STUDIES ON STREAMING

I. Inhibition of Protoplasmic Streaming and Cytokinesis of *Chaos chaos* by Adenosine Triphosphate and Reversal by Magnesium and Calcium Ions

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ABSTRACT

In *Chaos chaos* streaming, motility, and cytokinesis were inhibited nearly 100% for several hours by 2.5–5 mM sodium adenosine triphosphate (ATP)[1] added to culture fluid. All three effects were completely prevented by the addition of equimolar Mg^{++} or Ca^{++} ions but not Na^{+} to the ATP/culture fluid solution. The effects of ATP were not reproduced by EDTA, EGTA, colchicine, or AMP. Sodium pyrophosphate produced about 50% inhibition at 5 mM. Studies with ^{14}C-ATP showed that 5×10^{-5} to 5×10^{-4} mmole of ATP was firmly associated with each milliliter of packed cells after an hour's incubation at 24°C. Labeling studies also showed that prevention of the ATP effects by Mg^{++} ions was not due to a decrease in the amount of ATP associated with the cells.

INTRODUCTION

Adenosine triphosphate has been shown to produce several striking effects on the cytoplasmic streaming systems of *Physarum polycephalum*, *Amoeba proteus*, and *Chaos chaos* and to be present in *Physarum* (Hatano and Takeuchi, 1960). Effects in vivo and on in vitro preparations have been reported (Kriszat, 1950; Loewy, 1952; Marsland, 1956; Kamiya, 1959, 1964; Nakajima, 1960; Goldacre, 1964; Wolpert et al., 1964; Nachmias, 1968). However, the role of ATP in normal streaming is still uncertain.

During the course of some experiments on the inhibition of streaming of *Chaos* by pinocytosis inducers, it was found that ATP also inhibited streaming, and that this inhibition appeared to be released by calcium salts (Nachmias, 1968). The purpose of the present paper is to document these findings in more detail, and to show that not only calcium, but also magnesium salts antagonize the effect of ATP. An antagonism of this sort has not been previously reported, as far as the author is aware, yet it might have interesting implications for the regulation of ATP action in streaming and cytokinesis.

[1] Abbreviations used in this paper are as follows: ATP, adenosine triphosphate; AMP, adenosine monophosphate; ITP, inosine triphosphate; EDTA, ethylene diamine tetraacetate; EGTA, ethyleneglycol-bis-(β-amino ethyl ether) *N*,*N*′ tetraacetate; cf, culture fluid.

MATERIAL AND METHODS

Chaos chaos was cultured on *Paramecium aurelia*. The culture fluid (cf) contained 5×10^{-4} M $Ca(NO_3)_2$; 5×10^{-5} M $MgSO_4$; 1.6×10^{-4} M K_2HPO_4; 1.1×10^{-4} M KH_2PO_4 (pH 6.8).

Cytokinesis Experiments

Cells judged to be at stages between early prophase and late metaphase (Kudo, 1947; Roth and Daniels, 1962; Feldherr, 1966) were selected with braking pipets and gently deposited singly in depression slides containing cf alone or with appropriate additions. 3 hr later the depressions were scored for cell divisions. Since *Chaos* undergoes plasmotomy into 2–8 individuals, only the occurrence of cell division was scored, not the total number of daughter cells produced. Fifteen cells were used for each condition in each experiment; one group of 15 cells was run as a control each day. Only experiments in which 50% or more of control cells divided were used to obtain data.

Studies of Streaming

20 vigorously streaming cells were removed from 2-day starving cultures with braking pipets and deposited in 0.02 ml of cf in siliconed 1-ml watch glasses. 1 ml of cf alone or with appropriate additions was added, dispersing the cells. The cells were then recentered in the dish and examined every 5 min for 30 min with a low power binocular microscope illuminated so as to give a good view of the streaming in the cytoplasm. Cells were rechecked at 90 or 120 min. Cell shape, round and smooth, round with short, abortive pseudopods, monopodal, polypodal, was noted as well as the presence or absence of visible streaming. In control cells, streaming was typically visible in all cells throughout the experiment and cells were all either polypodal or, rarely, monopodal.

