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THE ROLE OF CALCIUM IONS IN THE PERMEABILITY CHANGES PRODUCED BY EXTERNAL ATP IN TRANSFORMED 3T3 CELLS

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External ATP causes a rapid increase in passive permeability to nucleotides and phosphate esters in transformed cell lines, such as 3T6 mouse fibroblasts. However, untransformed lines, such as 3T3, do not show a similar sensitivity to external ATP. Ca^{2+} inhibits permeabilization, but only at concentrations approaching those of external ATP. In contrast, La^{3+} and Tb^{3+} inhibit ATP-dependent permeabilizatian at one-fifth the concentration of external ATP. Considering reports that lanthanides can substitute for calcium ion in many enzymatic reactions, often with a higher affinity, it would appear that Ca^{2+} plays a specific role in the maintenance of a passive membrane permeability barrier and in opposing the effects of external ATP.

Other data suggest a regulatory role for the Ca^{2+}-calmodulin complex in the permeabilization process. Trifluoperazine, chlorpromazine and W-7, compounds which inhibit cellular functions dependent on the Ca^{2+}-calmodulin complex, are able to enhance the effect of external ATP. Thus, a dramatic stimulation of nucleotide permeability occurs with concentrations of external ATP and inhibitor that are ineffective when added alone. Calmodulin antagonists and low concentrations of external ATP increased membrane permeability to Na^+ and K^+ as was previously shown for permeabilization with ATP alone. Earlier studies have shown that energy inhibitors which reduce intracellular ATP levels greatly increase the sensitivity of transformed cells to external ATP. However, the Ca^{2+}-calmodulin antagonists used in the present study exert their effects at concentrations which do not alter intracellular ATP levels.

Introduction

A curious effect of external ATP on cultures of certain transformed cell lines was reported in a series of publications from this and another laboratory [1–8]. Addition of ATP to the medium surrounding transformed 3T3 cells (3T6, SV3T3, PY3T3, 3T12) or HeLa cells in culture produced abnormal permeability changes in the plasma membrane after 3–5 min. As a result, there occurred an efflux of nucleotide pools and an entry of normally impermeant phosphorylated compounds. The permeability change required an alkaline medium (pH 7.6–7.8) and a low divalent cation concentration. The permeability barrier could be restored upon shifting the medium pH to neutrality and by increasing the divalent cation concentration. Untransformed cells, including 3T3 and secondary cultures of mouse embryo fibroblasts, did not respond to external ATP.

Abbreviations: 3T6 and 3T12, Spontaneously transformed 3T3 cell lines: SV3T3, 3T3 cell line transformed by simian vacuolating virus 40; PY3T3, 3T3 cell line transformed by polyoma virus; HeLa, Cell line derived from human cervical carcinoma; W-5, *N*-(6-aminohexyl)-1-naphthalene sulfonamide; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide; Medium A, 0.1 M Tris-HCl (pH 7.8), 0.05 M NaCl, 50 μM $CaCl_2$ and 5 mg/ml Dextran T-500; Medium I, 0.03 M Hepes (pH 7.8), 0.12 M NaCl, 0.05 M KCl and 5 mg/ml Dextran T-500; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

In a previous study, it was reported that reduction of intracellular ATP levels by starvation or by a variety of energy inhibitors greatly increased the sensitivity of transformed cells to external ATP [3]. Recent work has shown that an immediate influx of Na^+ and efflux of K^+ occurred in response to external ATP [9] prior to the increase in passive permeability to normally impermeant molecules. Reduction of intracellular ATP levels was associated with the ion fluxes.

