INCREASED PHOSPHOLIPASE A ACTIVITY AND FORMATION OF COMMUNICATIVE CONTACTS BETWEEN *ACANTHAMOEBA CASTELLANII* CELLS

Effect of 3',5'-Cyclic AMP

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SUMMARY

1. Exogenous 1-palmitoyl-*sn*-glycero-3-phosphorylcholine becomes incorporated into the membrane of *A. castellanii* within 2 min of incubation.
2. Homogenates of *A. castellanii* are shown to contain phospholipase A activity.
3. The phospholipase A activity is dependent on the population density of the culture.
4. An increased phospholipase A activity in older cultures of *A. castellanii*, due to an increased population density, induces aggregation of these cells in a way that communicative contacts are formed.
5. At pH 5.5, the pH value at which the cells were grown, the phospholipase A activity is stimulated by cAMP; at pH 8.0 no stimulation occurs.

Aggregation of *Acanthamoeba castellanii* cells into multicellular masses can be generated by addition of exogenous lysolecithins or short chain lecithins [1]. The cells within these masses form close junctions [1] and are electrically coupled [2]. Spontaneous aggregation of *A. castellanii* cells occurs in older cultures. Again electrotonic coupling is measured [2]. It was tempting to correlate the spontaneous aggregation and formation of communicative contacts in older cultures to the phenomena artificially induced by exogenous lysolecithin.

3',5'-Cyclic adenosine monophosphate (cAMP), an intermediate in the action of many hormones [3] has been reported to be involved in the regulation of cellular growth by inducing respectively restoring contact inhibition of cell proliferation [4–15]. Further-more, cAMP plays a significant part in the control of intercellular communication by influencing the permeability properties of the junctional and non-junctional membrane parts [16], while lysophospholipids are able to induce the formation of communicative contacts [2]. Thus it was appropriate to study the influence of cAMP on the phospholipase A activity, which is indeed stimulated at pH 5.5 in the presence of cAMP.

MATERIALS AND METHODS

Cells

A. castellanii was grown axenically in an optimal growth medium as described by Neff [17]. At a given population density (cells/ml growth medium), the cells were harvested from the growth medium by centrifugation at 600 g for 5 min and washed 3 times in minimal medium by resuspension and centrifugation. The minimal medium was a 67 mM so-

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dium phosphate buffer of pH 5.5 to which 83 mM D(+)-glucose and 0.5 mM $CaCl_2$ had been added. The final pellet was suspended to a concentration of 2×10^7 cells/ml in a 10 mM Tris-HCl buffer of the required pH value. After swelling for 15 min the cells were broken by some gentle strokes with a tight-fitting Dounce homogenizer. The suspension of broken cells was used as enzyme source.

Incubation procedures

(a) *Phospholipase A activity.* The incubation mixture consisted of 10 mM Tris-HCl of the required pH values, 10 mM $CaCl_2$, 10 mM $MgCl_2$, and an aliquot of the suspension of homogenized *A. castellanii* cells in a total volume of 5 ml. Different samples were obtained by adding either 1 mM cAMP, 0.1 mM theophyllin or 1 mM cAMP+0.1 mM theophyllin. After 20 min incubation at 37°C in a shaking water bath the reactions were stopped by addition of 3 vol of chloroform/methanol (1 : 2, v/v).

(b) *Lysolecithin incorporation into the membrane.* The amoebae were washed twice in minimal medium and then suspended in that medium to a concentration of 25×10^6 cells/ml. One ml of this suspension was mixed with a dispersion of 1-palmitoyl-*sn*-glycero-3-phosphoryl-[^{14}C] choline in minimal medium of various concentrations. The incubation time at room temperature was varied. The reaction was stopped by centrifugation of an aliquot of the suspension for 30 sec at 600 g in a Beckman 152 microfuge. One tenth ml of the supernatant as well as 0.1 ml of the original suspension was transferred into different scintillation vials and dissolved in a scintillation mixture consisting of insta-gel (Packard) and water. Radioactivity measurements were done in a Packard scintillation spectrometer Model 2425.

