

# Mechanism of cAMP-induced H<sup>+</sup>-efflux of *Dictyostelium* cells: a role for fatty acids

H FLAADT\*, R SCHALOSKE and D MALCHOW<sup>†</sup>

Faculty of Biology, University of Konstanz, D-78457 Konstanz, Germany

\*Present address: Differentiation Epitheliale, Ecole Normale Supérieure, 46 rue d'Ulm, F-75005 Paris, France

<sup>†</sup>Corresponding author (Fax, 49-7531-88 2966; Email, Ralph.Schaloske@uni-konstanz.de).

Aggregating *Dictyostelium* cells release protons when stimulated with cAMP. To find out whether the protons are generated by acidic vesicles or in the cytosol, we permeabilized the cells and found that this did not alter the cAMP-response. Proton efflux in intact cells was inhibited by preincubation with the V-type H<sup>+</sup> ATPase inhibitor concanamycin A and with the plasma membrane H<sup>+</sup> ATPase blocker miconazole. Surprisingly, miconazole also inhibited efflux in permeabilized cells, indicating that this type of H<sup>+</sup> ATPase is present on intracellular vesicles as well. Vesicular acidification was inhibited by miconazole and by concanamycin A, suggesting that the acidic vesicles contain both V-type and P-type H<sup>+</sup> ATPases. Moreover, concanamycin A and miconazole acted in concert, both in intact cells and in vesicles. The mechanism of cAMP-induced Ca<sup>2+</sup>-fluxes involves phospholipase A<sub>2</sub> activity. Fatty acids circumvent the plasma membrane and stimulate vesicular Ca<sup>2+</sup>-efflux. Here we show that arachidonic acid elicited H<sup>+</sup>-efflux not only from intact cells but also from acidic vesicles. The target of regulation by arachidonic acid seemed to be the vesicular Ca<sup>2+</sup>-release channel.

## 1. Introduction

The cellular slime mould *Dictyostelium discoideum* is a model organism for studies on cell motility, chemotaxis, differentiation and evolutionary aspects of these processes (Devreotes and Zigmond 1988; Newell *et al* 1995; Kessin 1997; Maeda *et al* 1997; Söderbom and Loomis 1998). Chemotactic migration is accompanied by enhanced orientation and motility of the cells (Gerisch 1971; Devreotes and Zigmond 1988). Chemotactic stimulation of *Dictyostelium* cells elicits an efflux of protons (Malchow *et al* 1978a, b), and an increase in the intracellular pH (pH<sub>i</sub>, Aerts *et al* 1987). The increase in pH<sub>i</sub> seems to promote locomotion since the weak acid 5,5-dimethyl-2,4-oxazolidinedione (DMO), which reduces the attractant-induced increase in pH<sub>i</sub>, decreases chemotactic locomotion (Van Duijn and Inouye 1991). A change in pH<sub>i</sub> influences the pH-dependent binding of several actin-binding proteins to

actin filaments (Edmonds *et al* 1995 and references therein), and affects the localization of hisactophilin (Hanakam *et al* 1996). In human neutrophils chemotactic responses are accompanied by an increase in pH<sub>i</sub> as well as increased locomotion (Simchovitz and Cragoe 1986).

Extracellular cAMP not only stimulates proton fluxes but also activates an influx of Ca<sup>2+</sup> and an efflux of K<sup>+</sup> across the plasma membrane (Newell *et al* 1995; Aeckerle *et al* 1985). cAMP-stimulated Ca<sup>2+</sup> fluxes are due to activation of an IP<sub>3</sub>-sensitive Ca<sup>2+</sup> store and acidic vesicles (Rooney and Gross 1992; Flaadt *et al* 1993a, b). The latter pump Ca<sup>2+</sup> with the aid of a 2,5-di-(tert-butyl)-1,4-hydroquinone (BHQ)-sensitive Ca<sup>2+</sup> ATPase and a V-type H<sup>+</sup> ATPase. The resulting H<sup>+</sup> gradient serves to exchange H<sup>+</sup> for Ca<sup>2+</sup> ions. The plasma membrane has been reported to contain an electrogenic proton pump which is sensitive to diethylstilbestrol and miconazole (Pogge-von Strandmann *et al* 1984).

**Keywords.** Acidic vesicles; arachidonic acid; Ca<sup>2+</sup> transport; concanamycin A; H<sup>+</sup>-efflux; miconazole

Abbreviations used: AA, arachidonic acid; BHQ, 2,5-di-(tert-butyl)-1,4-hydroquinone; CMA, concanamycin A; DMO, 5,5-dimethyl-2,4-oxazolidinedione, fura-2 (1-[2-(5-Carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy)-ethane-N,N,N',N'-tetraacetic acid; IP<sub>3</sub>, inositol 1,4,5 trisphosphate; pH<sub>i</sub>, intracellular pH.

Acidic vesicles (e.g. endosomes and the contractile vacuoles) and/or the cytosol could be a source of the protons (Aubry *et al* 1993). By permeabilizing cells and the use of vesicles we found that acidic vesicles, not the cytosol, are a main source of protons for the cAMP-induced  $H^+$ -efflux.

## 2. Materials and methods

Filipin, NBD-Cl (7-chloro-4-nitrobenz-2-oxa-1,3-diazole), and miconazole were purchased from Sigma (USA). Concanamycin A (CMA) and sodium arachidonate were obtained from Fluka (Switzerland), fura-2 (1-[2-(5-Carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxyl]-2-(2'-amino-5'-methylphenoxy)-ethane-N,N,N',N'-tetraacetic acid, sodium), FM4-64 and tetramethylrhodamine dextran (MW 70000) from Mobi Tec (Germany).

### 2.1 Culture conditions and induction of the development of *D. discoideum*

Strain Ax-2 was grown and induced for differentiation as described by Bumann *et al* (1984). Differentiated cells were washed and resuspended in 5 mM Tricine buffer containing 5 mM KCl, pH 7.0.

### 2.2 $Ca^{2+}$ and pH measurements and permeabilization of the cells

cAMP-induced ion fluxes were measured with a  $Ca^{2+}$ -sensitive macro- or minielectrode (ETH 1001) as described by Bumann *et al* (1984), or with a pH electrode (Metrohm) and a Metrohm E 510 pH meter as previously reported (Malchow *et al* 1978a). Cells were permeabilized with the polyene antibiotic filipin as described by Flaadt *et al* (1993a). Up to 15–16  $\mu\text{g ml}^{-1}$  of a freshly prepared filipin solution (10 or 20  $\text{mg ml}^{-1}$  in DMSO) was required to permeabilize 70–90% of a suspension at a density of  $5 \times 10^7$  cells  $\text{ml}^{-1}$  at  $t_5$ . The degree of permeabilization was assessed in each experiment with methylene blue (4  $\text{mg ml}^{-1}$ , 10 min at 4°C). If necessary, further small amounts of filipin were added. The pore size of permeabilized cells allows penetration of methylene blue (356 Da) and lucifer yellow (522 Da), but not of FITC-Dextran (4100 Da). In some experiments cells were synchronized before induction of differentiation by shaking the cells in medium for 1 day at 8°C.

