methanol and 1.95 mM of the ionpairing agent tetrabutylammonium hydrogen sulphate as eluate [18] and a flow velocity of 1 ml/min. ATP was detected as the absorbance at 254 nm, and the absolute ATP content was estimated from chromatograms of a 20 μM ATP standard. The ATP retention time in samples was estimated using ATP as internal standard.

2.7.2. cAMP

The cellular cAMP content was detected by reversed phase high-pressure liquid chromatography using a C18-Superpac Sephasil column (4× 250 mm; 5 μm particles) as previously described [19].

2.8. Statistical evaluation

Unless otherwise indicated, the values are presented as the mean ± S.E.M. with the number of independent experiments indicated. Statistical significance was tested with a Student's *t*-test, and the significance level used was $P < 0.05$, unless noted otherwise.

3. Results

3.1. Release of nucleotides from Ehrlich cells after exposure to mechanical stress

Fig. 1A demonstrates that addition of 50 μl/ml supernatant from Ehrlich cells that have been centrifuged gently (700×*g*, 45 s), to non-stimulated Ehrlich cells leads to a transient increase in the intracellular concentration of free, cytosolic Ca^{2+}, $[Ca^{2+}]_i$, expressed as the ratio of fluorescence emission detected after excitation at 360 and 390 nm, respectively (see Section 2). Fig. 1 also demonstrates that addition of 50 μl/ml supernatant from mechanically stressed Ehrlich cells induced a transient in-

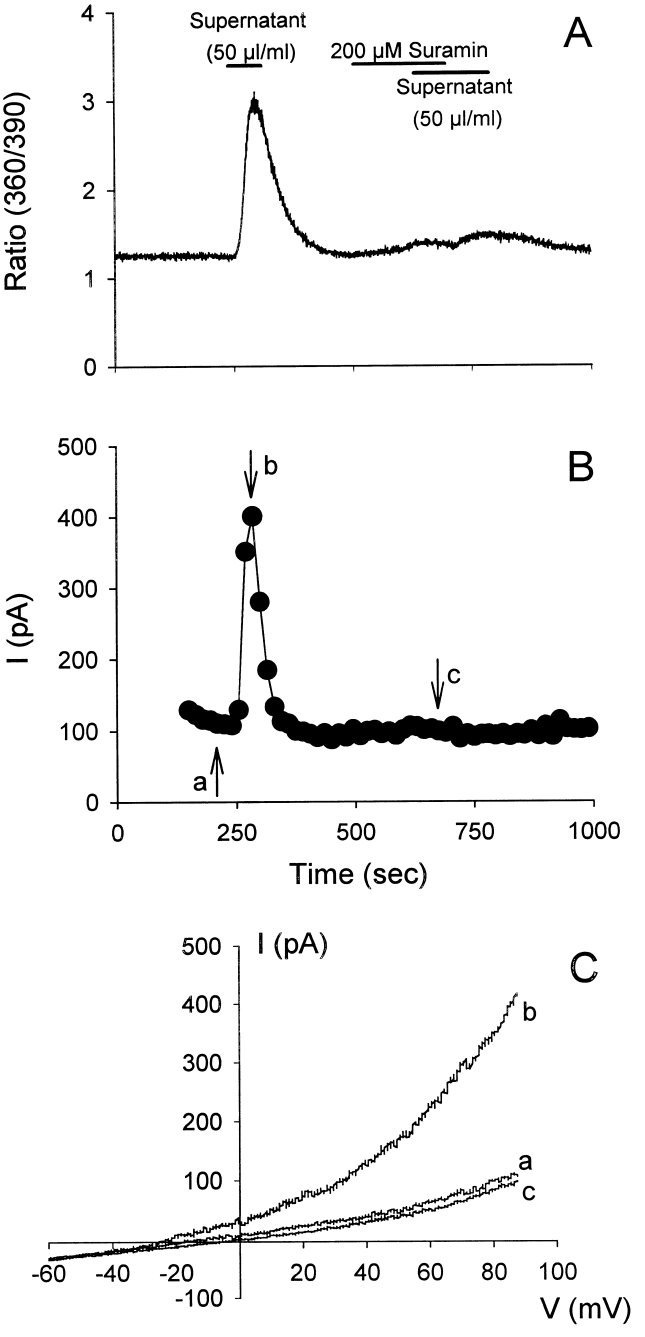

Fig. 1. Effect of the supernatant from mechanically stressed Ehrlich cells on $[Ca^{2+}]_i$ and whole-cell current in unperturbed cells. Ehrlich cells were suspended in Krebs medium, mounted on the microscope stage and subsequently superfused with either Krebs medium or Krebs medium containing 50 μl/ml supernatant from Ehrlich cells exposed to mechanical stress in the form of mild centrifugation (700×*g* for 45 s) and/or 200 μM suramin, as indicated by the bars. (A) The ratio of fluorescence emission from a single fura-2 loaded Ehrlich cell after 360 and 390 nm excitation, respectively, was followed over time. (B) The average whole-cell current at +100 mV as a function of time recorded from the same cell in the perforated patch configuration. (C) The reconstructed I-V curves for the whole-cell current recorded at the time points a, b and c, as indicated by the arrows in (B). E_K and E_{Cl} were estimated at −79 and −32 mV, respectively. The data represent four independent experiments.

Table 1
Release of ATP from Ehrlich cells exposed to mechanical stress

	Extracellular [ATP] (μM)	*P*
Control	2.6 ± 0.2	
Glibenclamide	0.7 ± 0.5	0.022
Verapamil	0.4 ± 0.2	0.004

Ehrlich cells (cyt 6%) were exposed to mechanical stress in the form of a mild centrifugation (700×*g*, 45 s) and an aliquot of the supernatant was used for estimation of the extracellular concentration of ATP. Glibenclamide (25 μM) or verapamil (30 μM) were added to the cell suspension 5 min prior to centrifugation. Values are given as the mean of four independent sets of experiments. *P* indicates the level of significance in a Student's paired *t*-test against the control values.