Extraction, fractionation and determination of the phospholipids

Lipids were extracted according to the method of Bligh & Dyer [18]. The chloroform extract was dried under reduced pressure and the residue was taken up in a known volume of chloroform/methanol (2 : 1, v/v). The separation of the lipid extract was achieved by way of two-dimensional thin layer chromatography on silica gel HR 60, with 2 % (w/w) Mg-silicate (Merck, Darmstadt). The dimensions of the thin-layer plates was 20×20 cm. The solvent system in the first direction consisted of chloroform/methanol/ammonia/water, 90 : 54 : 5.5 : 5.5 (v/v/v/v) In the second direction chloroform/methanol/acetic acid/water, 90 : 40 : 12 : 2 (v/v/v/v) was used. The TLC-solvents were run 17 cm in each direction. Lipids were made visible by staining with iodine and ninhydrin. Special attention was payed to identify the lyso-PC spot using a synthetic 1-palmitoyl-*sn*-glycero-3-phosphoryl[^{14}C] choline as a tracer. The added tracer could only be detected in the lyso-PC spot and not in the other spots. A quantitative phosphorous analysis of the single spots was obtained by applying a modified method of Fiske & Subbarow [19].

Isolation of plasma membranes

Plasma membranes of *A. castellanii* were isolated following the procedure described by Ulsamer et al. [20].

Electron microscopy

The cells were fixed for 1 h in a trialdehyde–DMSO fixative in 0.1 M NaH_2PO_4–Na_2HPO_4 buffer with pH 7.4 (3 % glutaraldehyde; 2 % formaldehyde; 1 % acrolein and 2.5 % DMSO). The fixed cells were rinsed in phosphate buffer—four changes over 1 h—and postfixed in 1 % OsO_4 containing the same phosphate buffer. Fixation and washing were carried out at room temperature.

Dehydration was done in graded acetone and embedding in Araldite. Thin sections were cut on a Reichert OM U-2 ultramicrotome and stained for 5 min with a saturated solution of uranyl acetate followed by 1 min staining with lead citrate (50 mg in 25 ml 0.01 N NaOH). The membrane fractions from *A. castellanii* were prepared for electron microscopy in the same way as the whole cells. Electron micrographs were taken with a Philips EM 200.

Substrates

1-Palmitoyl-*sn*-glycero-3-phosphoryl-[^{14}C] choline was obtained by degration of a synthetic 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoryl-[^{14}C]choline with phospholipase A from pancreas, kindly supplied by Professor G. H. de Haas (Utrecht), and purified from the reaction mixture by thin layer chromatography, using the same procedure as described above.

Adenosine 3′,5′-cyclic-monophosphoric acid as well as 1,3 dimethyl-xanthine (theophyllin) were purchased from Sigma Chemical Co.

Diisopropylphosphofluoridate (DFP) was obtained from Aldrich-Europe, Belgium.

RESULTS AND DISCUSSION

If *A. castellanii* cells are incubated with 1 500 μg palmitoyllysolecithin/25×10^6 cells an equilibrium state is reached within 2 min in which 50 % of the added lysophospholipid is incorporated into the membrane, and 50 % still stays in the supernatant. This equilibrium state lasts for at least 30 min (fig. 1). If *A. castellanii* is incubated with different concentrations of the labelled lysophospholipid the same equilibrium state with a partition coefficient of 1 is always reached (fig. 2). A change in cell concentration gives the same results (fig. 3). However, if the lysolecithin concentration increases above 2 000 μg pal-
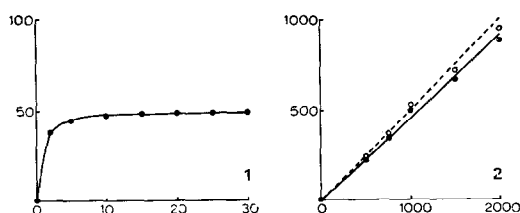
Fig. 1. Abscissa: time (min); *ordinate:* % LPC incorporated.

The percentage of palmitoyllysolecithin (LPC) incorporated into the membrane of *A. castellanii* is plotted against time. 25×10^6 cells are suspended in an aqueous dispersion of 1-palmitoyl-*sn*-glycero-3-phosphoryl-[^{14}C]choline (1 500 μg/25×10^6 cells). Total volume: 3 ml.

Fig. 2. Abscissa: μg LPC/25×10^6 cells; *ordinate:* μg LPC incorporated. ●, Uncorrected; ○, corrected for cell lysis.

The amount of palmitoyllysolecithin (LPC) incorporated into the membrane of *A. castellanii* is plotted against the concentration of LPC. 25×10^6 cells are suspended in an aqueous dispersion of 1-palmitoyl-*sn*-glycero-3-phosphoryl-[^{14}C]choline of different concentrations. Incubation time: 20 min. Total volume: 1.5 ml.