### 2.3 Preparation of vesicles and measurement of acidification

Cells (30 ml of a  $2 \times 10^7$  cells  $\text{ml}^{-1}$  suspension) were washed once in ice cold 20 mM Hepes buffer, pH 7.2, resus-

pended at  $2 \times 10^8$   $\text{ml}^{-1}$  and lysed by passage through nuclepore filters. Then 3% sucrose, 50 mM KCl, 1 mM  $\text{MgCl}_2$ , 20  $\mu\text{g ml}^{-1}$  leupeptin, 1  $\mu\text{g ml}^{-1}$  aprotinin and 2.5 mM DTT were added (final concentration). After centrifugation for 5 min at 3000 g the supernatant was further fractionated by centrifugation for 20 min at 12000 g. The sediment (P1) was resuspended in 1 ml of the above buffer yielding a protein concentration of about 2  $\text{mg ml}^{-1}$ . The supernatant (S1) contained 3 to 4  $\text{mg protein ml}^{-1}$ . Endosomal content was determined by fluid phase uptake of tetramethylrhodamine dextran. A suspension of 3.5 ml  $2 \times 10^8$  cells  $\text{ml}^{-1}$  was incubated for 20 min at 23°C in 15 mM Hepes buffer, pH 7.2, at a dye concentration of 7  $\text{mg ml}^{-1}$  (Hacker *et al* 1997). Contractile vacuoles were stained with the cell permeant dye FM4-64 as described by Heuser *et al* (1993). A suspension of 30 ml  $2 \times 10^7$  cells  $\text{ml}^{-1}$  in 17 mM phosphate buffer, pH 6.0, was shaken with 30  $\mu\text{l}$  (1  $\text{mg ml}^{-1}$ , dissolved in ethanol) FM4-64 for 30 min at 23°C. Before cell lysis cells were washed twice in 20 mM Hepes buffer. S1 was free of mitochondria and plasma membrane vesicles as measured by fluorescence labelling with rhodamine 123 for mitochondria and lucifer yellow staining of plasma membrane vesicles during cell breakage. Plasma membrane fragments reseal and lucifer yellow is thereby enclosed within plasma membrane vesicles. In addition, plasma membrane-bound folate deaminase activity was determined and found to be localized to P1 ( $90.6 \pm 0.7\%$ ,  $n = 2$ ). Proton pump activity was measured by fluorescence quenching of the weak base acridine orange (480 nm excitation, 528 nm emission) using a fluorimeter (Perkin Elmer, 650-10S) according to Rooney and Gross (1992). Usually 80 to 150  $\mu\text{g protein}$  were assayed in 1 ml buffer containing 10 mM Hepes buffer, pH 7.2, 50 mM KCl, 3% sucrose, 2 mM  $\text{MgCl}_2$ , 2  $\mu\text{M}$  acridine orange, and 6  $\mu\text{g ml}^{-1}$  antimycin A, 6  $\mu\text{g ml}^{-1}$  oligomycin A, 100  $\mu\text{M}$   $\text{NaN}_3$  in order to inhibit mitochondrial activity. One mM ATP was added where indicated.

### 2.4 Preparation of mitochondria and respiration measurements

Mitochondria were prepared as described by Troll *et al* (1992). Oxidation rates were measured according to Estabrook (1967) using a Clark-type oxygen electrode (2.120 ml test volume) with 0.5 M succinate as substrate and 200 mM  $\text{PO}_4^{3-}$  and  $\text{MgCl}_2$  as cofactors. To ensure a precise P/O stoichiometry 2 mM rotenone was given to block electron transfer from  $\text{NADH}_2$  to ubiquinone. The oxidation rate was stimulated by addition of 10 mM ADP yielding an ADP/O ratio of 2. One hundred mM ADP was given to elicit the maximal rate of oxidative phosphorylation. The amount of total protein in the reaction vessel

was 0.11 to 0.18 mg and succinate dehydrogenase activity was 285 mU mg<sup>-1</sup> protein.

### 2.5 Biochemical assays

Plasma membrane-bound folate deaminase and succinate dehydrogenase were measured as described by Wurster *et al* (1981) and Brdiczka *et al* (1968), respectively. Protein was determined using the method of Bradford (1976) with bovine serum albumin as standard.

## 3. Results

Previously, we have shown that cAMP-stimulation of starved *Dictyostelium* cells resulted in a transient proton efflux concomitant with a Ca<sup>2+</sup>-influx (Bumann *et al* 1986). A simultaneous measurement of both ion fluxes is shown in figure 1. To determine whether the proton fluxes are generated in the cytosol, intracellularly, or at both locations, we permeabilized the cells to eliminate the contribution of the plasma membrane. As shown in figure 2, similar cAMP-induced responses were obtained using intact or permeabilized cells except that, as noted previously (Flaadt *et al* 1993a), Ca<sup>2+</sup>-efflux from Ca<sup>2+</sup> storage compartments preceding Ca<sup>2+</sup> uptake became more prominent. In general, permeabilized cells yielded a somewhat smaller H<sup>+</sup>-efflux than intact cells (92 ± 13%, n = 17), and in one case the percentage was as low as 58%. Nevertheless, in 7 out of 17 experiments we found no reduction in H<sup>+</sup>-release indicating that the protons are not generated in the cytosol.

### 3.1 cAMP-induced H<sup>+</sup>-efflux in intact cells

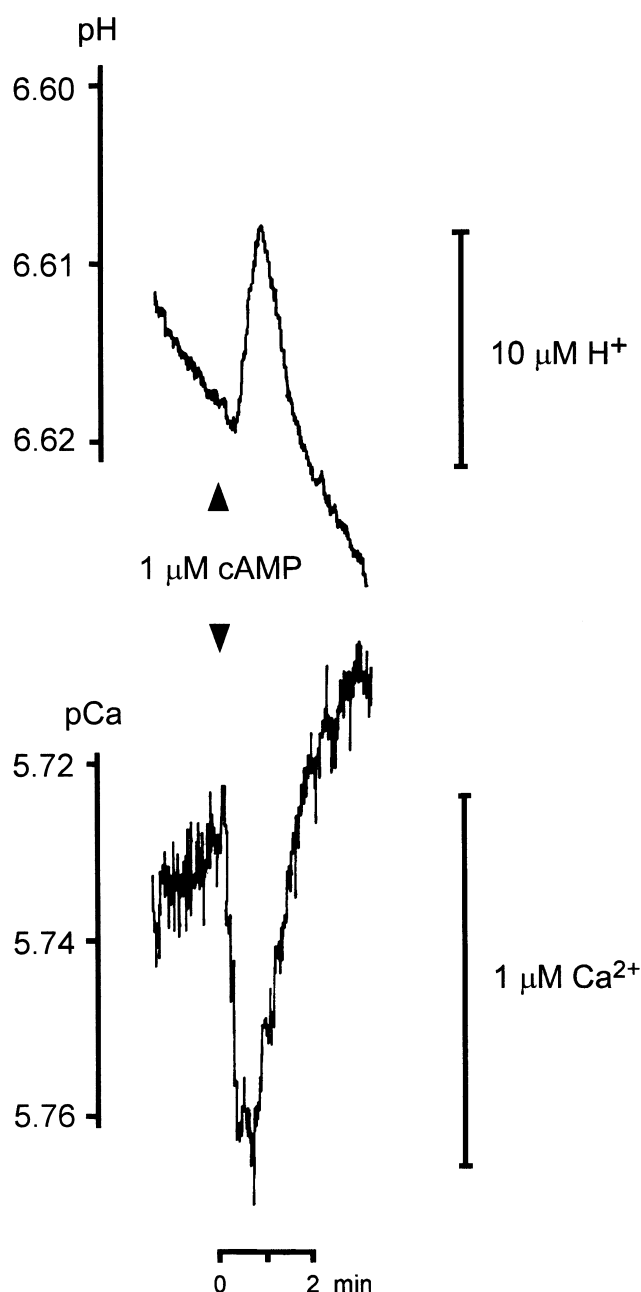
CMA is a potent inhibitor of V-type H<sup>+</sup> ATPases. A 5 μM concentration at 3 × 10<sup>6</sup> cells ml<sup>-1</sup> was reported to block completely vacuolar H<sup>+</sup> ATPase activity of acidic vesicles in *Dictyostelium* (Temesvari *et al* 1996). Figure 3A shows that 10 μM CMA at 5 × 10<sup>7</sup> cells ml<sup>-1</sup> inhibited about 60% of the cAMP-induced H<sup>+</sup>-release after 15 min of incubation.