crease in the average whole-cell current recorded at +100 mV (Fig. 1B), activated an outwardly rectifying whole-cell current (Fig. 1C), and shifted the reversal potential (V_{rev}) from −6 ± 4 to −32 ± 6 mV ($n=4$; Fig. 1C). Since E_K is −79 mV and E_{Cl} is −32 mV, the observed shift in V_{rev} is likely to reflect an increase in K^+ as well as in Cl^- permeability and possibly co-activation of a non-selective cation channel reversing at more positive potentials, which has previously been described in Ehrlich cells [20]. These findings indicate that Ehrlich cells exposed to centrifugation release an autocrine substance, which mobilizes Ca^{2+} and activates a whole-cell current when added to unperturbed cells. As seen in Fig. 1, these effects were blocked in the presence of 200 μM suramin ($n=4$), a non-specific P2 receptor antagonist [21], indicating that the autocrine messenger is a nucleotide, presumably ATP or UTP, which exert its effect via P2 receptors. From Table 1 it is seen that Ehrlich cells exposed to mechanical stress indeed released ATP. The ATP content in the supernatant was estimated at 2.6 ± 0.2 μM by HPLC analysis, which would correspond to a concentration at 0.1–0.2 μM ATP in the experimental situation shown in Fig. 1. Furthermore, Table 1 demonstrates that the mechanical stress-induced release of ATP from Ehrlich cells was significantly inhibited by 25 μM glibenclamide as well as by 30 μM verapamil, inhibitors of the cystic fibrosis transmembrane conductance regulator (CFTR) and P-glycoprotein, respectively (see [22,23]). This indicates that the release of ATP from Ehrlich cells after exposure to mechanical stress is, indeed, due to activation of an as yet unidentified

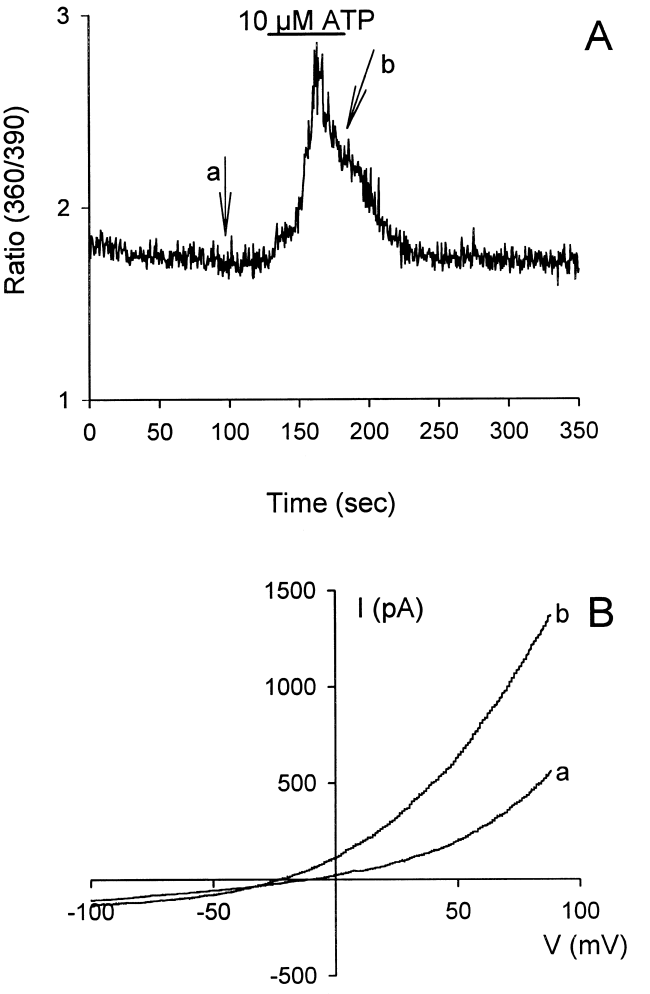

Fig. 2. The effect of ATP on $[Ca^{2+}]_i$ and whole-cell current. (A) $[Ca^{2+}]_i$ was followed over time in a single fura-2 loaded Ehrlich cell. The cell was superfused with either Krebs medium, or Krebs medium containing 10 μM ATP, as indicated in the figure. (B) The whole-cell current recorded from the same cell superfused with Krebs medium (a) or Krebs medium containing 10 μM ATP (b), at the time points indicated by the arrows in (A). E_K and E_{Cl} were calculated at −79 and −32 mV, respectively. The data represent three independent sets of experiments.

transport mechanism and not caused by simple cell lysis. This notion is further supported by the observation that less than 0.5% of the cells were stained blue after exposure to trypan blue (data not shown). In order to clarify whether the supernatant-induced effects could be ascribed to ATP-induced activation of P2 receptors, we investigated whether these effects could be mimicked by extracellularly applied ATP.