Table 1. *Lipid composition of whole amoebae cells as a percentage of total phospholipids*

These results are the mean values of four determinations. In parentheses: S.D.

	4-day culture	8-day culture
Origin	0.5 %	1.9 %
Lyso-PC	1.3 % (1.0)	7.6 % (2.3)
Lyso-PE	1.3 % (0.4)	3.1 % (0.6)
PC	42.7 % (5.4)	41.8 % (10.9)
PE	30.4 % (1.0)	18.4 % (1.2)
PI	3.6 % (2.0)	5.7 % (3.2)
PS	8.3 % (0.7)	7.3 % (1.1)
O1	1.9 % (1.1)	6.8 % (1.9)
O2	2.0 % (1.6)	1.9 % (0.9)
O3	1.0 % (0.1)	1.8 % (0.7)
F1–F3	7.0 % (2.0)	3.7 % (1.7)

For an overall identification of the spots see [28].

mitoyllysolecithin/25×10^6 cells a considerable amount of cell lysis is induced as can be seen from the decline in fig. 3. Spies et al. [1] assayed for viability of the *A. castellanii* cells after treatment with different lysolecithin concentrations. The results were used for cell lysis correction.

Aggregation into cell clumps of cells of a 4-day-old culture of *A. castellanii* occurred
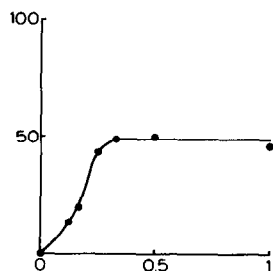

Fig. 3. Abscissa: cells/ml (25×10^6); *ordinate:* % LPC incorporated.

The percentage of palmitoyllysolecithin (LPC) incorporated into the membrane of *A. castellanii* is plotted against the cell concentration. Different amounts of *A. castellanii* cells are suspended in an aqueous dispersion containing 500 μg 1-palmitoyl-*sn*-glycero-3-phosphoryl-[^{14}C]choline. Incubation time: 20 min. Total volume: 1.5 ml.

after treatment with sublytical concentrations of palmitoyllysolecithin. Electrophysiological experiments revealed the existence of electrotonic spread within such a multicellular mass of aggregated cells. This means that palmitoyllysolecithin not only enables *A. castellanii* cells to aggregate, but also that communicative contacts are formed which allow ions to pass from one amoeba to the other as a result of an increased permeability at these specific contacts. The morphological basis of the contacts is rather similar to tight, focal-tight, and close junctions.

The phenomena due to incubation of *A. castellanii* with lysophospholipids occur, however, spontaneously in cultures which reached a high population density after 7 to 8 days growth (2×10^6 cells/ml). In order to investigate whether hydrolysis of endogenous phospholipids is at the basis of the spontaneous aggregation and formation of communicative contacts, the phospholipid composition of cultures grown for 4 days was compared with that of cultures grown for 8 days. The separation of the total lipid extracts by two-dimensional thin layer chromatography is demonstrated in fig. 4, while the results of the quantitative analysis of the
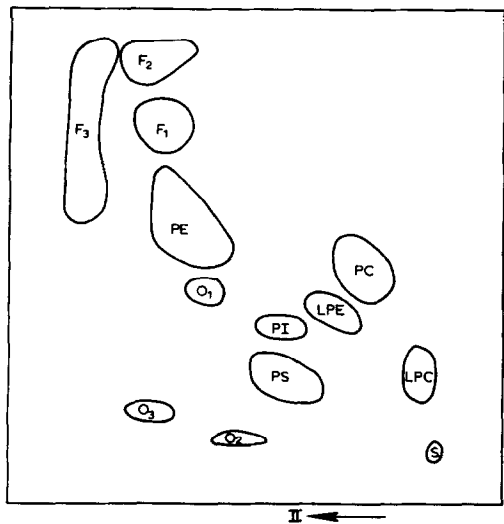
Fig. 4. Two-dimensional TLC on silica gel HR 60, with 2% (w/w) Mg-silicate was applied to separate the lipid extracts. *s*, origin; *LPC*, lysophosphatidylcholine; *LPE*, lysophosphatidylethanolamine; *PC*, phosphatidylcholine; *PE*, phosphatidylethanolamine; *PI*, phosphatidylinositol; *PS*, phosphatidylserine; O_1, O_2 and O_3: unknown; F_1, F_2 and F_3: unknown.

lipid composition of whole amoebae cells are shown in table 1 and fig. 5. The difference in the amount of lysophosphatidylcholine as well as lysophosphatidylethanolamine in these two groups is significant. Apparently the lysophospholipid content in cultures in the logarithmic multiplication state is low, while during the phase of population-growth deceleration, increased levels are found. Furthermore it is notable that the amount of

phosphatidylethanolamine decreases as the population density increases.