A saturating dose of miconazole, another H<sup>+</sup> pump inhibitor in yeast and *Dictyostelium*, blocked receptor-mediated H<sup>+</sup>-efflux faster than CMA but to a similar extent (figure 3B). Forty-five μM miconazole inhibited the response to cAMP as efficiently as 37 μM miconazole (data not shown). CMA and miconazole applied sequentially inhibited H<sup>+</sup>-efflux almost completely (figure 3C).

### 3.2 cAMP-induced H<sup>+</sup>-efflux in permeabilized cells

In *Dictyostelium* miconazole is known to inhibit plasma membrane H<sup>+</sup> ATPase activity (Pogge-von Strandmann

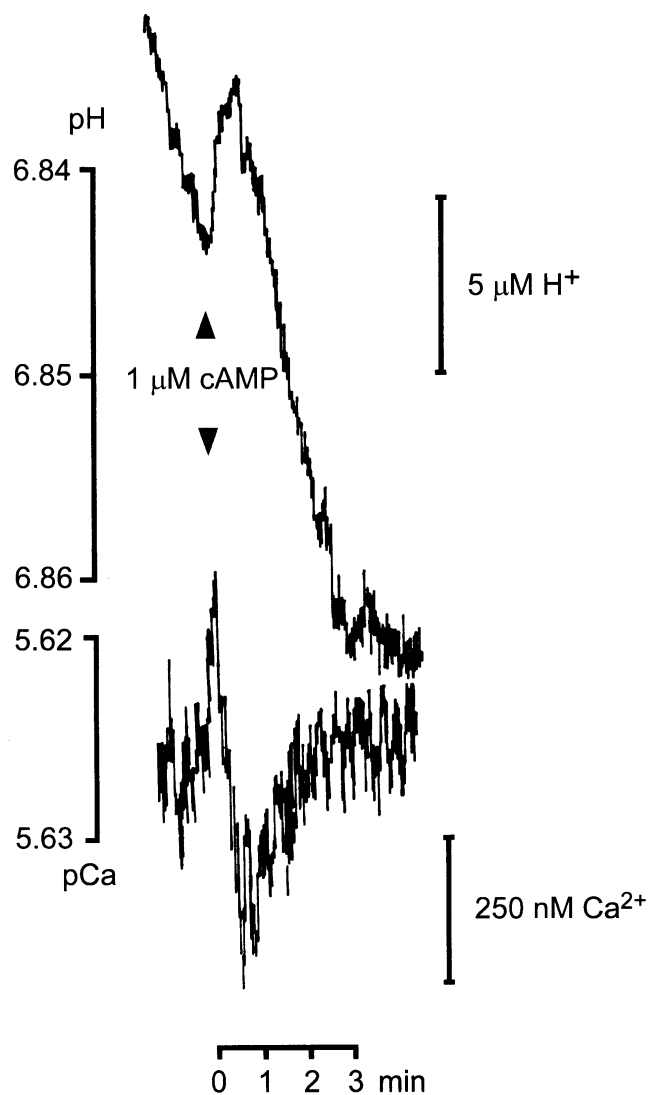
*et al* 1984). Surprisingly, miconazole blocked H<sup>+</sup>-release in permeabilized cells also, indicating that the target of miconazole is not limited to the plasma membrane (figure 4A). Since the vacuolar H<sup>+</sup> ATPase of *Dictyostelium* was reported to be sensitive to NBD-Cl (Padh *et al* 1989) we assayed this drug as well, and found a strong transient inhibition of the cAMP-induced H<sup>+</sup>-efflux



**Figure 1.** cAMP-stimulated H<sup>+</sup>-efflux and Ca<sup>2+</sup>-influx in intact cells. Measurements were made in a starved (6 h) cell suspension of 5 × 10<sup>7</sup> cells ml<sup>-1</sup> using ion-sensitive electrodes. One of six independent experiments is shown.

by 10  $\mu\text{M}$  NBD-Cl (figure 4B). These concentrations of miconazole and NBD-Cl did not affect the oxidation rate of isolated mitochondria (data not shown).

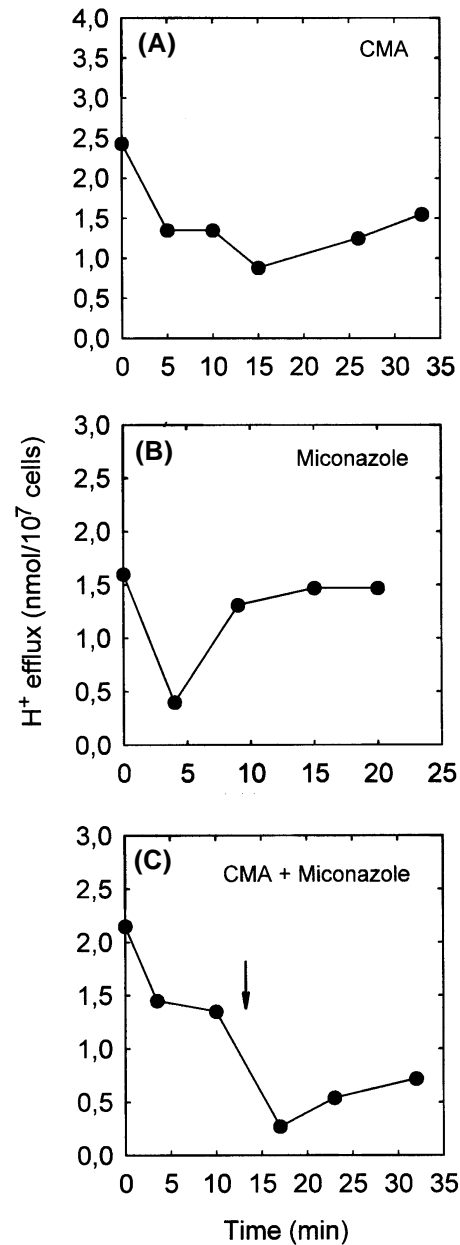
We tested CMA on permeabilized cells, too. We had to use a new batch of CMA which turned out to be less potent than the ones we used throughout the work. We found that CMA inhibited  $\text{H}^+$ -efflux in permeabilized cells to a similar extent and with the same time course as in intact cells, albeit at the higher concentration of 50  $\mu\text{M}$ . The inhibition amounted to  $68.5 \pm 4.5\%$  ( $n = 2$ ).



