EXTRACELLULAR ATP, ECTO-ATPase AND CALCIUM INFLUX IN *DICTYOSTELIUM DISCOIDEUM* CELLS

Roger W. PARISH and Monika WEIBEL

Cytology, Plant Biology Institute, University of Zürich, Zollikerstrasse 107, CH-8008 Zürich, Switzerland

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1. Introduction

There is evidence that a protein kinase is present on the surface of *Dictyostelium discoideum* cells [1–3] and is capable of phosphorylating certain plasma membrane proteins [3]. Such phosphorylation may be involved in membrane permeability and transport, cell movement, and the activation of cyclic AMP receptors which occurs during early differentiation [4]. External ATP induces cell aggregation, decreases the chemotactic sensitivity of cells to a cyclic AMP gradient and inhibits intercellular contact sites outside the aggregation center [1,5].

We found that ATP (0.1–0.8 μM produced at 1.5×10^7 cells/ml) is present extracellularly in cell suspensions. This reflected a steady state concentration since a Mg^{2+}-dependent ecto-ATPase was continually hydrolyzing extracellular ATP. The ecto-ATPase was inhibited by suramin and 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDAC), but not by oligomycin or ouabain. Ecto-ADPase, -AMPase and -nucleotide diphosphokinase activities were also detected. Calcium influx was inhibited by suramin, 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDAC), KCN, dinitrophenol, carbonyl-*m*-chlorophenylhydrazone (CCCP), adenyl imidodiphosphate, adenyl methylene diphosphate, and by adding hexokinase or apyrase to split extracellular ATP. Addition of ATP (optimum level 10^{-6} M) to cell suspensions stimulated calcium influx and partially overcame the KCN-induced inhibition of this influx. Glucose uptake was not affected by suramin or apyrase.

2. Materials and methods

D. discoideum cells (Ax-3) were grown axenically in HL-5 medium [6]; NC-4 cells were grown in liquid medium with *Escherichia coli* [7]. To assay ATP, cells were washed and suspended in 0.01 M MOPS buffer (3-(*N*-morpholino)-propane sulfonic acid, brought to pH 7.4 with conc. NaOH), 10 mM $MgSO_4$ (1.6×10^7 cells/ml) and shaken in a rotary shaker. Aliquots were removed at intervals, centrifuged with a Beckman microfuge and the ATP in supernatants determined using the luciferin–luciferase assay as modified [8]. ATP hydrolysis was measured by shaking cells (2×10^7/ml) in 0.02 Tris maleate buffer, 5 mM $MgCl_2$ (pH 7.0) with 2×10^{-8} M adenine-[U-^{14}C]ATP (Amersham, 275 mCi/mmol) at 23°C. Aliquots were removed at intervals and cells sedimented in a Beckman microfuge. EDTA (pH 7, final conc. 1 mM) was added to the supernatant and the products separated on PEI cellulose chromatograms using 0.25 M LiCl and formic acid (97:3). Radioactivity was detected with a scanner (Berthold) and % radioactivity in the products calculated.

To measure calcium transport cells (1×10^7/ml) were incubated with 0.02 mM Tris maleate (pH 7.0), 5 mM $MgCl_2$ and $^{45}Ca^{2+}$ (Amersham; 40 mCi/mg). Aliquots were centrifuged in a Beckman microfuge, resuspended in 1 mM EGTA, centrifuged, the sediments solubilized in Triton X-100 and radioactivity measured. Silicone oil centrifugation [9] was also used and gave similar results.

3. Results

At 1.5×10^7 NC-4 cells/ml, 0.1–0.25 μM extracellular ATP was produced (fig.1). Suspensions of the axenic strain Ax-2 contained 2–3-fold higher ATP concentrations. The ATP levels measured actually reflect a steady state concentration. When inhibitors such as KCN and dinitrophenol were added the con-

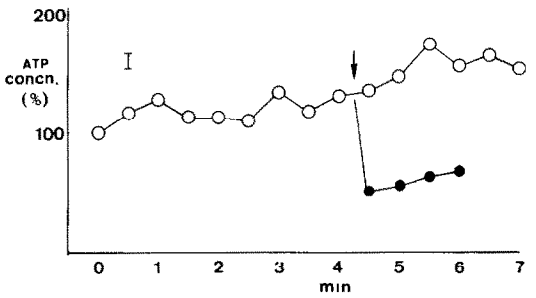

Fig.1. Variations in the extracellular ATP concentration of a *D. discoideum* (strain Ax-3) suspension. [ATP] is given in %, the initial measurement (equiv. 5.2×10^{-7} M) was taken as 100%: (○) control; (●) [ATP] following addition of 1 mM KCN at 4.25 min (arrow)

centration of extracellular ATP dropped immediately (fig.1).