Labeling Experiments with Individual Cell Technique

For ^{14}C-ATP, experiment 1, 25 or 50 actively streaming cells in, respectively, 0.02 and 0.04 ml of cf were added to 5 mM ATP in cf or cf + 5 mM Ca Cl_2 containing ^{14}C-ATP and were then incubated undisturbed at room temperature for 80 min. The cells were then removed to the cold room to arrest streaming and to facilitate handling. The cells were washed three times in 30 ml of cf at 5°C by allowing the individual cells to fall through the solution. The cells were retrieved from the bottom of the 50-ml beaker and recollected in 0.04 ml of cf. This procedure was repeated twice more. Efficiency of the wash was tested by counting the third wash solution; the count

TABLE I a

Typical Experiment on the Inhibition of Streaming by ATP and the Effect of Na^+, Ca^{++}, and Mg^{++} Ions

ATP and all salts were present at 5 mM, pH 6.8. 18–25 cells were used for each condition. S = % streaming, polypodal; NS = % rounded, not streaming.

	cf alone		cf + ATP		cf + ATP + Na		cf + ATP + Mg		cf + ATP + Ca	
Time	S	NS	S	NS	S	NS	S	NS	S	NS
min										
10	100	0	6–11	94–89	5–10	95–90	96	4	100	0
20	100	0	0	100	0	100	96	4	100	0
90	100	0	6	94	0	100	118*	0	100	0
Lysed	0		0	0	0	0	0		0	

* NS cell was in mitosis; divided into 4.

TABLE I b

Temporary Inhibition of Streaming by 5 mM EDTA and 5 mM EGTA (Mean of Two Observations Each on 20 Cells)

S = % cells streaming, polypodal; NS = % cells rounded with no or very slight streaming; Lysed = % cells ruptured; no recovery in normal cf.

	cf alone			5 mM EDTA			5 mM EGTA		
Time	S	NS	Lysed	S	NS	Lysed	S	NS	Lysed
min									
5	100	0	0	0	95	5	95	0	5
10	100	0	0	52.5	42.5	5	0	95	5
20	100	0	0	85	5	10	95	0	5
90	100	0	0	65	17	17	85	0	15

was equal to that of background. The cells were then lysed as described below.

Labeling Experiments with Packed Cells

For ^{14}C-ATP, experiments 2–4, mass cultures of starving cells were collected, washed several times in cold cf, and packed at low centrifugal speed at 5°C. Then 25 or 50 lambdas of packed cells were slowly removed with prechilled pipets and added to flasks containing 2 ml of cf alone or with added divalent ions. The flasks were then returned to room temperature. After the cells had resumed normal streaming (10–20 min), ATP containing 0.5–1.8 μC ^{14}C ATP was added to give a final concentration of 5 mM ATP. The flasks were then shaken (80 rotations/minute) or tilted (20 tilts/minute) for 60–120 min. In three experiments the same amount of labeled ATP was added to control flasks at the end of the incubation period. In one experiment a control flask was kept in the cold room throughout the incubation period. At the end of the incubation, 10 ml of 5°C cf were rapidly added to each flask, the cells were collected in the cold room by a low speed centrifugation, and supernatant was removed down to 0.1 ml. This wash was repeated twice more. The final wash was again checked for residual counts, and the count was indistinguishable from that of the background. The last wash was removed to 0.1 ml and the packed cells were lysed by the addition of 0.1 ml of 1 M NaOH. After 5 min, 10 ml of dioxane scintillation fluid (Bray, 1960) were added. Total ^{14}C was counted in a Packard model 314 EX scintillation spectrometer (Packard Instruments Co., Inc., Downers Grove, Ill.). Samples were counted three times and the three readings were averaged for each vessel. A check with a toluene standard showed an efficiency of 46% and absence of quenching of an internal standard.