**Figure 2.** cAMP-induced  $\text{H}^+$ - and  $\text{Ca}^{2+}$ -changes in permeabilized cells. The cell suspension of figure 1 was permeabilized with filipin and ion fluxes were recorded simultaneously in a suspension of  $5 \times 10^7$  cells  $\text{ml}^{-1}$  7 h after the start of differentiation. One of six independent experiments is shown.

### 3.3 Vesicle acidification

In cellular homogenates we detected acidic vesicles and vesicles that became acidic after ATP addition as shown by Rooney and Gross (1992). By differential centrifuga-



**Figure 3.** Time course of inhibition of cAMP-induced  $\text{H}^+$ -efflux in intact cells. To 6–7 h starved cells ( $4\text{--}5 \times 10^7$  cells  $\text{ml}^{-1}$ ) 10  $\mu\text{M}$  CMA (A) or 37.5  $\mu\text{M}$  miconazole (B) were added at time zero. The response to 1  $\mu\text{M}$  cAMP was measured about 3–5 min before (control) and at the indicated times after application of the drug. In the bottom panel (C) 10  $\mu\text{M}$  CMA was given at zero time and 37.5  $\mu\text{M}$  miconazole several minutes later as indicated by the arrow. *Dictyostelium* inactivates drugs rapidly. Therefore, the inhibition often is of a transient nature. One of three independent experiments is shown.

tion we obtained a pellet (P1), which included most of the plasma membrane ( $90 \pm 0.7\%$ ,  $n = 2$ ), the contractile vacuoles ( $77 \pm 5.2\%$ ,  $n = 3$ ) and the endosomes ( $66 \pm 4.7\%$ ,  $n = 4$ ). It also contained about 90% of the acidic vesicles. As shown in figure 5 quenching of acridine orange fluorescence occurred to a large extent in the absence of ATP in P1 and increased by about 12% with ATP. In different experiments this increase was variable (compare table 1). By contrast, the supernatant (S1) contained no acidic

vesicles (not shown) and displayed only some acidification in the presence of ATP (figure 5). As a control inactive S1, obtained after prolonged incubation on ice, is shown. Since S1 contributed very little to vesicle acidification we studied drug action in P1.

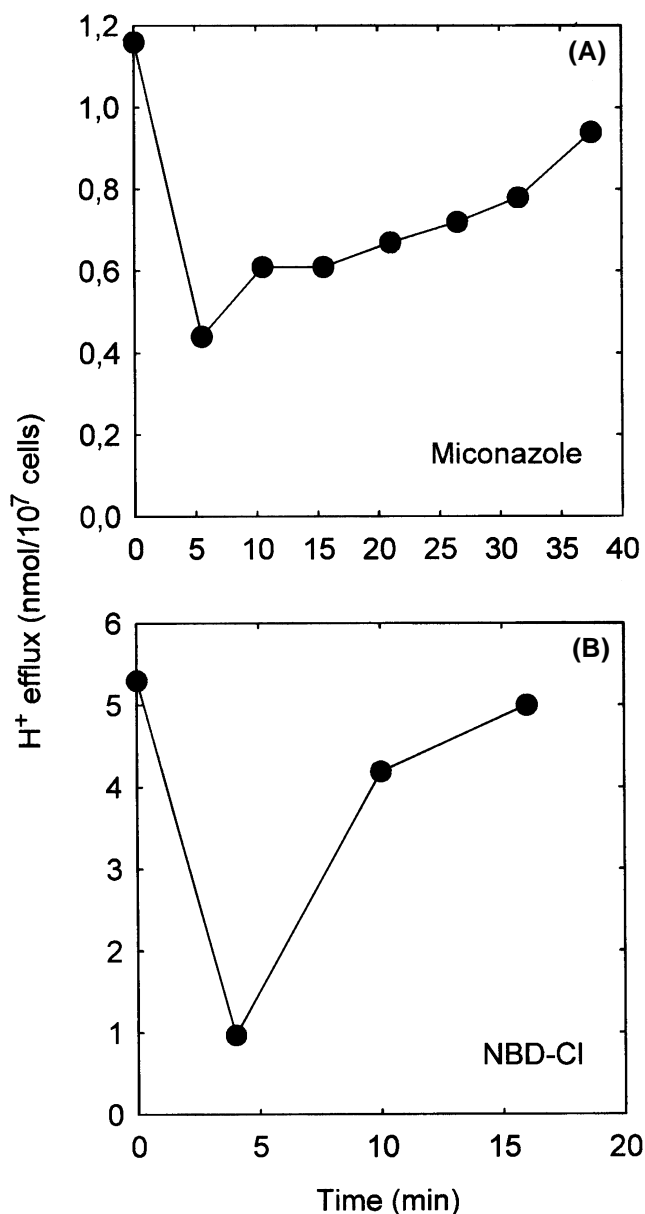
Acidification of P1 was inhibited by miconazole or CMA (table 1). The degree of inhibition was greater in experiment I containing a smaller percentage of ATP-independent acid vesicles (table 1). This result indicates that acid vesicles are more resistant to drug action, as expected, since the H<sup>+</sup> ATPase was operative before drug addition. Pumping itself is prevented by the drugs. As found for intact cells, the combination of miconazole and CMA resulted in stronger inhibition than for each drug alone (table 1). Thus, our results show that cAMP-induced H<sup>+</sup>-efflux is sensitive to miconazole not only in intact cells but also in permeabilized cells and that vesicular acidification is sensitive to the same drug concentration. Moreover, CMA and miconazole acted in concert, both in intact cells and in vesicles.

#### 3.4 Arachidonate induces H<sup>+</sup>-release in intact cells

Previously we have shown that cAMP-induced Ca<sup>2+</sup>-influx depends on phospholipase A<sub>2</sub>-activity. Among the products of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) activity fatty acids like arachidonic acid (AA) elicited Ca<sup>2+</sup>-influx and induced Ca<sup>2+</sup>-release from acidic vesicles in a dose-dependent manner (Schaloske and Malchow 1997; Schaloske *et al* 1998). If the cAMP-induced proton-efflux originates from acidic vesicles and is required for part of the Ca<sup>2+</sup> uptake we should expect AA to cause proton-efflux as well. As shown in figure 6 this was indeed the case. Following addition to the cell suspension arachidonate caused a slight alkalization followed by proton-efflux similar to the response induced by cAMP (figure 6). The dose response curve was slightly biphasic with a half maximal value at 6  $\mu$ M arachidonate (figure 6). Ten  $\mu$ M CMA reduced the rate of AA-induced proton-efflux by  $51 \pm 6.9\%$  ( $n = 3$ ), and both miconazole (37.5  $\mu$ M) and CMA (10  $\mu$ M), by  $75 \pm 7\%$  ( $n = 3$ ). The values for cAMP were  $59 \pm 7.2\%$  ( $n = 4$ ) for CMA and  $78 \pm 7.2\%$  ( $n = 4$ ) for CMA and miconazole.