The formation of lysophospholipids might be achieved by the action of aspecific hydrolysing enzymes or by a specific phospholipase A activity. Incubation of homogenized *A. castellanii* cells at pH 5.5 and 8.0 revealed that addition of diisopropylphosphofluoridate (DFP) did not inhibit hydrolysis of the phospholipids suggsting that this effect is indeed reached by a specific phospholipase A activity [21]. The phospholipase A activity at pH values between 5.5 and 8.0 is almost the same. This phospholipase A activity, however, seems to be correlated with the population density of the culture as shown in fig. 6. This effect is seen at both pH values but it is more pronounced if 10 mM sodium deoxycholate is added to the incubation medium.

Ulsamer et al. [22] also analysed the lipid composition from the whole amoeba cell. They concluded that only 0.5% of the total phospholipid present was lysophosphatidylcholine. No lysophosphatidylethanolamine could be detected. Except for these amounts of lysophospholipid our results agree with the results of Ulsamer et al. The population density of the cultures used for chemical analyses also agree with each other. Besides, Ulsamer et al. [20] made an extensive analysis of the plasma and phagosome mem-
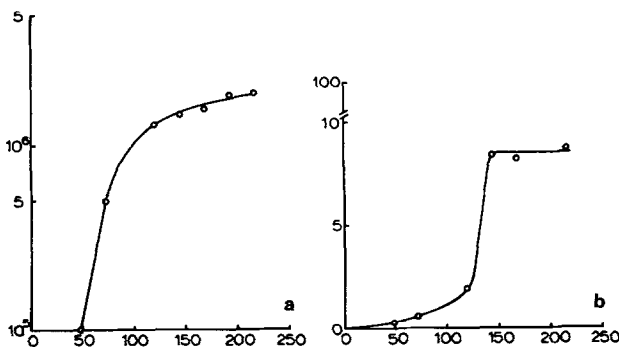

Fig. 5. Abscissa: culture age (hours); *ordinate:* (*a*) population density; (*b*) % lyso-PC.

The population density of a 10 l culture is plotted against the culture age;

The percentage of lysophosphatidylcholine (LPC) present in the lipid extract of whole amoebae cells is plotted against the culture age. From the 10 l culture at different times an aliquot was taken in order to determine the phospholipid composition.
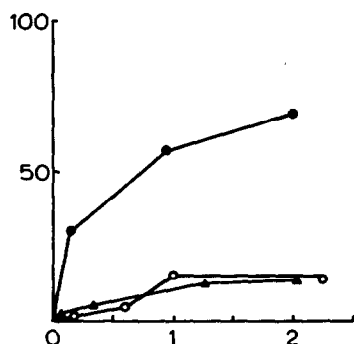
Fig. 6. *Abscissa:* population density (cells/ml × 10⁶); *ordinate:* % of hydrolysed endogenous PC.

The percentage of endogenous phosphatidylcholine hydrolysed in homogenates of *A. castellanii* is plotted against the population density (cells/ml growth medium) of the culture. The incubation mixture consisted of 10 mM Tris-HCl of a particular pH value, 10 mM CaCl₂, 10 mM MgCl₂. pH: ○, 5.5; △, 8.0; ●, 8.0. Besides 10 mM sodium deoxycholate was added to the incubation mixture. After a 20 min incubation at 37°C the incubation was stopped by the addition of 3 vol of chloroform/methanol (1 : 2, v/v).