Other work in this laboratory has shown that Ca^{2+} and La^{3+} inhibit the permeabilization process [10]. The data presented here extend these findings and demonstrate that Ca^{2+} inhibits ATP-dependent permeabilization only at a concentration equivalent to that of external ATP. This result could suggest that inhibition by Ca^{2+} is due to chelation of the external ATP. However, La^{3+} inhibits the ATP-dependent permeabilization process at concentrations at which only a fraction of the external ATP would be chelated. Reports that lanthanides can substitute for Ca^{2+} in a number of biological processes, often with a higher affinity [11–14], suggest a specific role for Ca^{2+} in preventing the permeability changes resulting from treatment of cells with ATP. Other evidence for a specific role for Ca^{2+} in the regulation of ATP-dependent permeabilization is that antagonists of the Ca^{2+}-calmodulin complex enhance the effect of external ATP. These complexes have been shown to mediate many biological effects of Ca^{2+} [15–16].

Materials and Methods

Cells. Stock cultures of 3T6 and 3T3 cells [17] were grown in Dulbecco's modified Eagle's medium, supplemented with 5% fetal calf serum (Gibco, Grand Island, NY), 100 units/ml penicillin and 100 μg/ml streptomycin (growth medium), in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Cells were subcultured into 35 mm Falcon dishes in growth medium. The plating density was usually $1 \cdot 10^5$ cells per dish, and experiments were carried out 3–4 days after plating as the cells approached confluency. The protein concentration at confluency varied from 500 to 600 μg/dish. There was no detectable mycoplasma contamination when cultures were tested on mycoplasma agar.

Chemicals. ATP was purchased from Sigma or Boehringer-Mannheim. All other compounds were of the highest purity available from commercial sources. [6-^{3}H]uridine and [2-^{3}H]adenosine were obtained from New England Nuclear.

Measurement of efflux of labeled acid-soluble pools. The acid-soluble pool was labeled with [^{3}H]uridine or [^{3}H]adenosine (1 μM, 0.5 μCi/ml) in 1.0 ml growth medium for 2 h in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C to obtain a stable nucleotide pool. After labeling, the cells were washed three times with 0.15 M NaCl containing 50 μM $CaCl_2$ and once with medium A followed by incubation at 37 °C under conditions specified in figure and table legends. A fraction of the supernatant solution (efflux fluid), usually 0.8 ml, was sampled when indicated, mixed with 8 ml of 'Liquiscint' (National Diagnostics, Somerville, NJ), and counted in a Beckman LS7000 scintillation counter. Efflux is expressed as a percentage of the total labeled acid-soluble pool. The total labeled acid-soluble pool, which varied between 100000 and 200000 cpm/dish, was obtained by extracting a control dish with 1 ml of cold 5% trichloroacetic acid for 20 min at the start and finish of the experiment.

Analytical procedures. Polyethyleneimine-coated cellulose column chromatography was performed as previously described [18] with minor modifications [9].

The concentration of ATP in aqueous samples was measured by the luciferase method [19]. Protein was assayed by the method of Lowry et al. [20].

Na^+ and K^+ quantitation was performed as previously described [9].

Results

Lanthanum and terbium inhibit ATP-dependent permeabilization

It was reported earlier [10] that divalent and trivalent cations inhibited the increase in membrane permeability caused by external ATP. We have confirmed and extended these observations by carefully titrating La^{3+}, Tb^{3+} and Ca^{2+} against a permeabilizing concentration of external ATP. For these experiments, $CaCl_2$ was omitted from medium A and 10 μM EGTA was added to chelate

endogenous Ca^{2+}. Fig. 1A shows that either 40 μM La^{3+} or 40 μM Tb^{3+} effectively inhibited the efflux of labeled nucleotides caused by 150 μM ATP. By contrast, a concentration of 200 μM Ca^{2+} was required to inhibit ATP-dependent permeabilization completely (Fig. 1B).

Antagonists of the Ca^{2+}-calmodulin complex stimulate the ATP-dependent efflux of nucleotides

Studies have shown that certain phenothiazines, such as trifluoperazine and chlorpromazine, can bind to calmodulin and block various biological effects of Ca^{2+}-calmodulin complexes [15–16,21–22]. We recently observed that phenothiazines greatly enhanced the ability of low concentrations of external ATP to permeabilize transformed cells. Thus, a combination of 50 μM trifluoperazine and 25 μM ATP caused an increase in permeability to nucleotides in 3T6 cells (Fig. 2). Neither compound was effective alone at these concentrations. However, at higher concentrations, trifluoperazine caused significant permeabilization in the absence of external ATP (Fig. 3). Chlorpromazine (30 μM) also enhanced permeabilization of 3T6 cells by low concentrations of external ATP (unpublished results).