#### 3.5 Vesicular H<sup>+</sup>-release

AA could act on the plasma membrane or intracellularly on acidic vesicles. It is known that ion channels in the plasma membrane are activated by AA (Chyb *et al* 1999; Mignen and Shuttleworth 2000). If this were the case in *Dictyostelium* we should find proton influx upon stimulation with AA, because the external medium was acidic (pH 6.7) compared to a cytosolic pH of 7.2 (figure 6). By contrast, we observed proton-efflux. The latter could result if AA stimulated the H<sup>+</sup>ATPase of the plasma

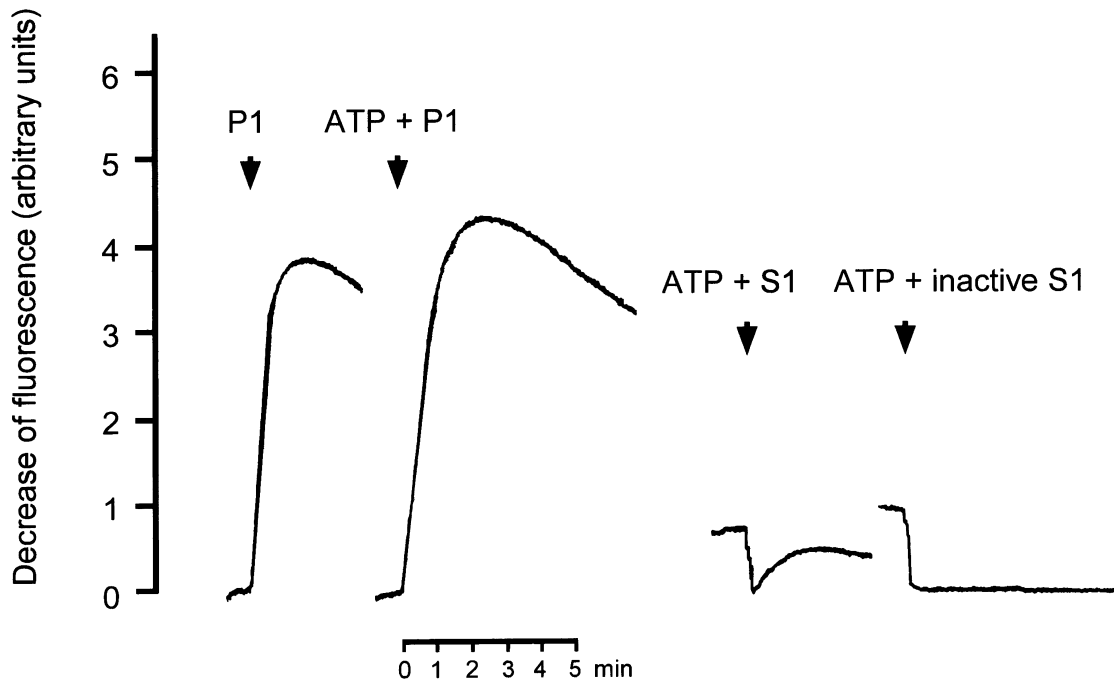


**Figure 4.** Time course of inhibition of cAMP-induced H<sup>+</sup>-efflux in permeabilized cells. Cells ( $5 \times 10^7$  cells ml<sup>-1</sup>) starved for 6–9 h were permeabilized and the response to 1  $\mu$ M cAMP was measured before and after addition, at zero time, of 37.5  $\mu$ M miconazole (A,  $n = 3$ ) or 10  $\mu$ M NBD-CI (B,  $n = 2$ ).

membrane. However, as reported previously (Schaloske *et al* 1998), we found no significant activation or inhibition of H<sup>+</sup>ATPase activity by AA.

Alternatively, AA could act on acidic vesicles. In figure 7 we show a hypothetical mechanism for stimulated proton-efflux. cAMP-binding to cell surface receptors causes the production of fatty acids (FA) by stimulation of

PLA<sub>2</sub> (Schaloske and Malchow 1997). The fatty acids could regulate, directly or indirectly, the H<sup>+</sup>ATPase, a Ca<sup>2+</sup>-release channel that is permeable for H<sup>+</sup> as well, a Ca<sup>2+</sup>-ATPase that countertransports Ca<sup>2+</sup> for H<sup>+</sup> or a Ca<sup>2+</sup>/H<sup>+</sup> exchanger. As shown in table 2, AA stimulated acridine orange release from acidic vesicles. This indicates that either the Ca<sup>2+</sup>-channel, the Ca<sup>2+</sup>ATPase or the



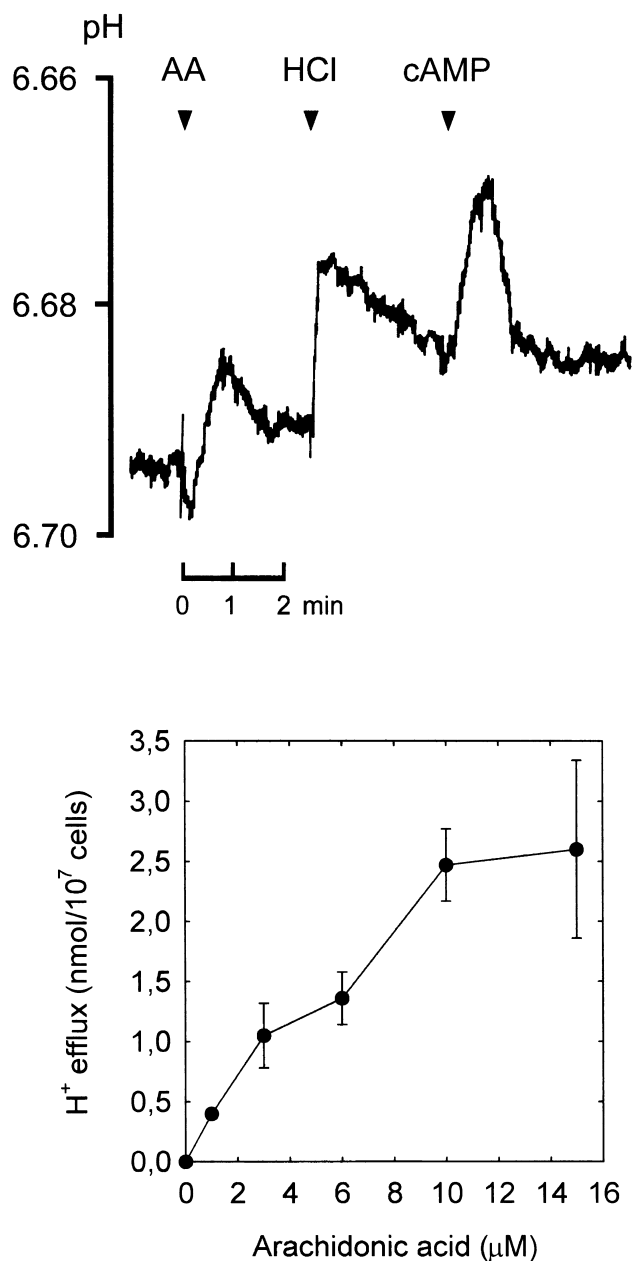
**Figure 5.** Acidification of vesicles P1 and S1. Quenching of acridine orange fluorescence was used to measure acidification of vesicular preparations as described in methods. P1 exhibited acidification without addition of ATP and a further increase with ATP. S1 displayed acidification only in the presence of ATP. Addition of S1 caused an increase in fluorescence. S1 kept for 6 h on ice was inactive and thus devoid of acidification. One of 10 independent experiments is shown.