Chemicals

Adenosine triphosphate and ethylene diamine tetraacetate were purchased as the disodium salts from Sigma Chemical Co., St. Louis, Mo. ATP was stored dessicated at −20°C. Stock 0.05 M solutions (neutralized to pH 6.8) were stored at −20°C in aliquots. EGTA (ethyleneglycol-bis-(β-amino-ethyl ether) *N,N'*-tetraacetic acid) was a product of Lamont Laboratories. All salts were reagent grade. Ion-free water was used throughout, both for cultures and for all solutions. All test solutions were made up in culture fluid from neutralized stock solutions. Tetralithium ^{14}C-ATP (uniformly labeled) was a product of New England Nuclear Corp., Boston, Mass., and had a specific activity of 418 mC/mmole. It was stored as a 50 mC/ml solution in 50% ethanol at −20°C. On the basis of a destruction rate of 0.5% per month, a maximal destruction of 5% could have occurred during the course of the experiments.

RESULTS

Table Ia summarizes a typical experiment in which the effect of ATP on streaming in *Chaos* was observed. Table II gives the results of experiments in which the effects of ATP and other compounds on cell division were scored. These two tables show clearly that 2.5 or 5 mM ATP in cf strongly inhibited normal streaming, pseudopod formation, and cytokinesis. The inhibition was nearly 100% effective and lasted for several hours without

TABLE II

Cytokinesis Experiments

All concentrations are expressed as millimolar. 15 cells were used for each condition.

Expt No.	Conditions	% cytokinesis	% lysis
1	cf	60	0
	2.5 NaATP	0	0
2	cf	53	0
	2.5 NaATP	6.7	0
	2.5 NaATP + 2.5 Mg^{++}	67	0
	2.5 NaATP + 2.5 Mg^{++} + 1 EGTA	40	0
3	cf	87	0
	2.5 NaATP	0	7
	2.5 NaATP + 2.5 Ca^{++}	60	0
	2.5 NaATP + 2.5 Sr^{++}	53	0
4	cf	53	0
	2.5 Na EGTA	73	0
	5 Na EGTA	80	0
5	cf	87	0
	2.5 Na EDTA	60	40
	2.5 Na EGTA	60	0
Mean cytokinesis in controls of NaATP experiments		66%	
Mean cytokinesis with 2.5 mM ATP		2.2%	
Mean cytokinesis with 2.5 mM ATP + divalent ions		60%	
Mean % inhibition by ATP	$\frac{cf - ATP}{cf} \times 100 = 96.6\%$		
Mean % inhibition in all EGTA experiments = 5.5%			
6	cf	66*	0
	2.5 AMP	64*	0
	5 AMP	44*	0
7	cf	71	0
	2.5 Na pyrophosphate	38	0
	5 " "	27	0
8	cf	73.5*	0
	2.5 colchicine	76.5*	0

* Mean of two experiments

producing lysis. Frequently a large vacuole was seen in the cells after an hour. This finding is in agreement with previous observations on the effect of ATP on streaming in amebae (Kriszat, 1950; Marsland, 1956), although the author is not aware that the effect on cytokinesis has been previously noted. Both manifestations of the ATP effect were prevented by equimolar Mg^{++} or Ca^{++} ions as also shown in Tables I and II. Although a stepwise titration of the effect has not been done, it was found in the streaming assay that 0.5 mM Mg^{++} was ineffective in reversing 5 mM ATP, although 1.25 mM Mg^{++} did reverse the effect of 2.5 mM ATP. Thus a strict 1:1 molar ratio is not necessary. It was also found that 5 mM Mg^{++}, Ca^{++} or Sr^{++} ions alone when added to cf produced no discernible effect on streaming. Preliminary results on the movement of amebae in a controlled negative phototaxis situation also indicated that ATP prevented attachment and movement, whereas added equimolar Mg^{++} restored the ability of the cells to move away from the light at a normal rate.