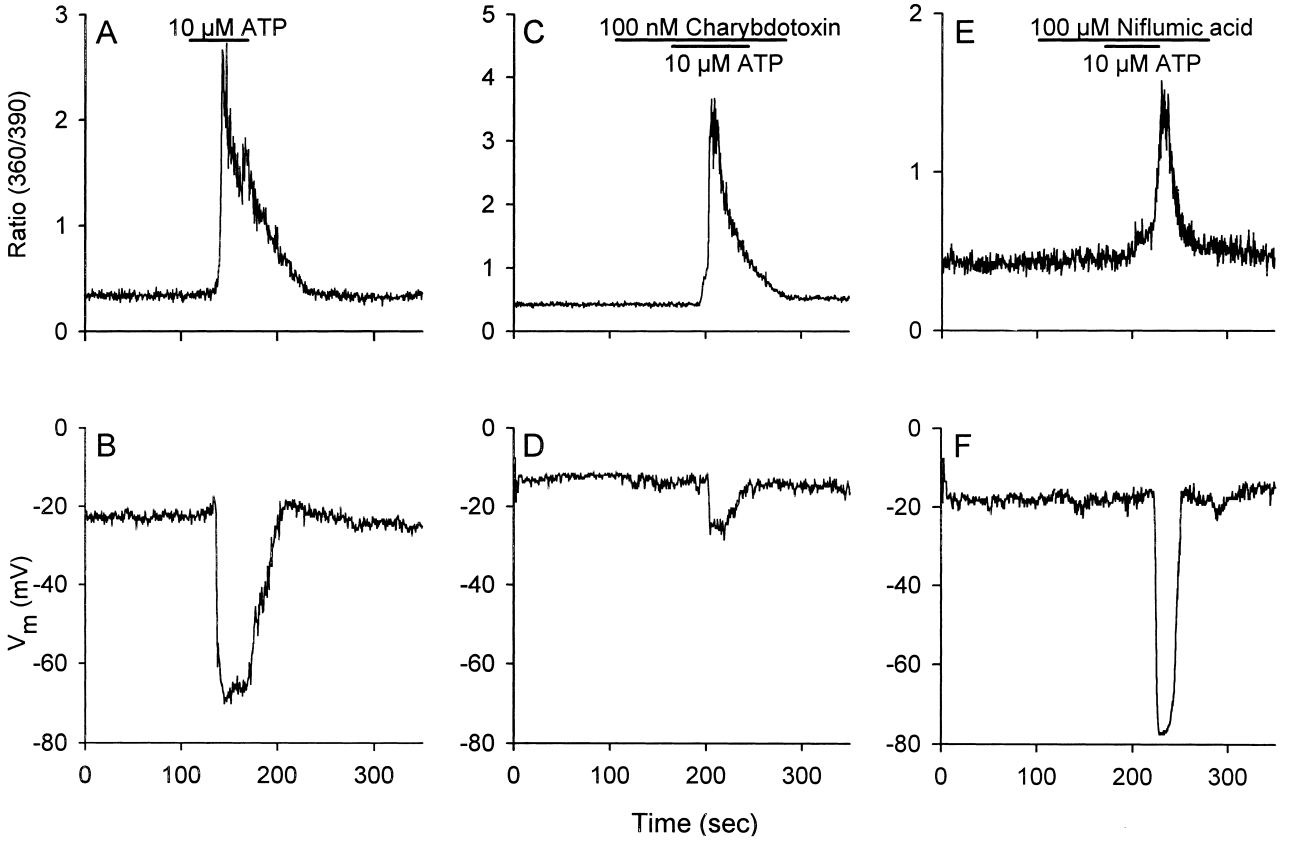

Fig. 3. The effect of ATP on $[Ca^{2+}]_i$ and V_m, and the sensitivity of these responses to ion channel blockers. Simultaneous measurements of $[Ca^{2+}]_i$ and V_m in cells suspended in Krebs medium. (A, C, E) The fluorescence ratio (360/390) as a function of time in a fura-2 loaded Ehrlich cell superfused with Krebs medium containing either 10 μM ATP (A), 10 μM ATP and 100 nM charybdotoxin (C) or 10 μM ATP and 100 μM niflumic acid (E), as indicated by the bars. (B, D, F) The corresponding traces of V_m recorded using the current clamp technique (see Section 2). At the times indicated by the bar, the cells were superfused with Krebs medium containing either 10 μM ATP (B), 10 μM ATP and 100 nM charybdotoxin (D) or 10 μM ATP and 100 μM niflumic acid (F). E_K and E_{Cl} were estimated at −79 and −34 mV, respectively. The traces in (C) and (D) represent four independent experiments, whereas the traces in (A), (B), (E) and (F) represent three independent experiments.

3.2. ATP-induced effects in the Ehrlich cells

Fig. 2 demonstrates that addition of 10 μM ATP to a single, fura-2 loaded Ehrlich cell induced a transient increase in $[Ca^{2+}]_i$ and evoked an outwardly rectifying whole-cell current, leading to a shift in the reversal potential from 0 ± 6 to -28.5 ± 5 mV ($n = 4$). This effect of ATP on $[Ca^{2+}]_i$ and whole-cell current indicates that P2 receptors are present in the Ehrlich cells. The ATP-induced effects were essentially similar to those induced by addition of supernatant from mechanically stressed cells (see Fig. 1).

From Fig. 3 it is seen that stimulation with 10 μM ATP leads to an increase in $[Ca^{2+}]_i$ (Fig. 3A) as well as to a hyperpolarization of the plasma membrane (Fig. 3B). The resting membrane potential in an unstimulated Ehrlich cell was estimated at -19.3 ± 2 mV ($n = 3$). The resting membrane potential in the Ehrlich cells was estimated at not very negative values (see Figs. 1–3) both in voltage clamp and current clamp experiments (see Section 2). This could be a consequence of a very positive equilibrium potential for Na^+, combined with the relatively high Na^+ conductance previously described in these cells [26]. Addition of 10 μM ATP to the Ehrlich cells resulted in a hyperpolarization of the plasma membrane of -34.7 ± 7 to a value of -54.7 ± 8 mV ($n = 3$), indicating that ATP increases the permeability for K^+ as well as for Cl^-, and apparently no co-activation of a

cation channel reversing at much more positive potentials as suggested in the description of Figs. 1 and 2.

Charybdotoxin (ChTX) inhibits various types of K^+ channels, including some Ca^{2+}-activated K^+ channels [27,28]. When an Ehrlich cell was exposed to 10 μM ATP in the presence of 100 nM ChTX, an increase in $[Ca^{2+}]_i$ was still induced (Fig. 3C), whereas the ATP-induced hyperpolarization was reduced to -8.9 ± 3 mV, resulting in a V_m of -23 ± 3 mV ($n = 4$; Fig. 3D). Although the ATP-induced hyperpolarization of the plasma membrane did not seem to be completely inhibited in the presence of ChTX (Fig. 3D), the V_m was not significantly different from the resting membrane potential under these conditions. This is taken to indicate that ChTX-sensitive, Ca^{2+}-activated K^+ channels are stimulated in the Ehrlich cells after addition of ATP, and that these channels are the major contributors to the ATP-induced hyperpolarization of the plasma membrane.

It has previously been demonstrated that niflumic acid inhibits Ca^{2+}-activated Cl^- channels in the Ehrlich cells [29] as well as in other cell types [30,31]. From Fig. 3E and F it is seen that even in the presence of 100 μM niflumic acid, addition of 10 μM ATP induced an increase in $[Ca^{2+}]_i$ as well as a hyperpolarization of the plasma membrane of -45.8 ± 10 mV, leading to a V_m of -63 ± 11 mV ($n = 3$), which is not significantly different from the control situation (compare with Fig. 3A and B). This indicates that the niflumic acid-sensitive Ca^{2+}-activated Cl^- channels are not activated upon stimulation of Ehrlich cells with ATP. However, the equilibrium potential for Cl^- (E_{Cl}) under the conditions used in Fig. 3 was estimated at -34 mV, suggesting that the minor hyperpolarization induced by ATP in the presence of 100 nM ChTX (Fig. 3D) reflects activation of a Cl^- current. This notion is further supported by the finding that addition of ATP to Ehrlich cells elicited cell shrinkage due to K^+ and Cl^- loss [32].