**Table 1.** Inhibition of acidification in P1 by CMA and miconazole.

	Acidification	Inhibition of acidification		
	Decrease of fluorescence (relative units)	Miconazole (%)	CMA (%)	Both (%)
Experiment I				
without ATP	2.8			
with ATP	6.4 = 100%	85	75	94
Experiment II				
without ATP	4.2			
with ATP	4.5 = 100%	44	32	70

Measurements were performed as described in § 2. 37.5 µM miconazole and/or 10 µM CMA were added and the assay was started with the addition of P1. Control experiments without drugs were done immediately before and afterwards. A total of three experiments were performed.

exchanger are regulated by AA. To address the question whether cytosolic Ca<sup>2+</sup> is required for H<sup>+</sup>-release, we tested the AA response in the presence of the Ca<sup>2+</sup>-chelator BAPTA and found that the rate of both basal and stimulated efflux was reduced 2–3-fold (table 2), but the ratio of stimulated versus basal efflux was maintained.



**Figure 6.** Arachidonate-induced proton-efflux in intact cells. *Upper panel:* 3 μM sodium arachidonate (AA), 10 μM HCl or 1 μM cAMP were applied as indicated. The proton efflux induced by AA was preceded by a rise in pH due to the alkalinity of the sodium salt. One representative trace of four experiments is shown. *Lower panel:* Dose-response curve. Data represent means ± SEM, 3–5 separate experiments were performed.

#### 4. Discussion

Chemotactic stimulation of *Dictyostelium* cells elicits Ca<sup>2+</sup>-influx and H<sup>+</sup>-efflux. Where do the protons come from? In various cell types Ca<sup>2+</sup> and H<sup>+</sup> pumping activities are directly connected via Ca<sup>2+</sup>/H<sup>+</sup> exchange mechanisms. Vercesi *et al* (1994) described a Ca<sup>2+</sup>/H<sup>+</sup> ATPase system pumping Ca<sup>2+</sup> into an acidic vacuole, the acidocalcisome, of *Trypanosoma brucei*. Energized Ca<sup>2+</sup> uptake into vacuoles of *Saccharomyces cerevisiae* also occurs via Ca<sup>2+</sup>/H<sup>+</sup> exchange (Ohsumi and Anraku 1983). We reasoned that acidic vacuoles could be a likely source of protons in *Dictyostelium*, too. This was demonstrated to be the case with the following rationale:

(1) The proton fluxes were not generated in the cytosol, since the H<sup>+</sup>-release, even if reduced, persisted in permeabilized cells. The reduction in proton flux observed in several cases can be explained in two ways: The permeabilization procedure uses filipin which complexes with cholesterol and thereby produces pores in the plasma membrane. A portion of the acidic vesicles is derived from endosomes and hence from the plasma membrane. It is therefore conceivable that filipin could act on the acidic vesicles, provided that cholesterol was still present in their membrane. In this case, their activity could be diminished, especially if higher concentrations of filipin were required to achieve the desired degree of permeabilization. Alternatively, higher concentrations of filipin may be deleterious to the signal transduction cascade that begins at the cAMP receptor and leads to an enhanced activity of the H<sup>+</sup> ATPase. Ferber *et al* (1970) found that filipin inhibits phospholipase A<sub>2</sub> activity in *Dictyostelium* with an IC<sub>50</sub> of 50 μg/ml. Phospholipase A<sub>2</sub> activity is required for cAMP-induced Ca<sup>2+</sup>-influx (Schaloske *et al* 1998) and possibly for H<sup>+</sup>-efflux, too.

(2) The V-type H<sup>+</sup> ATPase inhibitors NBD-Cl and CMA blocked the cAMP-response in permeabilized cells. An

**Table 2.** Release of acridine orange by AA.

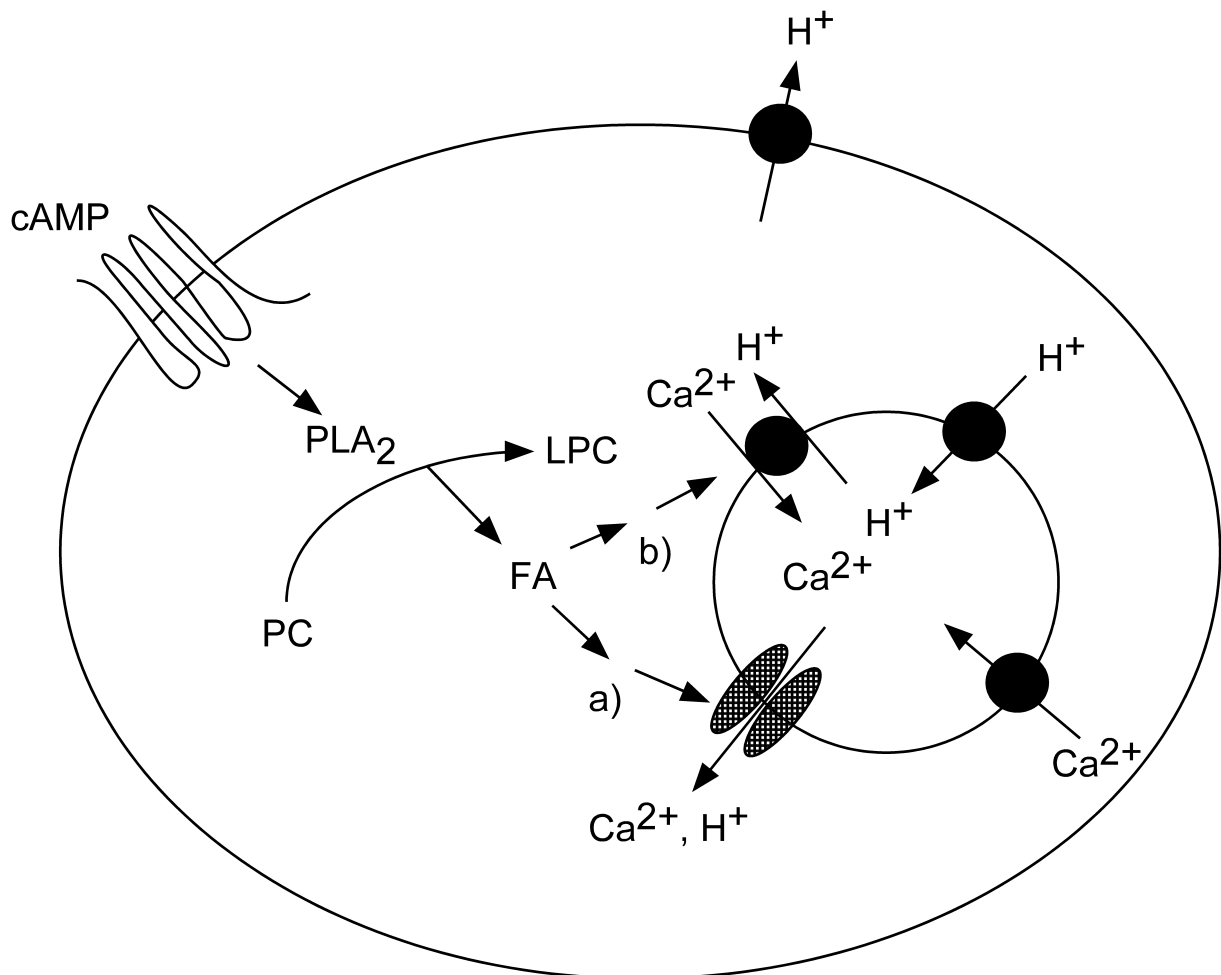
BAPTA	AA	Rate of release (a.u./min)
–	–	0.41 ± 0.14 (n = 5)
–	+	1.20 ± 0.32 (n = 5)
+	–	0.14 ± 0.05 (n = 3)
+	+	0.51 ± 0.16 (n = 3)