branes of *A. castellanii*. They did not detect any lysophospholipids in the plasma membranes of cells of 5- to 7-day-old cultures, whereas about 4.5 % lysophosphatidylcholine was present in the phospholipids of the phagosome membranes. Preliminary results of our analyses of the plasma membrane of *A. castellanii*, isolated in the same way as described by Ulsamer (see fig. 7), show high (up to 20 %) percentages of lysophospholipid present in that membrane, dependent on the population density. We want to emphasize, however, that it was impossible to block the enzyme activities during the long isolation procedure of the plasma membranes. Incubation of exogenous radioactive lecithin (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoryl-[¹⁴C]choline) at pH 5.5 with an aliquot of the plasma membrane fraction during 20 min at 37°C resulted in 40–50% hydrolysis if 10 mM sodium deoxycholate was present. This obviously demonstrates that a phospholipase A is present, whose activity is significantly dependent on the population density. A pos-

sible explanation for the differences between our results and those obtained by Ulsamer et al. may be found in differences in culture conditions. The differences in plasma membrane composition might also be related to minor preparation differences, especially in the homogenization step, which could lead to fractions with increased phospholipase A content and differences in lipid composition. Besides, the separation procedure of the phospholipid extract was quite different. In the present study the separation was achieved by way of two-dimensional thin layer chromatography. Ulsamer et al. first used a column of silicic acid in order to get a rough separation between neutral and polar lipids; then one-dimensional thin layer chromatography was applied to separate within each class. The column chromatography may well be responsible for the failure to find lysophosphatidylcholine and lysophosphatidylethanolamine. To overcome problems posed by a possible formation of lysophospholipids during the extraction and separation procedure [¹⁴C]-labelled lecithin (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoryl-[¹⁴C]choline: spec. act. 88210 dpm/µl) was added to the cell/membrane suspension. No radioactivity whatsoever was detected in the lysolecithin-spot.

It is remarkable that, particularly in older cultures with a high population density, aggregation and formation of communicative contacts occur spontaneously. Initially this was explained as a result from lysosomal enzyme activity of lysed cells in older cultures. This might increase the lysophospholipid content of the medium and therefore of the cell membrane of the remaining intact cells [1, 2]. Our present data, however, clearly indicate that increased phospholipase A activity in older cultures, due to increase in population density, is responsible for an enhancement of the lysophospholipid content
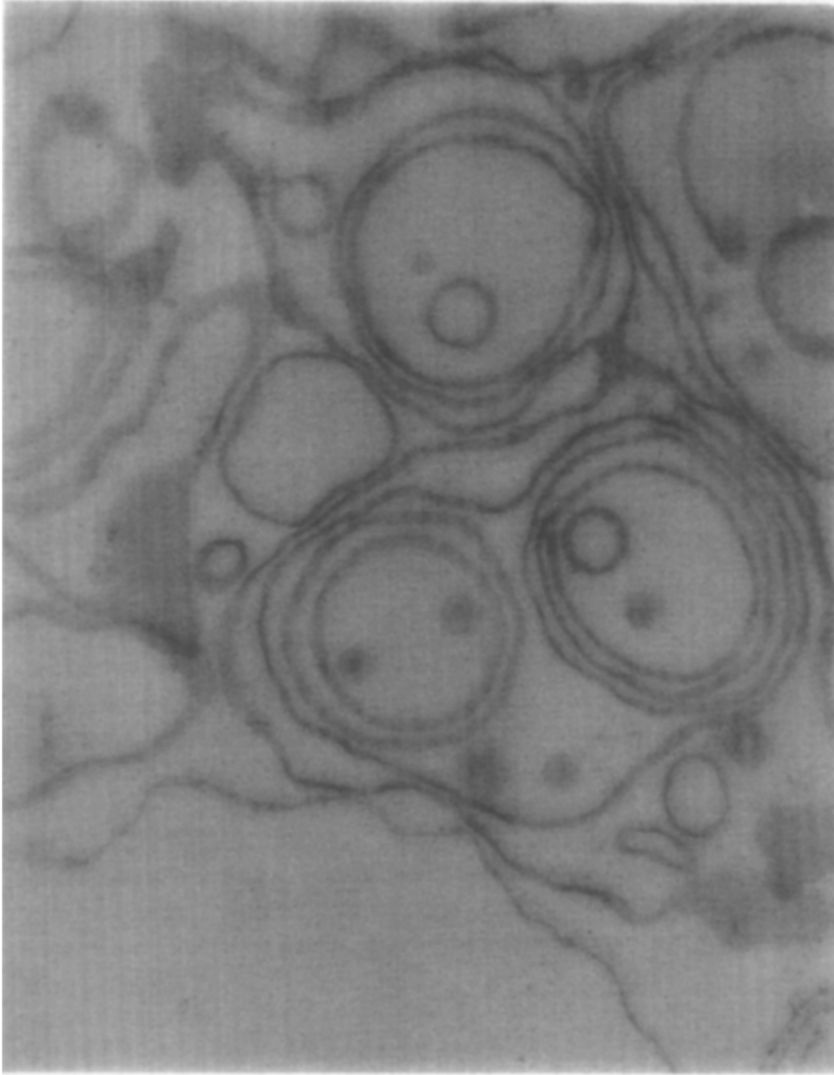
Fig. 7. Plasma membrane fraction of *A. castellanii.*

of the plasma membrane of *A. castellanii* cells in older cultures. This results in aggregation of these cells in a way that communicative contacts are formed.