Permeabilization obtained with trifluoperazine or chlorpromazine may represent a nonspecific hydrophobic effect [23–25]. Therefore, two derivatives of naphthalene sulfonamide, W-7 and W-5, were used to evaluate the effect of hydrophobicity on permeabilization. W-7 and W-5 are similar in both structure and hydrophobic properties, but W-7 is much more effective as a Ca^{2+}-calmodulin antagonist [26]. As indicated in Fig. 4, W-7 but not W-5, enhanced permeabilization of 3T6 cells by low concentrations of external ATP, suggesting that this effect was not due to hydrophobic properties of the antagonists.

In an earlier study [3], a variety of compounds

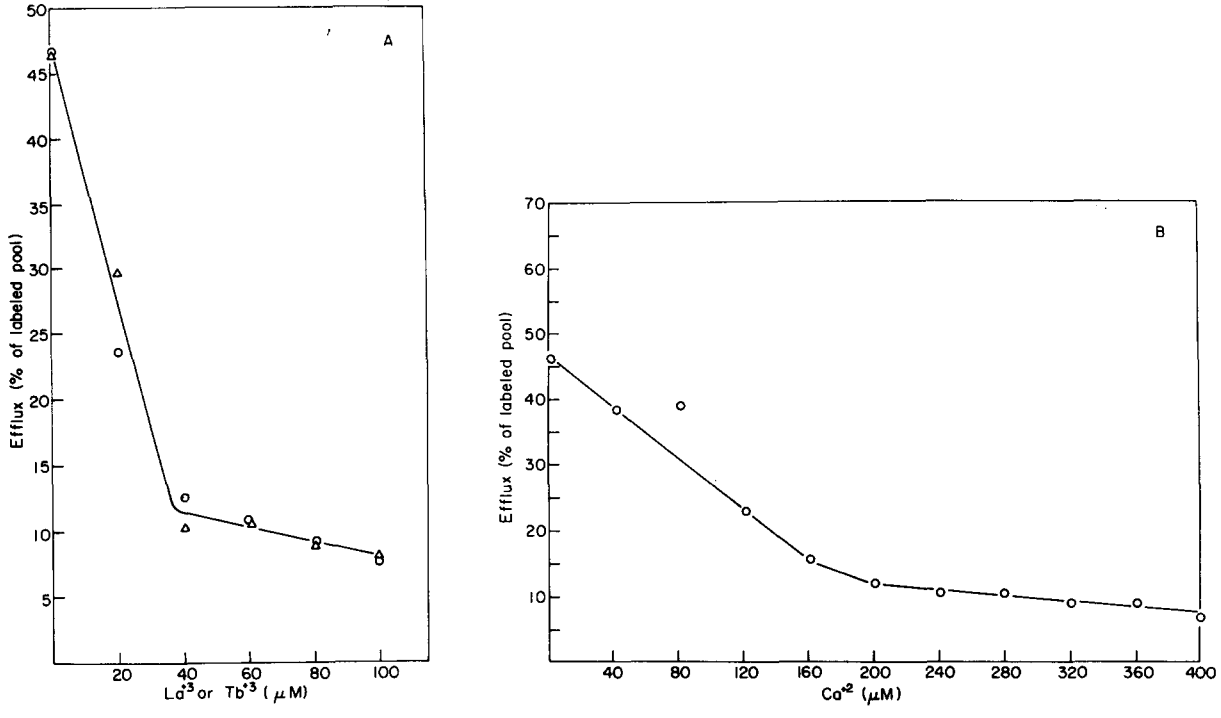

Fig. 1. Effect of La^{3+}, Tb^{3+} and Ca^{2+} on ATP-dependent efflux of nucleotides from 3T6 cells. The acid-soluble pool was labeled with [^{3}H]adenosine (see Materials and Methods). The cells were washed and incubated at 37°C in medium A (pH 7.8) containing 10 μM EGTA and no added $CaCl_2$. The medium also contained 150 μM ATP alone or with different concentrations of: (A) La^{3+}, ○, or Tb^{3+}, △; or (B) Ca^{2+}, ○. After 20 min, samples of 0.8 ml were removed for counting. La^{3+} or Tb^{+3} alone in incubation medium did not cause a permeability change (unpublished results).