Fig. 1 shows the typical appearance of three cells incubated for 1 hr with 5 mM ATP/cf and one cell incubated for 1 hr with 5 mM ATP/cf containing, in addition, 5 mM $MgCl_2$. The cells were fixed and then photographed together. The three rounded cells showed no progressive streaming, but small amounts of movement of the cytoplasm could be seen in some of the short pseudopodia.

Since ATP chelates both Ca^{++} and Mg^{++}, the effect of two highly efficient chelators, EDTA and EGTA, on streaming and cell division was also studied. Typical results are shown in Tables Ib and II. Both EDTA and EGTA produced a temporary inhibition of streaming which lasted only about 20 min in the case of EGTA, although a small effect of EDTA was found in some experiments after 90 min. About 15–20% of the EDTA-treated cells lysed, although most of the remaining

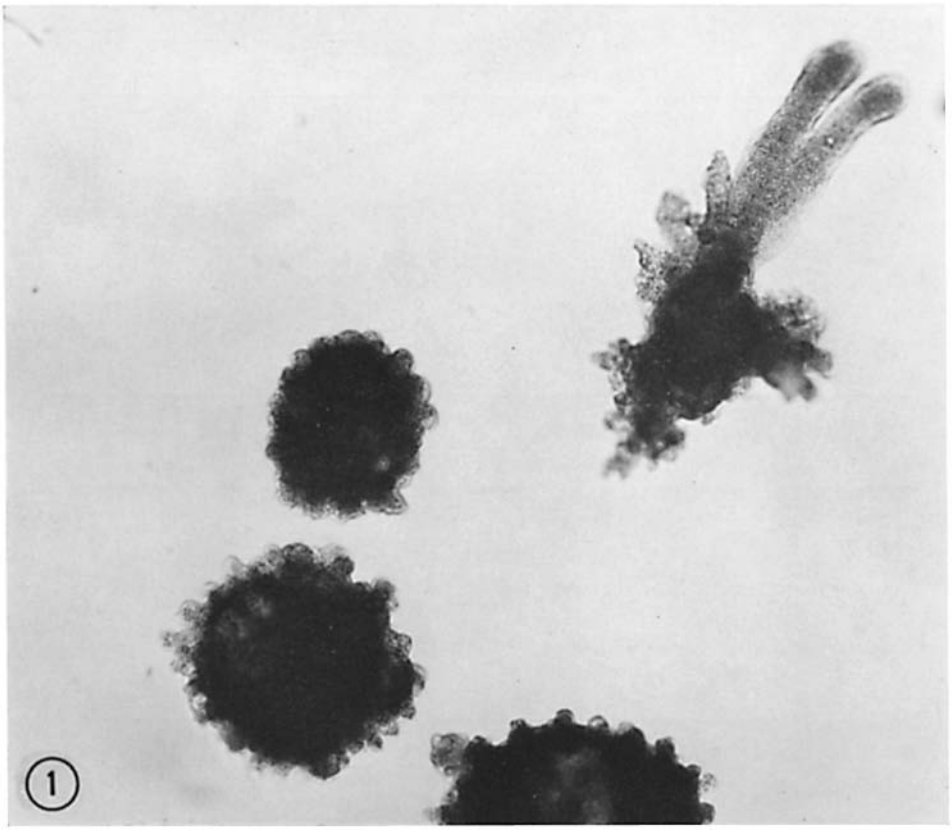

FIGURE 1 Cell at upper right incubated for 1 hr with 5 mM ATP/cf plus 5 mM $MgCl_2$. Three cells at lower left incubated with 5 mM ATP/cf alone. × 125.