The ATP-induced increase in $[Ca^{2+}]_i$ in a population of Ehrlich cells (see Section 2) was concentration-dependent (Fig. 4). The maximal detectable increase in $[Ca^{2+}]_i$ after addition of ATP, $[Ca^{2+}]_{i,max}$, and the EC_{50}, i.e. the concentration of ATP needed in order to induce an increase in $[Ca^{2+}]_i$ half the size of $[Ca^{2+}]_{i,max}$, were estimated at 204 nM Ca^{2+} and 0.5 μM ATP, respectively, in Ca^{2+}-containing medium (see legend to Fig. 4). Stimulation of Ehrlich cells with ATP in Ca^{2+}-free medium (2 mM EGTA) also induced a concentration-dependent increase in $[Ca^{2+}]_i$ (Fig. 4), which was significantly smaller at all concentrations ($P < 0.04$) than that seen in standard medium (1 mM Ca^{2+}). In the absence of external Ca^{2+}, $[Ca^{2+}]_{i,max}$ and EC_{50} were estimated at 132 nM and 0.8 μM ATP, respectively. Since no external Ca^{2+} was present in this situation, any increase in $[Ca^{2+}]_i$ seen after addition of ATP must be due to release of Ca^{2+} from intracellular stores. Thus, addition of ATP to the Ehrlich cells leads to an influx of Ca^{2+} as well as a mobilization of Ca^{2+} from intracellular stores.

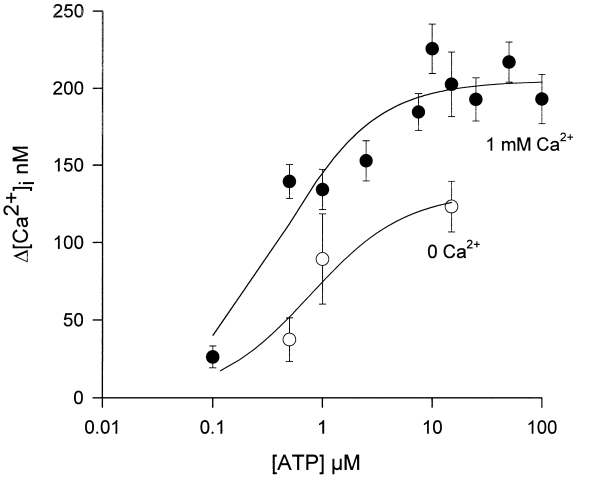

Fig. 4. Concentration-response curve for the ATP-induced increase in $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured in fura-2-loaded Ehrlich cells suspended in standard Ringer (see Section 2), and $\Delta[Ca^{2+}]_{i,max}$, i.e. the maximal increase in $[Ca^{2+}]_i$ detected after addition ATP, was determined after addition of increasing concentrations of ATP in the presence and absence of extracellular Ca^{2+}, respectively. The data were fitted to the Michaelis-Menten equation $(\Delta[Ca^{2+}]_i = (\Delta[Ca^{2+}]_{i,max} \cdot [ATP])/(EC_{50} + [ATP])$, where EC_{50} is the [ATP] needed to induce an increase in $[Ca^{2+}]_i$ half the size of $\Delta[Ca^{2+}]_{i,max}$. Each data point represents at least three independent experiments.

3.3. Receptor types in the Ehrlich cells and their agonist profiles

In order to determine the nature of the nucleotide receptor(s) in Ehrlich cells, the cells were exposed to various P1 and P2 receptor agonists. Addition of the P1 receptor agonist adenosine (ADO) in the concen-

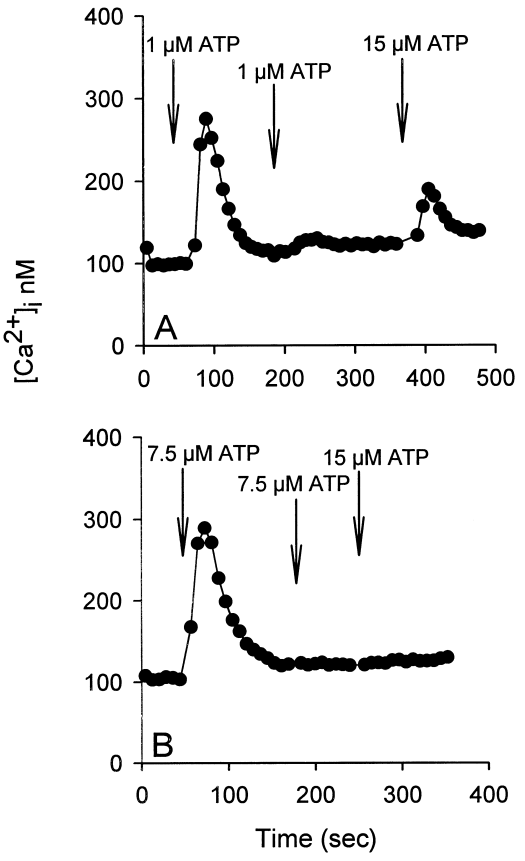

Fig. 5. The effect of successive additions of ATP on $[Ca^{2+}]_i$. Fura-2 loaded Ehrlich cells in suspension were exposed to repetitive stimulation with either sub-maximal and maximal concentrations of ATP (A), or only maximal ATP concentrations (B), as indicated by the arrow, and $[Ca^{2+}]_i$ was followed over time. The data represent five (A) and three (B) independent experiments, respectively.

tration range 1–100 μM, had no detectable effect on either $[Ca^{2+}]_i$ or intracellular pH, pH_i, or the intracellular level of cAMP ($n=3$; data not shown), indicating that receptors of the P1 type are not present in the Ehrlich cells. On the other hand, application of 10 μM ATP in the presence of 100 μM suramin, which acts as a non-specific inhibitor of the P2X and the P2Y receptors (see, e.g. [9,21]) completely abolished the 10 μM ATP-induced increase in $[Ca^{2+}]_i$ in the Ehrlich cells, as well as the ATP-induced hyperpolarization of the plasma membrane (data not shown). Thus, Ehrlich cells seem to possess P2X and/or P2Y receptors.