This hypothesis can be supported by the approach on the ultrastructural level of the above-mentioned phenomenon. *A. castellanii* cells from an aerated culture, grown for 3 days in the optimal growth medium, were allowed to stand for 2 h without shaking or aerating. These cells which are in fact actively dividing cells from the logarithmic multiplication state can now be induced to behave as cells from older populations, due to the artificially created increase of the population density. Fig. 8 shows sedimented amoebae in close contact. These contact areas can be fully compared with the close junctions which are found after treating the amoebae with lysolecithins. Our claim that
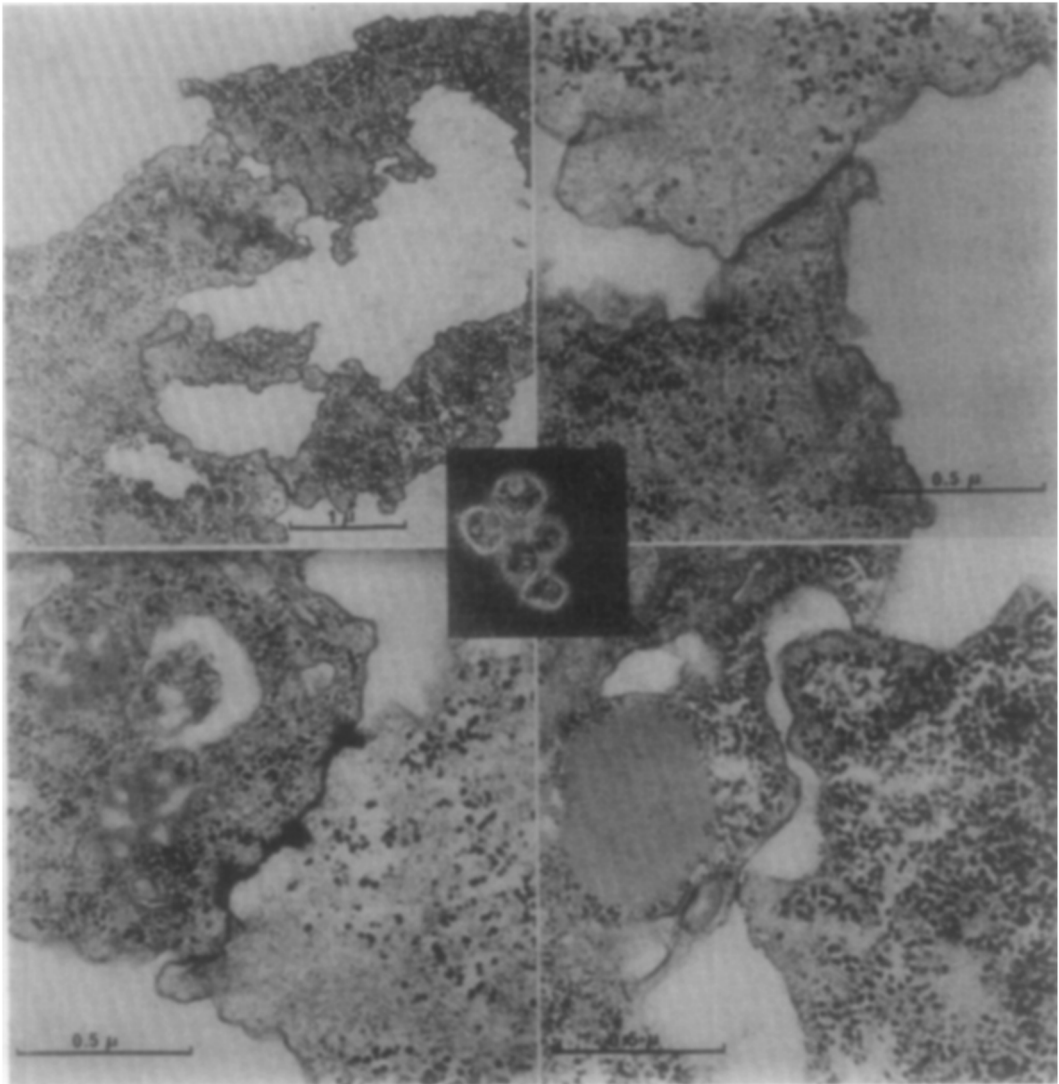
Fig. 8. A. castellanii are allowed to aggregate spontaneously due to an artificially induced increase in population density. Close junctions and focal tight junctions are formed at the contact area of two apposed cell membranes. *Inset:* a mass of aggregated *A. castellanii* cells as seen under a phase contrast microscope.