TABLE III

Uptake of ^{14}C-ATP by Cells Incubated in ATP/cf and in ATP/cf plus Divalent Ions

Expt No. 1 No. of cells	0.62 μc ^{14}C-ATP/flask Conditions	80 min incub 24°C Appearance	With occasional swirl	
			*cpm**	*cpm/10^3 cells/hr*
24	5 mM ATP/cf	rd, ō str	14	436
49	" "	" "	25	383
22	" "	" "	10	341
60	" "	" "	30	375
24	5 mM ATP/5 mM Ca^{++}/cf	pp, str	12	375
49	" "	" "	25	382

Mean cpm/10^3 cells/hr in ATP/cf: 384
Mean cpm/10^3 cells/hr in ATP/Ca cf: 378

Expt No. 2 No. of cells × 10^3‡	1 μc ^{14}C-ATP/flask Conditions	60 min incub 24°C Appearance	80 rotations/min	
			cpm	*cpm*/10^3 cells/hr*
1	5 mM ATP/cf	rd, ō str	365	343
2	" "	" "	848	413
2	5 mM ATP *cold* incub	" "	299	138
2	5 mM ATP added at end expt	pp, str	89	34
—	Wash solution No. 3	—	22	—

Expt No. 3 No. of cells × 10^3	0.6 μc^{14}/C-ATP/flask Conditions	60 min incub 24°C Appearance	80 rotations/min	
			cpm	*cpm*/10^3 cells/hr*
1	5 mM ATP/cf	rd, ō str	153	130
1	" "	" "	152	129
1	5 mM ATP/5 mM Mg/cf	pp, str	130	107
1	" "	" "	164	141
1	5 mM ATP at end	" "	43	20
1	" "	" "	70	47
—	Wash solution No. 3	—	23	—

Mean cpm/10^3 cells/hr in cf: 130; with Mg/cf: 124

Expt No. 4 No. of cells × 10^3	1.8 μc ^{14}C ATP/flask Conditions	120 min incub 24°C Appearance	15 tilts/min	
			cpm	*cpm*/10^3 cells/hr*
2	5 mM ATP/cf	rd, ō str	1003	244
2	" "	" "	1542	372
2	5 mM ATP + 5 mM Mg/cf	pp, str	978	238
2	" " cc	" "	1653	407
2	5 mM ATP added at end	" "	317	73
2	5 mM ATP/Mg cf added at end	" "	174	37
—	cf alone	—	26	—

Mean cpm/10^3 cells/hr with Mg: 308; with Mg: 322

* = cpm minus background.
‡ = direct counts showed mean of 400 cells/10λ packed cells; hence conversion of 10^3 cells/25λ used for mass cell experiments.
rd, ō str = cells rounded up, little or no streaming visible; after an hour large vacuole often visible.
pp, str = cells polypodal, streaming appears normal.

cells exhibited normal or nearly normal streaming for 1–2 hr thereafter. As Table II shows, EDTA and EGTA had only a slight effect, if any, on cytokinesis. EGTA did not produce any lysis in the cytokinesis experiments, and often produced no lysis in the streaming assay. Adenosine monophosphate and colchicine had no effect on cytokinesis at 2.5 mM, although sodium pyrophosphate produced about 50% inhibition (Table II).

Table III shows that, when cells were incubated in 5 mM ATP containing labeled ATP, a significant amount of radioactivity became associated with the cells and that it persisted after thorough washing of the cells. Up to 400 cpm per thousand cells were recovered after an hour of incubation. This works out to be approximately 5×10^{-4} to 5×10^{-4} M/L associated with the cells over the 1 hr period, if one employs a specific activity in the incubation medium of 0.2 μc/mmole and the efficiency figure of 46% found for the spectrometer.

Experiments 2, 3, and 4 of Table III also demonstrate the following controls:

(*a*) About a third as much ^{14}C was associated with cells incubated in the cold as with cells incubated at room temperature.

(*b*) Only a small amount (from a third to a tenth of the control amount) of ^{14}C was associated with cells when added for 5–10 min at the end of the experiment.

(*c*) The amount of ^{14}C associated with the cells was roughly proportional to the number of cells present.