Table 2 illustrates the effect of several P2 receptor agonists on $[Ca^{2+}]_i$ in Ehrlich cells suspended in standard medium (1 mM Ca^{2+}). Generally, when agonists induced an increase in $[Ca^{2+}]_i$ it was found to be concentration-dependent, which allowed us to estimate the $[Ca^{2+}]_{i,max}$ and EC_{50} values for the agonist-induced Ca^{2+} mobilization.

From Table 2 it is seen the P2 receptors in Ehrlich cells have a higher affinity for ATP ($EC_{50}=0.5$ μM) compared to UTP ($EC_{50}=5.1$ μM) and ADP ($EC_{50}=391$ μM). $[Ca^{2+}]_{i,max}$ and EC_{50} values were not determined for the 2-MeSATP-induced increase in $[Ca^{2+}]_i$ since very high concentrations (>300 μM) were needed in order to detect any effect on $[Ca^{2+}]_i$. Furthermore, Table 2 shows that α,β-MeATP and bzATP had no detectable effect on $[Ca^{2+}]_i$ in the Ehrlich cells. Of the nucleotides and nucleotide analogues capable of inducing an increase in $[Ca^{2+}]_i$ in the Ehrlich cells (Table 2), ADP and 2-MeSATP are both agonists at the $P2Y_1$ receptor, whereas UTP is an agonist at the $P2Y_2$ receptor (see [6]), indicating that $P2Y_1$ as well as $P2Y_2$ receptors could be present in the Ehrlich cells.

α,β-MeATP is a general agonist at the $P2X_1$, $P2X_3$

Table 2
Effect of different nucleotides on $[Ca^{2+}]_i$ in the Ehrlich cells

Agonist	Effect on $[Ca^{2+}]_i$	EC_{50} (μM)	$\Delta[Ca^{2+}]_{i,max}$ (nM)
ATP	Increase	0.5 ± 0.1[a]	204 ± 8
UTP	Increase	5.1 ± 0.8	277 ± 12
ADP	Increase	391 ± 132	198 ± 23
2-MeSATP	Increase	>500[b]	177 ± 37[b,c]
α,β-MeATP	None detected		
bzATP	None detected		

Ehrlich cells were treated and $[Ca^{2+}]_i$ estimated as described in Section 2. The P2X receptor agonists α,β-MeATP (15–500 μM) and bzATP (15 μM–1 mM), each tried at three or more concentrations ($n=3$) in the range indicated, have no detectable effect on $[Ca^{2+}]_i$ in the Ehrlich cells. The P2Y receptor agonists UTP, ADP, 2-MeSATP as well as ATP itself, induced a dose-dependent increase in $[Ca^{2+}]_i$, which allowed estimation of $[Ca^{2+}]_{i,max}$ and EC_{50} as described in the legend to Fig. 4. The data was obtained from at least five different concentrations, and each data point represent three to thirteen experiments.

[a]All data points were fitted to the Michaelis-Menten equation, and the obtained values are given as ± asymptotic standard errors.

[b]2-MeSATP did not elicit any detectable increase in $[Ca^{2+}]_i$ in the concentration range of 15–300 μM. In the concentration range of 500–1000 μM there was a linear relationship between [2-MeSATP] and the induced increase in $[Ca^{2+}]_i$.

[c]The value indicated is the mean ± S.E.M. following stimulation with 1000 μM 2-MeSATP.