When intact cells were incubated with [^{14}C]ATP at neutral pH, ADP and AMP were the products (table 1). (The enzymes were not found to be soluble in the incubation buffer.) At higher pH the ecto-alkaline phosphatase [10] metabolized AMP to adenosine. The most rapid step involved ADP formation, but the reaction never went to completion and after 1–2 min the [^{14}C]ATP level began to increase (fig.2a). The reappearance of [^{14}C]ATP was not observed with excess ADP present (fig.2a) or when plasma membranes were used in the assay, and we subsequently identified a nucleotide diphosphokinase on the cell surface. Hence, extracellular nucleotide triphosphates act as substrates and intact cells phosphorylase the [^{14}C]ADP. Similarly, when non-radioactive ATP, GTP or UTP were added to the plasma membranes, [^{14}C]ATP was synthesized from [^{14}C]-ADP (fig.2b).

Experiments using plasma membranes showed the

Table 1
The hydrolysis of [^{14}C]ATP by intact cells

Time (min)	Nucleotide conc. (% total counts)		
	ATP	ADP	AMP
1	37 (50)	57 (44)	6 (6)
2	26.5 (61)	68 (33)	5.5 (6)
4	14.5 (60)	72 (32)	13.5 (8)
8	22 (19)	61 (57.5)	17 (13.5)

Results of 1 expt. with strains Ax-3 and NC-4 (in parenthesis) are shown

ATPase was Mg^{2+}-dependent and Mg^{2+} could not be replaced by any other ion. The ATPase was inhibited by suramin and EDAC when these substances were added to plasma membranes or to intact cells (fig.2). Suramin is an impermeant anion which binds strongly to proteins and inhibits, for example, the ($Na^+ + K^+$)-activated ATPase of erythrocyte membranes [12] and the ecto-Mg^{2+}-ATPase of human granulocytes [13]. EDAC inhibits the erythrocyte membrane ATPase with some specificity [14]. The *Dictyostelium* ecto-enzymes alkaline phosphatase and cyclic AMP phosphodiesterase were not inhibited by EDAC or suramin (not shown). The ecto-ATPase was not inhibited by oligomycin or ouabain. Suramin did not inhibit the ecto-nucleotide diphosphokinase (fig.2b).

The K_m of the ecto-ATPase was difficult to determine accurately but levels of 1–25 μM were obtained.

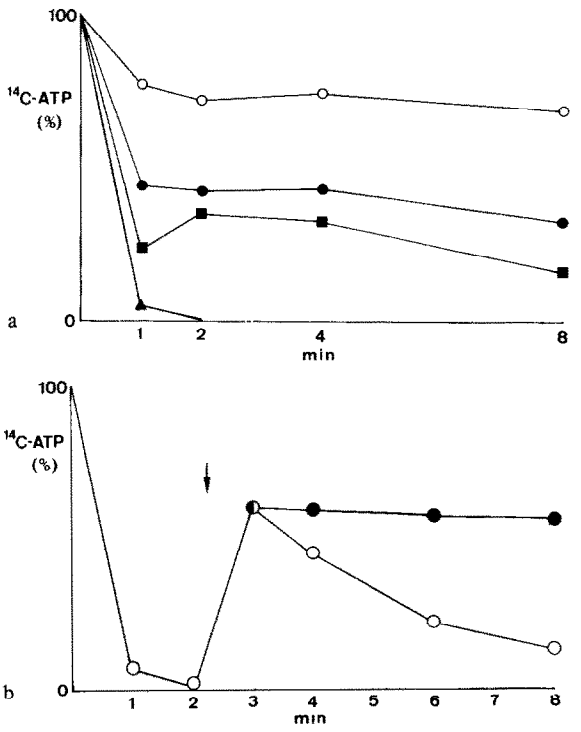

Fig.2. The hydrolysis of ATP by intact cells (strain NC-4) and purified plasma membranes [11]. Incubation conditions and separation of products were as in section 2, except 2 vol. acetone was added to the plasma membrane aliquots and proteins precipitated at −20°C. (a) Intact cells: (■) control; (○) 50 μM suramin; (●) 1 mM EDAC; (▲) 0.1 mM ADP. (b) Plasma membranes (100 μg protein/ml): (○) control; after 2.25 min, 1×10^{-5} M ATP (unlabelled) was added; (●) after 2.25 min, 1×10^{-5} M ATP plus 50 μM suramin was added.

The possibility that the ecto-ATPase is involved in calcium transport was examined using ATPase inhibitors and competitors or by reducing the extracellular ATP concentration. Both the uptake and release of Ca^{2+} from the cells was inhibited by KCN, CCCP and dinitrophenol (not shown). Calcium uptake was inhibited by EDAC, suramin and ATP-analogues containing non-hydrolysable β-γ-phosphate bonds (table 2). When apyrase of hexokinase plus glucose were added to the cell suspensions, calcium influx was also inhibited (table 2). (Glucose or glucose 6-phosphate alone had no effect; hexokinase alone did inhibit slightly as the cells release glucose.) The efflux of Ca^{2+} from preloaded cells was inhibited by EDAC, KCN and CCCP, but not by suramin, the ATP-analogues or hexokinase plus glucose (not shown).