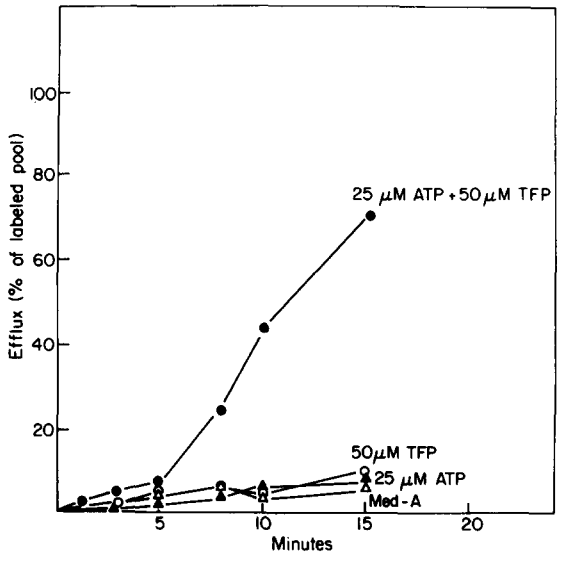

Fig. 2. Effect of trifluoperazine on ATP-dependent efflux of nucleotides from 3T6 cells. Cells were treated as in Fig. 1, except for the absence of EGTA in the incubation medium. Additions of ATP and trifluoperazine were made, as indicated, at the beginning of the incubation period. Medium A, △; 25 μM ATP, ▲; 25 μM ATP+40 μM trifluoperazine, ●; 50 μM trifluoperazine, ○. Samples of 0.8 ml were removed for counting at the indicated times, TFP, trifluoperazine; Med-A, medium A.

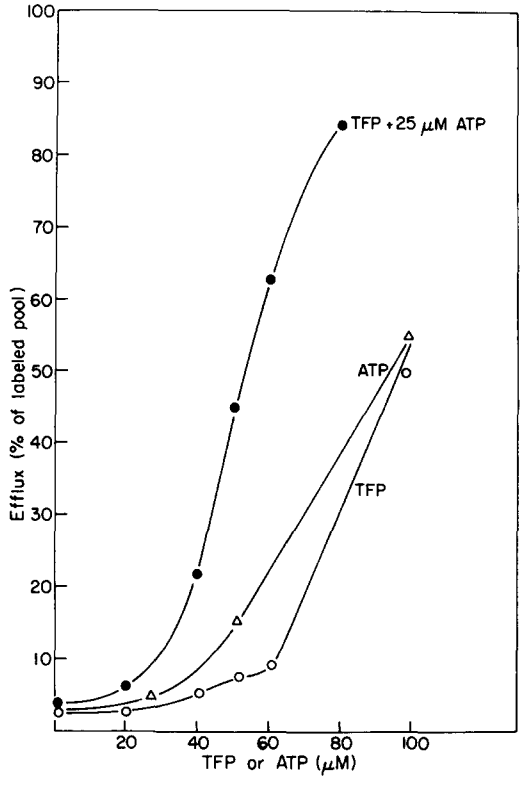

Fig. 3. Effect of trifluoperazine (TFP) concentration on sensitivity of 3T6 cells to external ATP. Cells were labeled with [^{3}H]adenosine (see Materials and Methods). Trifluoperazine was added, as indicated, for 1 h in growth medium. Cells were washed and ATP was added in medium A, as indicated, for 15 min at 37°C, after which the extracellular medium was sampled and counted. Additions were: trifluoperazine, ○; trifluoperazine+25 μM ATP, ●; ATP, △. The abscissa indicates variations in the concentration of ATP or trifluoperazine.