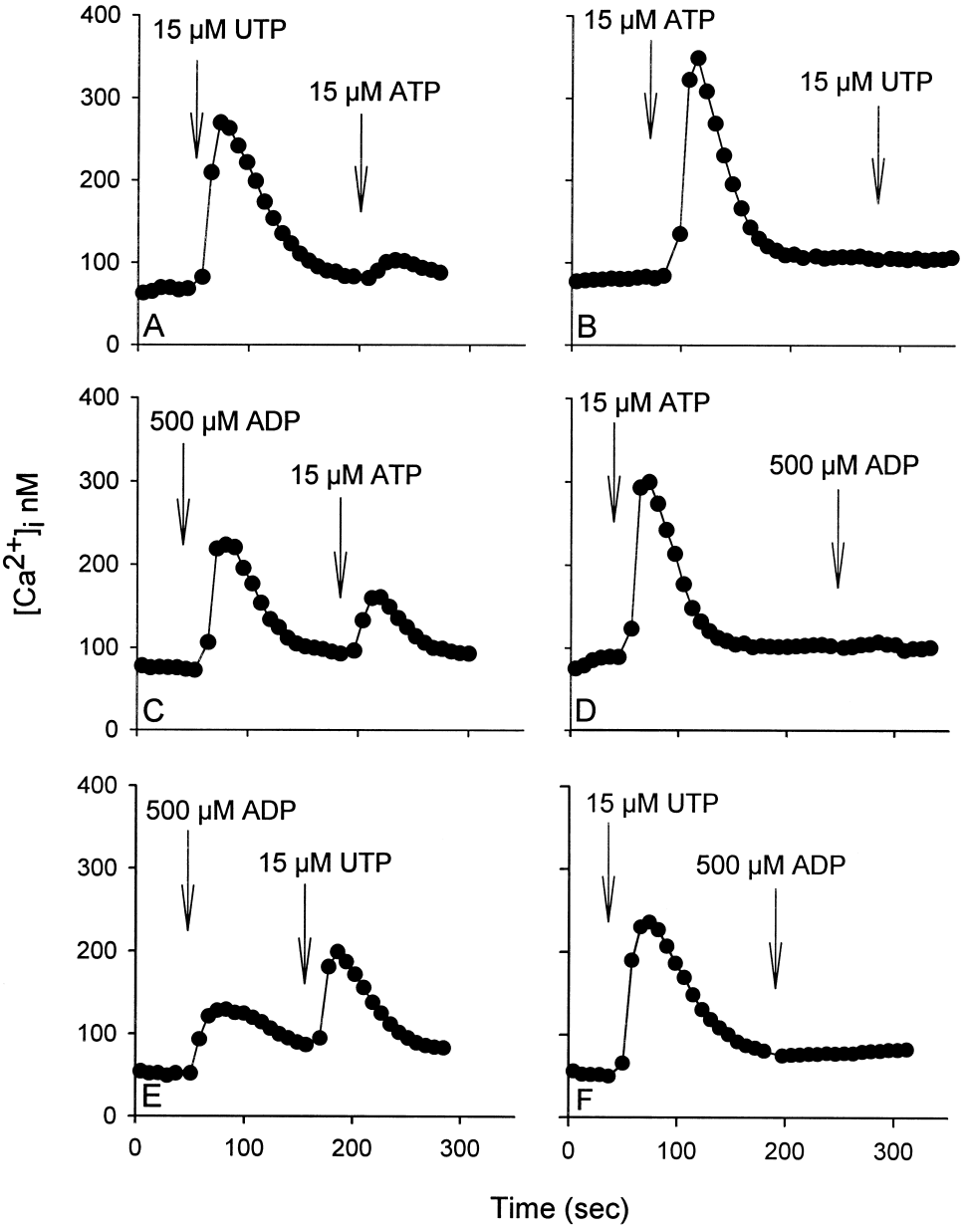

Fig. 6. Effect of repetitive stimulation with ATP, UTP and/or ADP on $[Ca^{2+}]_i$. A suspension of Ehrlich cells loaded with fura-2 was exposed to repetitive stimulation with either 15 μM UTP and 15 μM ATP (A), 15 μM ATP and 15 μM UTP (B), 500 μM ADP and 15 μM ATP (C), 15 μM ATP and 500 μM ADP (D), 500 μM ADP and 15 μM UTP (E) or 15 μM UTP and 500 μM ADP (F), as indicated by the arrows, and $[Ca^{2+}]_i$ was followed as a function of time. The data represent three (A, C, D, E and F) and four (B) independent experiments, respectively.

and some $P2X_2$ receptors (see [6,7]), and was found to have no detectable effect on $[Ca^{2+}]_i$ in the Ehrlich cells (concentration range 15–500 μM; Table 2), indicating that none of these receptor types are present in the Ehrlich cells. Furthermore, the $P2X_4$ and $P2X_6$ receptors are insensitive to suramin (see [7]), which is in contrast to the ATP-induced effects in the Ehrlich cells, suggesting that these P2X purinergic receptors are not present in the Ehrlich cells. BzATP, a specific agonist at the $P2X_7$ (P2Z) receptor (see [6,8]), had no detectable effect on $[Ca^{2+}]_i$ in the Ehrlich cells (concentration range 15 μM–1 mM; Table 2). Thus, it

seems unlikely that the Ehrlich cells possess P2X receptors.

From the observations listed in Table 2, the overall agonist profile of the P2Y receptors in the Ehrlich cells seems to be: ATP > UTP > ADP > MeSATP. As discussed above, this agonist profile probably reflects the responsiveness of two different P2Y receptors.

In Fig. 5A it is seen that when the Ehrlich cells are exposed to successive stimulation with sub-maximal concentrations of ATP, i.e. concentrations which induce an increase in $[Ca^{2+}]_i$ that is smaller than $[Ca^{2+}]_{i,max}$ (see Fig. 4 and Table 2), the primary addition of ATP induced an increase in $[Ca^{2+}]_i$, whereas the secondary addition of ATP did not lead to any detectable increase in $[Ca^{2+}]_i$. However, if the cells were subsequently stimulated with a maximal concentration of ATP, i.e. a concentration of ATP normally inducing a maximal increase in $[Ca^{2+}]_i$, an increase in $[Ca^{2+}]_i$ estimated at 33.2 ± 10 nM ($n = 7$) was observed, which is significantly smaller than $[Ca^{2+}]_{i,max}$ (204 nM, see Fig. 4, $P < 0.0001$). Fig. 5B demonstrates that when the Ehrlich cells were exposed to successive stimulation with maximal [ATP], the primary addition of ATP induced an increase in $[Ca^{2+}]_i$, whereas the subsequent additions of ATP did not. Taken together, the results in Fig. 5 would be compatible with the finding that two distinct P2Y receptors with different affinities are present in the Ehrlich cells.

To explore this possibility further, we exposed Ehrlich cells suspended in standard medium (1 mM Ca^{2+}) to successive stimulation with various agonists at the $P2Y_1$ and $P2Y_2$ receptors. In Fig. 6A it is seen that stimulation with a maximal [ATP] after exposure to a maximal concentration of the $P2Y_2$ receptor agonist, UTP, induced a small increase in $[Ca^{2+}]_i$, (20 ± 4 nM Ca^{2+}; $n = 3$), which was significantly smaller than $[Ca^{2+}]_{i,max}$ (see Fig. 6 and Table 2, $P < 0.0006$), whereas a secondary stimulation with 15 μM UTP had no detectable effect on $[Ca^{2+}]_i$ ($n = 3$; data not shown). In contrast, stimulation with a maximal [ATP] prior to addition of a maximal [UTP], resulted in no detectable effect of UTP on $[Ca^{2+}]_i$ (Fig. 6B). Similarly, it is seen in Fig. 6C, that following exposure to the $P2Y_1$ receptor agonist ADP, stimulation with a maximal [ATP] induced an increase in $[Ca^{2+}]_i$ at 76 ± 21 nM Ca^{2+} ($n = 6$) significantly lower than $[Ca^{2+}]_{i,max}$ (204 nM, see Fig. 4), whereas a secondary stimulation with 500 μM ADP induced an even more strongly reduced increase in $[Ca^{2+}]_i$ (30 ± 6 nM; $n = 3$). Stimulation of Ehrlich cells with ADP after a maximal [ATP] stimulation, did not induce any detectable increase in $[Ca^{2+}]_i$ (Fig. 6D). Finally, stimulation with a maximal [UTP], after a primary stimulation with 500 μM ADP, still induced an increase in $[Ca^{2+}]_i$ (Fig. 6E), although it was significantly reduced compared to $[Ca^{2+}]_{i,max}$ ($P < 0.02$, see Table 2). In contrast, from Fig. 6F it is seen that when the cells are stimulated with a maximal [UTP], a subsequent addition of ADP induced no detectable increase in $[Ca^{2+}]_i$. Taken together, these findings indicate that addition of UTP and ADP induce an increase in $[Ca^{2+}]_i$ via stimulation of two distinct types of P2Y receptors, whereas ATP activates both P2Y receptor types.