Acidic vesicles (P1) were loaded with acridine orange in the presence of ATP. Then AA (10 μM) or ethanol (0.1%) were added and the initial rate of acridine orange release (expressed in arbitrary units/min) was measured in the absence or presence of the Ca<sup>2+</sup>-chelator BAPTA (1 mM).

interesting twist in our results was provided by miconazole, which also inhibited  $H^+$ -efflux to a large extent (figures 3 and 4). Since miconazole was known as a blocker of the plasma membrane  $H^+$  ATPase of *Dictyostelium* we expected miconazole to be inactive in permeabilized cells and in the vesicular preparations. However, this was not the case. Miconazole inhibited both the cAMP response in permeabilized cells and vesicular acidification. This result too can be explained by the plasma membrane origin of the acidic vesicles. Endosomes still contain the cAMP-receptor and thus are likely to possess other plasma membrane components as well (Padh and Tanjore 1995). Recently, Moniakis *et al* (1999) described the occurrence of the  $Ca^{2+}$  pump PAT1 on plasma membranes and contractile vacuoles. An alternative possibility would be that miconazole also inhibits the V-type  $H^+$  ATPase of *Dictyostelium*. This would explain why

CMA or miconazole inhibited more than 50% of  $H^+$ -efflux. The latter result is in line with the hypothesis of plasma membrane  $H^+$  ATPases being present on endosomes if one assumes that the drug induces a conformational change of the respective  $H^+$  ATPase, thereby opening the  $H^+$ -entry pathway for passive leakage of protons. Thus, the formation of any proton gradient would be blocked.

(3) Arachidonic acid caused a similar  $H^+$ -efflux as cAMP in intact cells and this response, likewise, was inhibited by CMA and miconazole. Furthermore, we showed that AA did not act at the plasma membrane, but instead induced vesicular  $H^+$ -release. In addition, vesicular acidification was inhibited by CMA and miconazole at the same extent and the same concentrations as in intact cells. Therefore, we propose the following model for cAMP-induced  $H^+$ -efflux (figure 7): cAMP-binding to cell surface receptors



**Figure 7.** Hypothetical scheme for the mechanism of cAMP-induced  $H^+$ -efflux. For explanation see text. Pathway (a) is supported by the experiments.

activates PLA<sub>2</sub>. The fatty acids released cause proton efflux from acidic vesicles. Subsequently, the protons are transported to the extracellular medium by the plasma membrane H<sup>+</sup> ATPase.

There are four possible targets for the fatty acids: a Ca<sup>2+</sup>/H<sup>+</sup> exchanger, a Ca<sup>2+</sup>(H<sup>+</sup>)-release channel, H<sup>+</sup> and Ca<sup>2+</sup> ATPases. Previously, we have shown that AA neither significantly activated nor inhibited H<sup>+</sup> or Ca<sup>2+</sup> ATPase activity (Schaloske *et al* 1998). Thus possible targets reduce to (a) the Ca<sup>2+</sup>(H<sup>+</sup>)-release channel and (b) the Ca<sup>2+</sup>/H<sup>+</sup> exchanger. Since AA stimulated vesicular H<sup>+</sup>-efflux in the presence of the Ca<sup>2+</sup>-chelator BAPTA (table 2) the Ca<sup>2+</sup>/H<sup>+</sup> exchanger cannot mediate the response, because no Ca<sup>2+</sup> is present for Ca<sup>2+</sup>/H<sup>+</sup> exchange. Consequently, AA may act by binding to the Ca<sup>2+</sup>-channel. Indeed, Schaloske *et al* (1998) have shown that AA elicits vesicular Ca<sup>2+</sup>-release in *Dictyostelium*. It is conceivable that protons are released via the Ca<sup>2+</sup>-channel provided that the channel is not selective for Ca<sup>2+</sup>. Such a channel has been found in the brain (Waldmann *et al* 1997). Alternatively, AA could mediate H<sup>+</sup>-release directly. However, it has been reported that long chain FA are relatively inefficient H<sup>+</sup>-carriers (Gutknecht 1988). In the absence of BAPTA (table 2), the Ca<sup>2+</sup> released by AA activates the Ca<sup>2+</sup>/H<sup>+</sup> exchanger as well as the Ca<sup>2+</sup> pump and promotes further H<sup>+</sup>-efflux.

We did not find a constant ratio of Ca<sup>2+</sup>/H<sup>+</sup> exchange. In intact cells this ratio was almost 10, but could be as large as 30. This variation is likely due to the presence of a proton gradient-independent uptake of Ca<sup>2+</sup> by a Serca type Ca<sup>2+</sup> transport ATPase (Rooney *et al* 1994). On the other hand, the Ca<sup>2+</sup> estimate may be too low compared to the protons, because Ca<sup>2+</sup> buffering is very strong in the cytosol of *Dictyostelium* cells, whereas protons display an anomalously high mobility in aqueous solution (Marx *et al* 1999).

### Acknowledgements

We thank R Mutzel and C Schlatterer for stimulating discussions and J Breed for critical reading of the manuscript, V Ullrich for use of the Sigma II fluorimeter and the DFG for support.