Table III also shows that when Mg^{++} or Ca^{++} ions were added to ATP/cf there was no significant depression in the amount of ^{14}C associated with the cells as compared to cells incubated in ATP/cf alone.

DISCUSSION

The results presented in Tables I and II show that one can distinguish between an early, or transitory, effect on streaming of the cells (5–20 min) and a late or prolonged effect (20 min or longer). EDTA and EGTA, have respectively, mainly and entirely transitory effects on streaming and pseudopod formation, whereas ATP has both an early and a late effect. One can reasonably conclude that, although a chelating action may be part or all of the mode of action of ATP in the first 20 min, the late or delayed effect of ATP must be due to some other mechanism. Since the performance of cytokinesis in *Chaos* occurs 30 min or more after metaphase (Feldherr, 1966), it seems likely that the transitory effect of EDTA and EGTA could be over before the cells begin to divide. This could account for the lack of an effect of these agents on cytokinesis.

The effect of ATP on streaming and on cell shape has been previously described for amebae (Kriszat, 1950; Marsland, 1956). The effect has been considered to be a contraction of the cell, but it is not clear from the available evidence whether this effect should be considered a contraction or a relaxation. However, it is interesting to note the results of the elegant double chamber work of Kamiya. He found that, when ATP (10^{-3} M) was applied to only one side of *Chaos*, that side became the tail of the ameba and the cell streamed away from that side. Pyrophosphate was also effective but adenosine monophosphate and inorganic phosphate were not. A range of concentrations of ATP was not studied, so that we do not know whether there was a reversal of the effect at higher concentrations. It would also be very interesting to know whether divalent ions would prevent the action of ATP in such a double chamber experiment.

From the ^{14}C results, it seems very probable that under the conditions of these experiments a considerable amount of ATP actually enters the cells. The controls reported in experiments 2, 3, and 4 of Table III make it unlikely that some form of external binding of ATP could account for the ^{14}C associated with the cells. External binding like that found with polycations would be expected to reach saturation within 5–10 min and to be relatively unaffected by incubation at 5°C. However, we cannot rule out the possibility that the ATP is held in the membrane in some way. The term uptake used below is meant only to imply firm association that does not behave like external binding. It should be noted that uptake could result from a small amount of "permanent pinocytosis" such as that reported by Wohlfarth-Bottermann and Stockem (1966) for *Amoeba proteus*.

The ^{14}C experiments demonstrate the surprising finding that when Mg^{++} (and probably Ca^{++}) ions are added to ATP solutions and normal streaming is observed, there does not seem to be any significant inhibition in the amount of ^{14}C associated with the cells. Since the threshold for the ATP effect on streaming is below 2.5 mM (Nachmias, 1968), there would have to be a 50% decrease in

the amount of ^{14}C-ATP associated with the cells for this to be the sole cause of the antagonism. Of course, this assumes a linear relation between external ATP concentration and uptake.

Since no inhibition was found, it seems clear that the antagonism of the ATP effect, by Mg^{++} ions at least, does not proceed via inhibition of uptake of the ATP. This conclusion makes the antagonism reported here of considerable interest for the understanding of streaming and ameboid movement. This is so because an interaction between divalent ions and ATP that does not involve a change in uptake by the cells could play a role in regulating the action of endogenous ATP on the streaming process and on the performance of cytokinesis.

Two possible ways in which divalent ions could affect ATP action within the cells (including the cell membrane) can be envisaged at present. One is simply by altering the effective ATP concentration within the cell (or membrane), for example, by activating enzymes responsible for its destruction. A second, more intriguing possibility, but one that is more difficult to establish, is that divalent ions could be involved in regulating the formation of a magnesium-actin polymer like that reported for and purified from *Physarum* (Hatano et al., 1967). Inhibition of streaming by excess ATP has been reported for *Physarum* (Kamiya, 1959), and it will be interesting to see whether this inhibition can also be reversed by excess divalent ions. The author is now trying to make a suitable preparation from *Physarum* with which to study this phenomenon further.

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