It should be noted that when Ehrlich cells suspended in standard medium (1 mM Ca^{2+}) were stimulated with various concentrations of ATP, UTP or ADP, and subsequently with a maximal [ATP], the total increase in $[Ca^{2+}]_i$, i.e. the sum of the increases in $[Ca^{2+}]_i$ induced by the nucleotides added, did not vary significantly (tested by analysis of variance, ANOVA, $P < 0.05$). This could indicate that nucleotide-induced increases in $[Ca^{2+}]_i$ are additive, but that there is an upper limit with respect to the nucleotide-induced increase in $[Ca^{2+}]_i$. This notion is supported by the fact that the total nucleotide-induced increase in $[Ca^{2+}]_i$ was not significantly different from the increase in $[Ca^{2+}]_i$ induced by a maximal [ATP] in itself.

4. Discussion

4.1. Mechanical stimulation of Ehrlich cells induces release of ATP

It is demonstrated that Ehrlich cells exposed to mechanical stimulation in the form of a mild centrifugation respond by releasing ATP (Table 1) which is capable of eliciting an increase in $[Ca^{2+}]_i$ (Figs. 1A and 2A) as well as evoking a whole-cell current (Figs. 1B, 1C and 2B) in other Ehrlich cells. The mechanical stress-induced release of ATP was significantly reduced in the presence of glibenclamide as well as

in the presence of verapamil, which inhibit CFTR (see [22]) and P-glycoprotein [23], respectively, and both have been reported to inhibit volume-activated Cl^- channels in several cell types [24,25]. In this context, it is interesting that Nilius, Droogmans and Oike have shown direct permeation of ATP through volume sensitive Cl^- channels (personal communication). However, glibenclamide is also known to inhibit ATP-sensitive K^+ channels (see [22]) and has recently been reported to activate a Na^+,K^+,2Cl^- co-transporter [33], and verapamil also acts as a Ca^{2+} channel inhibitor [34]. Thus, the inhibitory effect of glibenclamide and verapamil is at present only taken to indicate that the ATP release involves a transport mechanism and is not due to cell lysis. Whether the release of ATP from Ehrlich cells induced by mechanical stress takes place via a membrane protein belonging to the ATP-binding cassette (ABC) family or via a volume- and mechano-sensitive Cl^- channel is a question for further investigation.

From Fig. 1 it is seen that the supernatant-induced effect on $[Ca^{2+}]_i$ was abolished in the presence of suramin, indicating that ATP mediates its effects via P2 receptors in Ehrlich cells. Generally, stimulation of P2 receptors with ATP induces an increase in $[Ca^{2+}]_i$ via release of Ca^{2+} from intracellular stores, influx of Ca^{2+} from the extracellular medium, or both (see, e.g. [1,6]). In agreement with previous findings [9], we find that stimulation of Ehrlich cells with ATP leads to a concentration-dependent, transient increase in $[Ca^{2+}]_i$ (Figs. 2–4 and Table 2). The ATP-induced increase in $[Ca^{2+}]_i$ is significantly reduced in the absence of extracellular Ca^{2+}, indicating that release as well as influx of Ca^{2+} takes place. For further characterization of the ATP-activated signaling pathway in Ehrlich cells see [32]. It is noted that in the experiment shown in Fig. 1 the supernatant is diluted 20 times prior to addition, i.e. the final [ATP] would be in the range 0.1–0.2 μM, which is sufficient to induce a detectable increase in $[Ca^{2+}]_i$ (see Fig. 4).

4.2. Activation of whole-cell current in the Ehrlich cells by ATP

Ehrlich cells possess ChTX-sensitive Ca^{2+}-activated K^+ channels as well as ChTX-insensitive K^+ channels [35]. The transient hyperpolarization of the plasma membrane following addition of ATP, is believed to be partly a result of activation of ChTX-sensitive Ca^{2+}-dependent K^+ channels, because the ATP-induced hyperpolarization is significantly smaller in the presence of ChTX (Fig. 3). The small ATP-induced hyperpolarization of the plasma membrane seen in Ehrlich cells even in the presence of ChTX (Fig. 3B) could be explained either by activation of the ChTX-insensitive, Ca^{2+}-independent K^+ channels or by stimulation of Cl^- channels. The observation that stimulation of Ehrlich cells with ATP only activates a ChTX-sensitive Ca^{2+}-dependent K^+ efflux [36], argues strongly for a concomitant stimulation of Cl^- channels. It has recently been demonstrated that Ca^{2+}-activated Cl^- currents in Ehrlich cells are elicited already at a $[Ca^{2+}]_i$ of 100 nM [29], which approximately equals the resting level of $[Ca^{2+}]_i$ in these cells (see Figs. 5 and 6), hence, indicating that these currents might be activated after a nucleotide-induced increase in $[Ca^{2+}]_i$. However, the ATP-induced hyperpolarization of the plasma membrane was unaffected by niflumic acid (Fig. 3E,F), an inhibitor of Ca^{2+}-activated Cl^- channels. Since niflumic acid inhibits Ca^{2+}-activated Cl^- channels more potently at positive potentials than at negative potentials [31], it is thus speculated that the nucleotide-induced hyperpolarization of the plasma membrane impairs the otherwise inhibitory effect of niflumic acid on Ca^{2+}-activated Cl^- channels seen in Ehrlich cells [29]. Additional investigations are, however, needed in order to reveal the nature of the putative ATP-evoked Cl^- current in Ehrlich cells.

ATP-induced stimulation of Ca^{2+}-activated K^+ and Cl^- efflux pathways in Ehrlich cells has previously been reported [11]. In addition, extracellular ATP has also been shown to stimulate Ca^{2+}-activated K^+ and Cl^- channels in various other cell types, e.g. in distal nephron epithelial cells [37] and in human coronary artery smooth muscle [38]. In addition, extracellular ATP acts as an important regulator of transepithelial ion transport in several epithelia, including both normal and cystic fibrosis human airway epithelium [39], mainly via stimulation of Ca^{2+}-activated ion channels. In contrast, in several other cell types P2 receptor-activated K^+ and Cl^- currents were found to be Ca^{2+}-independent. In rat hippocampal neurons, a P2 receptor-mediated Ca^{2+}-independent K^+ current is suggested to be di-

rectly G-protein-activated, possibly via direct action of the βγ subunits [40], and in rat submandibular gland acinar, in duct cells [41], as well as in the colon [42], a Ca^{2+}-independent Cl^- current is induced by P2 agonists.