those junctions are communicative contacts was confirmed by Pigon & Morita [23]. They showed exchange of macromolecular material between individual amoebae (*Hartmannella castellanii*) when these are in contact with each other. *Hartmannella castellanii* in contact constitute similar junctions as artificially or spontaneously aggregated *A. castellanii* cells.

As mentioned before, both cAMP and lysophospholipids are involved within the regulatory system of intercellular communication. In order to study the influence of cAMP on the phospholipase A activity, homogenates of *A. castellanii* were incubated at pH 8.0 with either cAMP, theophyllin, an inhibitor of phosphodiesterase activity [24], or both. No alteration of the phospholipase

Table 2. *Hydrolysis of endogenous phosphatidylcholine by A. castellanii cells*

The control incubation mixture consisted of 10 mM Tris-HCl of a particular pH-value, 10 mM $CaCl_2$ and contained when indicated 10 mM $MgCl_2$ or 10 mM sodium deoxycholate. The amount of cAMP, if present, was 1 mM; the amount of theophyllin 0.1 mM. The results are expressed as the ratio of the percentage of hydrolysed endogenous phosphatidylcholine in the sample and the percentage of hydrolysed phosphatidylcholine in the control

pH	Na-DOC	$MgCl_2$	%PC hydrolysed[sample]/ %PC hydrolysed[control]			
			control	cAMP	Theophyllin	cAMP + Theophyllin
8.0	−	−	1.0	0.97	—	1.06
8.0	+	−	1.0	1.01	1.04	1.01
8.0	−	+	1.0	0.97	1.25	1.06
5.5	−	+	1.0	1.30	1.05	1.34
5.5	−	+	1.0	1.32	1.10	1.37
5.5	−	+	1.0	1.49	1.10	1.61

A activity was obtained (table 2). Addition of 10 mM sodium deoxycholate to the incubation medium did increase the phospholipase A activity, but no particular stimulation by cAMP, theophyllin or cAMP + theophyllin could be observed. Also the addition of Mg^{2+}-ions had no effect. If, however, cell homogenates are incubated at pH 5.5—the pH value of the growth medium of *A. castellanii*—then addition of cAMP or the combined action of cAMP + theophyllin results in a stimulation of the phospholipase A activity by about 40 %. Addition of theophyllin alone gives a slight increase in activity (table 2). A stimulation of phospholipase A by cAMP was also shown by Chiappe de Cingolani et al. [25] for isolated fat cells of rats and for rat lung tissue by Imre [26]. Although the observed stimulation of the phospholipase A activity in *A. castellanii* as well as in the fat cells and lung tissue is quite low, it may have considerable physiological consequences with respect to the regulation of intercellular communication in organisms, especially if a local action on a particular part of the membrane is presumed. Within our hypothesis concerning the regulation of intercellular communication, the intracellular cAMP level is supposed to

be crucial. An increased cAMP concentration, due to hormonal activation of an adenyl cyclase system may indirectly and/or directly activate the conversion of an inactive precursor of a membrane-bound phospholipase A, situated on the contact area of two adjacent cells, into an active phospholipase A. The permeability of the junctional membrane part will increase due to the increase of the lysophospholipid content of that particular part of the membrane. This results in an increase of the intercellular communication. Within the control mechanism of cell communication, the concentration of divalent ions (Ca^{2+}- and Mg^{2+}-ions) plays a prominent part in the concerted action of the involved enzymes. Increased levels of these ions, due to mobilization from intracellular pools by high cAMP concentration, will act as a feed-back inhibitor of further adenyl cyclase activation [27]. Conversion of lysophospholipids by (an) acyltransferase(s) will induce a decrease in intercellular communication under these circumstances.

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