### References

- Aeckerle S, Wurster B and Malchow D 1985 Oscillations and cyclic AMP-induced changes of the K<sup>+</sup> concentration in *Dictyostelium discoideum*; *EMBO J.* **4** 39–43
- Aerts R J, De Wit R J W and Van Lookeren Campagne M M 1987 Cyclic AMP induces a transient alkalinization in *Dictyostelium*; *FEBS Lett.* **220** 366–370
- Aubry L, Klein G, Martiel J-L and Satre M 1993 Kinetics of endosomal pH evolution in *Dictyostelium discoideum* amoebae; *J. Cell Sci.* **105** 861–866
- Bradford M M 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding; *Anal. Biochem.* **72** 255–260
- Brdiczka D, Pette D, Brunner G and Miller G 1968 Kompartimentierte Verteilung von Enzymen in Rattenlebermitochondrien; *Eur. J. Biochem.* **5** 294–304
- Bumann J, Malchow D and Wurster B 1986 Oscillations of Ca<sup>++</sup> concentration during the cell differentiation of *Dictyostelium discoideum*; *Differentiation* **31** 85–91
- Bumann J, Wurster B and Malchow D 1984 Attractant-induced changes and oscillations of the extracellular Ca<sup>2+</sup> concentration in suspensions of differentiating *Dictyostelium* cells; *J. Cell Biol.* **98** 173–178
- Chyb S, Raghu P and Hardie R C 1999 Polyunsaturated fatty acids activate the *Drosophila* light-sensitive channels TRP and TRPL; *Nature (London)* **397** 255–259
- Devreotes P N and Zigmond S H 1988 Chemotaxis in eukaryotic cells: a focus on Leukocytes and *Dictyostelium*; *Annu. Rev. Cell Biol.* **4** 649–686
- Estabrook R W 1967 Mitochondrial respiratory control and the polarographic measurement of ADP : O ratios; *Methods Enzymol.* **10** 41–47
- Edmonds B T, Murray J and Condeelis J 1995 pH regulation of the f-actin binding properties of *Dictyostelium* elongation factor 1a; *J. Biol. Chem.* **270** 15222–15230
- Ferber E, Munder P G, Fischer H and Gerisch G 1970 High phospholipase activities in amoebae of *Dictyostelium discoideum*; *Eur. J. Biochem.* **14** 253–257
- Flaadt H, Jaworski E, Schlatterer C and Malchow D 1993a Cyclic AMP- and Ins(1,4,5)P<sub>3</sub>-induced Ca<sup>2+</sup> fluxes in permeabilised cells of *Dictyostelium discoideum*: cGMP regulates Ca<sup>2+</sup> entry across the plasma membrane; *J. Cell Sci.* **105** 255–261
- Flaadt H, Jaworski E and Malchow D 1993b Evidence for two intracellular calcium pools in *Dictyostelium*: the cAMP-induced calcium influx is directed into a NBD-Cl- and 2,5-di-(tert-butyl)-1,4-hydroquinone-sensitive pool; *J. Cell Sci.* **105** 1131–1135
- Gerisch G 1971 Periodische Signale steuern die Musterbildung in Zellverbänden; *Naturwissenschaften* **58** 430–438
- Gutknecht J 1988 Proton conductance caused by long-chain fatty acids in phospholipid bilayer membranes; *J. Membr. Biol.* **106** 83–93
- Hacker U, Albrecht R and Maniak M 1997 Fluid-phase uptake by macropinocytosis in *Dictyostelium*; *J. Cell Sci.* **110** 105–112
- Hanakam F, Albrecht R, Eckerskorn C, Matzner M and Gerisch G 1996 Myristoylated and non-myristoylated forms of the pH sensor protein hisactophilin II: intracellular shuttling to plasma membrane and nucleus monitored in real time by a fusion with green fluorescent protein; *EMBO J.* **15** 2935–2943
- Heuser J, Zhu Q and Clarke M 1993 Proton pumps populate the contractile vacuoles of *Dictyostelium* amoebae; *J. Cell Biol.* **121** 1311–1327
- Kessin R H 1997 The evolution of the cellular slime molds; in *Dictyostelium – A model system for cell and developmental biology* (eds) Y Maeda, K Inouye and I Takeuchi (Tokyo: Universal Academy Press) pp 3–13
- Maeda Y, Inouye K and Takeuchi I 1997 *Dictyostelium – A model system for cell and developmental biology* (Tokyo: Universal Academic Press)
- Malchow D, Nanjundiah V and Gerisch G 1978a pH oscillations in cell suspensions of *Dictyostelium discoideum*: their relation to cyclic-AMP signals; *J. Cell Sci.* **30** 319–330

- Malchow D, Nanjundiah V, Wurster B, Eckstein F and Gerisch G 1978b Cyclic AMP-induced pH changes in *Dictyostelium discoideum* and their control by calcium; *Biochim. Biophys. Acta* **538** 473–478
- Marx D, Tuckerman M E, Hutter J and Parrinello M 1999 The nature of the hydrated excess proton in water; *Nature (London)* **397** 601–604
- Mignen O and Shuttleworth T J 2000 IARC, a novel arachidonate-regulated, noncapacitative  $\text{Ca}^{2+}$  entry channel; *J. Biol. Chem.* **275** 9114–9119
- Moniakakis J, Coukell M B and Janiec A 1999 Involvement of the  $\text{Ca}^{2+}$ -ATPase PAT1 and the contractile vacuole in calcium regulation in *Dictyostelium discoideum*; *J. Cell Sci.* **112** 405–414
- Newell P C, Malchow D and Gross J D 1995 The role of calcium in aggregation and development of *Dictyostelium*; *Experientia* **51** 1155–1165
- Ohsumi Y and Anraku Y 1983 Calcium transport driven by a proton motive force in vacuolar membrane vesicles of *Saccharomyces cerevisiae*; *J. Biol. Chem.* **258** 5614–5617
- Padh H, Lavasa M and Steck T L 1989 Characterization of a vacuolar proton ATPase in *Dictyostelium discoideum*; *Biochim. Biophys. Acta* **982** 271–278
- Padh H and Tanjore S 1995 Localization of cyclic-AMP receptors with acidosomes in *Dictyostelium discoideum*; *FEBS Lett.* **368** 358–362
- Pogge-von Strandmann R, Kay R R and Dufour J-P 1984 An electrogenic proton pump in plasma membranes from the cellular slime mold *Dictyostelium discoideum*; *FEBS Lett.* **175** 422–427
- Rooney E K and Gross J D 1992 ATP-driven  $\text{Ca}^{2+}/\text{H}^{+}$  antiport in acid vesicles from *Dictyostelium*; *Proc. Natl. Acad. Sci. USA* **89** 8025–8029
- Rooney E K, Gross J D and Satre M 1994 Characterisation of an intracellular  $\text{Ca}^{2+}$  pump in *Dictyostelium*; *Cell Calcium* **16** 509–522
- Schaloske R and Malchow D 1997 Mechanism of cAMP-induced  $\text{Ca}^{2+}$  influx in *Dictyostelium*: role of phospholipase  $\text{A}_2$ ; *Biochem. J.* **327** 233–238
- Schaloske R, Sonnemann J, Malchow D and Schlatterer C 1998 Fatty acids induce release of  $\text{Ca}^{2+}$  from acidosomal stores and activate capacitative  $\text{Ca}^{2+}$  entry in *Dictyostelium discoideum*; *Biochem. J.* **33** 541–548
- Simchovitz L and Cragoe E J Jr 1986 Regulation of human neutrophil chemotaxis by intracellular pH; *J. Biol. Chem.* **261** 6492–6500
- Söderbom F and Loomis W F 1998 Cell-cell signalling during *Dictyostelium* development; *Trends Microbiol.* **6** 402–406
- Temesvari L A, Rodriguez-Paris J M, Bush J M, Zhang L and Cardelli J A 1996 Involvement of the vacuolar proton-translocating ATPase in multiple steps of the endo-lysosomal system and in the contractile vacuole system of *Dictyostelium discoideum*; *J. Cell Sci.* **109** 1479–1495
- Troll H, Malchow D, Müller-Taubenberger A, Humbel B, Lottspeich F, Ecke M, Gerisch G, Schmid A and Benz R 1992 Purification, functional characterization, and cDNA sequencing of mitochondrial porin from *Dictyostelium discoideum*; *J. Biol. Chem.* **267** 21072–21079
- Van Duijn B and Inouye K 1991 Regulation of movement speed by intracellular pH during *Dictyostelium discoideum* chemotaxis. *Proc. Natl. Acad. Sci. USA* **88** 4951–4955
- Vercesi A E, Moreno S N J and Docampo R 1994  $\text{Ca}^{2+}/\text{H}^{+}$  exchange in acidic vacuoles of *Trypanosoma brucei*; *Biochem. J.* **304** 227–233
- Waldmann R, Champigny G, Bassilana F, Heurteaux and Lazdunski M 1997 A proton-gated cation channel involved in acid-sensing; *Nature (London)* **386** 173–177
- Wurster B, Bek F and Butz K 1981 Folic acid and pterin deaminases in *Dictyostelium discoideum*: Kinetic properties and regulation by folic acid, pterin and adenosine 3',5'-phosphate; *J. Bacteriol.* **148** 183–192

MS received 19 May 2000; accepted 18 July 2000

Corresponding editor: VIDYANAND NANJUNDIAH