4.3. P2 receptors in the Ehrlich cells

Generally, P2X receptor activation induces a depolarization of the plasma membrane due to activation of cation channels, which results in an influx of Ca^{2+} (see, e.g. [6,7]). The fact that ATP apparently induces an increase in $[Ca^{2+}]_i$ prior to the hyperpolarization (Fig. 3), and that the Ehrlich cells seem to be insensitive to α,β-MeATP and bzATP (Table 2), therefore, strongly argues against the presence of P2X receptors in the Ehrlich cells.

From the data shown in Table 2 and in Figs. 5 and 6, we suggest that the Ehrlich cells possess a $P2Y_1$ receptor with the agonist profile ATP > ADP > 2-MeSATP, and a $P2Y_2$ receptor with the agonist profile UTP ≥ ATP. The presence of two P2Y receptor types is also strongly suggested by recent results, in which it was demonstrated that the non-specific P2Y receptor antagonists suramin and DIDS have different potencies as inhibitors of the ATP- and UTP-induced effects on pH_i, respectively [32]. Further characterization of the P2 receptors on the molecular level is needed to clarify this point. It should be noted that it has been suggested that the ADP-induced effects on $[Ca^{2+}]_i$ in the Ehrlich cells are due to conversion of ADP to ATP via an ecto-adenylate kinase [9]. However, in the present study very different effects are seen of ADP and ATP, which makes ecto-adenylate kinase activity unlikely to be responsible for the observed effects of ADP.

It is noted that presence of ecto-ATPase activity on the cell surface of the Ehrlich cells has previously been demonstrated [43], and the activity of such enzymes might interfere with and blur the nucleotide-induced effects (see [44]). However, in the present study, we add the nucleotide in question to a highly diluted cell suspension (a cytocrit of 0.5%) and look at the induced effects within such a short time interval after addition that degradation of the nucleotides should be minimal. Concerning the supernatant, this is rapidly removed from the cells and added to a highly diluted cell suspension within minutes after centrifugation, thus, any ecto-ATPase activity should be minimized.

4.4. Repetitive stimulation of P2Y receptors in the Ehrlich cells

Homologous desensitization of the $P2Y_1$ receptors as well as heterologous desensitization of $P2Y_2$ receptors upon stimulation has been reported in a wide variety of cell types, e.g. in endothelial cells [45], in MDCK cells [46] and in neuroblastoma-glioma hybrid cells [47]. The $P2Y_2$ receptors in Ehrlich cells apparently undergo heterologous desensitization upon stimulation, because activation of these receptors with UTP prevents any subsequent mobilization of Ca^{2+} via the $P2Y_1$- or $P2Y_2$ receptors, by addition of ADP and ATP, respectively (see Figs. 5 and 6). The $P2Y_1$ receptors in Ehrlich cells, on the other hand, seem to undergo only homologous desensitization, since stimulation of the $P2Y_1$ receptor with ADP has no desensitizing effect on the $P2Y_2$ receptor-mediated effects of UTP or ATP (see Figs. 5 and 6). However, one should be careful about making such statements since it is not clear to what extent the observations are caused by competition of the nucleotides at the receptors, lack of activation due to receptor occupancy or true desensitization of the receptors.

In a previous study, repetitive stimulation with 25 μM ATP did not result in desensitization of the ATP-induced effects on $[Ca^{2+}]_i$ in the Ehrlich cells [9]. This apparent discrepancy could be due to the fact that, in the present study, the interval between the successive additions of ATP to the Ehrlich cells is not longer than five minutes, whereas in [9], this interval was approximately ten minutes, which could be enough time for the receptors to recover completely, or for the added ATP to have been removed completely by ecto-enzymes.

4.5. Physiological relevance of ATP release from Ehrlich cells exposed to mechanical stress

From the above discussion, it seems likely that the ATP released from Ehrlich cells upon mechanical stimulation exerts its effects via P2Y. Hence, the current evoked by the messenger released is probably due to stimulation of a Ca^{2+}-activated K^+ and Cl^-

currents (see Fig. 3), and the increase in $[Ca^{2+}]_i$ most likely is due to release from intracellular Ins(1,4,5)P_3-sensitive stores as well as activation of CCE.

Release of nucleotides after exposure to various forms of stress has been reported in many other cell types. It is well known that endothelial cells respond to an increase in fluid shear stress by releasing ATP and endothelin, which in turn play important roles in regulation of vessel tone [2]. Release of ATP after exposing cells to mechanical stress has also been demonstrated in rat basophilic cells [48], in human astrocytoma cells [49], in hepatocytes [50], as well as in an epithelial cell line [3]. Furthermore, mechanical stimulation of human astrocytoma cells was recently demonstrated to induce release of UTP from the cells. In addition, in a rat hepatoma cell line, swelling was found to induce a release of ATP, mediated via a ATP-binding cassette protein, resulting in accelerated recovery from cell swelling [4,51]. Similarly, changes in hydrostatic pressure resulted in release of ATP from rabbit urinary bladder [52]. Thus, ATP and UTP seem to be ubiquitous autocrine signaling molecules, and it is tempting to suggest a role for ATP and/or UTP in cell volume regulation in Ehrlich cells. However, in Ehrlich cells, swelling results in a depolarization of the plasma membrane [26], whereas ATP induces a hyperpolarization (Fig. 3). In addition, ATP elicits a transient increase in $[Ca^{2+}]_i$ (Figs. 1–3) which is not seen during volume regulation in Ehrlich cells [53], and ATP induces ChTX-sensitive K^+ efflux, whereas cell swelling induces ChTX-insensitive K^+ efflux [36]. Thus, although ATP and/or UTP appear as important autocrine messengers after mechanical stimulation they are not the major signaling molecules mediating normal volume regulation in Ehrlich cells. It is, however, still possible that very low concentrations of ATP or UTP might be involved in swelling activation of the volume-sensitive Cl^- channel without inducing any detectable increase in $[Ca^{2+}]_i$.

In conclusion, the present study demonstrates that mechanical stimulation of Ehrlich cells leads to release of nucleotides acting as autocrine messengers via separate $P2Y_1$ and $P2Y_2$ receptors on the cells. The activation of these receptors results in a concentration-dependent increase in $[Ca^{2+}]_i$, hyperpolarization of the plasma membrane and an increase in the whole-cell K^+ and Cl^- currents.

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