When 10^{-6} M ATP was added to cells the initial rate of calcium influx was stimulated (fig.3). Increasing [ATP] up to 0.1 mM did not further stimulate the calcium influx. The addition of KCN to cells led to an immediate reduction of intra- and extra-cellular ATP (cf. fig.1) and calcium influx was reduced (fig.3). The addition of 10^{-6} M ATP with KCN partially overcame the inhibition of calcium influx (fig.3). These results further implicate extracellular ATP in calcium influx. Glucose uptake by cells was not affected by suramin or apyrase (not shown).

Table 2
Uptake of $^{45}Ca^{2+}$ by NC-4 cells

Treatment	$^{45}Ca^{2+}$ uptake (%)	
	5 min	15 min
Control	23	100
Suramin (100 μM)	10	43
Apyrase (1 mg/ml)	7	12
Apyrase (10 μg/ml)	12	56
Adenylyl imidodiphosphate (1 mM)	32	65
Adenylyl methylene diphosphate (1 mM)	28	55
Hexokinase (0.2 U/ml)	14	91
Hexokinase (0.2 U/ml) + glucose (1 mM)	10	59
Hexokinase (1 U/ml) + glucose (1 mM)	5	22

Maximum uptake (15 min) by controls is taken as 100% and corresponds to 2.6 μg $Ca^{2+}/10^7$ cells (variability ±5%). Apyrase (Sigma grade II) contained ATPase and ADPase activity of ~0.1 unit/mg

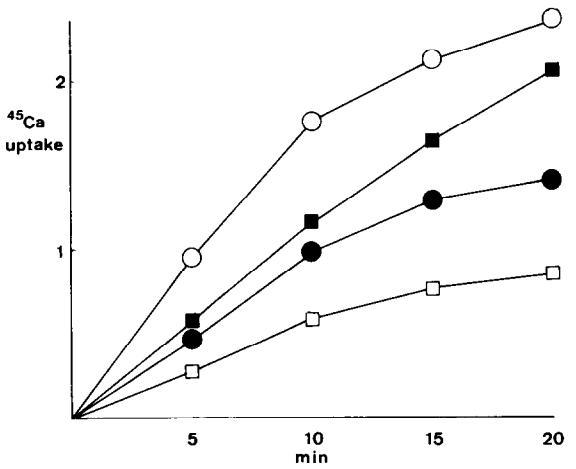

Fig.3. Uptake of $^{45}Ca^{2+}$ ($\mu g/10^7$ NC-4 cells): (■) control; (○) 10^{-6} M ATP; (□) 1 mM KCN; (●) 10^{-6} M ATP + 1 mM KCN.

4. Discussion

The results show that ATP occurs extracellularly in *D. discoideum* cell suspensions and enzymes are present on the cell surface capable of metabolizing this ATP to adenosine. We do not know whether the ATPase and the protein kinase are two different proteins. Rahmsdorf et al. [15] have questioned the presence of an ecto-protein kinase. Protein phosphorylation in cell suspensions incubated with [γ-^{32}P]-ATP may be due, for example, to a low percentage of damaged cells. However, we have found using autoradiography that all cells are labelled (unpublished).

The inhibition of calcium uptake by suramin and EDAC supported the idea that the ecto-ATPase might be involved in ion transport, although the specificity of these compounds as ecto-ATPase inhibitors is questionable. Nevertheless, suramin did not inhibit the surface AMPase, cyclic AMP phosphodiesterase, alkaline phosphatase or nucleotide disphophokinase. (Luciferase used in the ATP assay was inhibited.) Furthermore, continuous removal of the extracellular ATP from cultures by adding hexokinase or apyrase and competition with non-hydrolysable ATP analogues also inhibited calcium uptake. Neither suramin nor apyrase affected glucose uptake.

Wick et al. [16] found stimulation of *Dictyostelium* cells with cyclic AMP induced a transient calcium influx. However, we were unable to detect changes in the extracellular ATP levels or ecto-ATPase activity on the addition of cyclic AMP (not shown). The role

of the surface nucleoside diphosphokinase is unclear although it would ensure all extracellular nucleotide triphosphates are utilized to form ATP from the ADP produced by the ecto-ATPase. Guccione et al. [17] suggest the surface nucleoside diphosphokinase of platelets may transfer high-energy phosphate across the membrane to phosphorylate extracellular ADP. Hence, ATP could be synthesized extracellularly or released from *Dictyostelium* cells by a mechanism resembling cyclic AMP release [18,19].

The extracellular metabolism of nucleotide phosphates is apparently important in *D. discoideum* differentiation since ecto-AMPase is strikingly localized in pre-stalk cells at the culmination stage of development [20].

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