was reported to act synergistically with external ATP to increase the permeability of 3T6 cells. Although their mode of action differed, they had in common the ability to reduce levels of intracellular ATP. By contrast, treatment of 3T6 cells with 50 μM trifluoperazine, 30 μM chlorpromazine, 25 μM W-7 or 25 μM W-5 did not significantly alter intracellular ATP levels (unpublished results).

The permeability change caused by low concentrations of external ATP in the presence of trifluoperazine was reversible, as was observed previously for treatment with higher concentrations of ATP alone [2]. We have found that 3T6 cells treated with trifluoperazine and ATP could be resealed by shifting to a neutral medium containing divalent cations (unpublished results). In addition, permeabilization did not require the continued presence of trifluoperazine and ATP. Therefore, 3T6 cells exposed to medium A containing 50 μM trifluoperazine and 25 μM ATP continued to efflux the labeled pool when subsequently incubated in medium lacking ATP and trifluoperazine.

Trifluoperazine and chlorpromazine induce influx of Na^+ and efflux of K^+ in the presence of low concentrations of external ATP

We have observed earlier [9] that immediate influx of Na^+ and efflux of K^+ occurs in 3T6 cells in response to 250 μM external ATP, prior to the increase in membrane permeability to nucleotides. Therefore, we determined whether permeability changes induced by calmodulin antagonists and low concentrations of external ATP also increased Na^+ and K^+ permeability. Table I shows that trifluoperazine or chlorpromazine alone did not affect levels of intracellular Na^+ and K^+. However, these inhibitors in the presence of 50 μM external ATP caused a 2.5-fold increase in intracellular Na^+ levels and a 2-fold decrease in

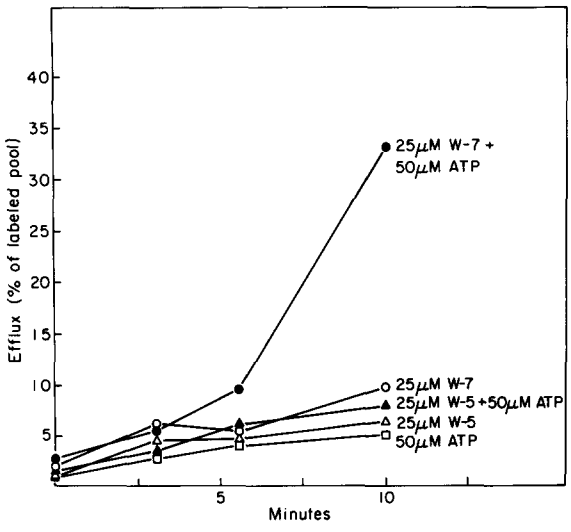

Fig. 4. Effect of W-7 and W-5 on ATP-dependent efflux of nucleotides from 3T6 cells. Experimental conditions were as in Fig. 2. Additions of ATP, W-5 and W-7 were made, as indicated, at the beginning of the incubation period. W-5, △; W-5 + ATP, ▲; W-7, ○; W-7 + ATP, ●; ATP, □.

TABLE I

EFFECT OF ATP AND CALMODULIN ANTAGONISTS ON THE FLUX OF Na^+ AND K^+ AND THE EFFLUX OF LABELED NUCLEOTIDES IN 3T6 CELLS

Confluent cultures of 3T6 cells were labeled with [^{3}H]adenosine (see Materials and Methods). Trifluoperazine, chlorpromazine and ATP were added in medium I, as indicated, for 10 min at 37°C after which the extracellular medium was sampled and counted. The cells were washed six times with 0.1 M $MgCl_2$, and a trichloroacetic acid-soluble pool was obtained. The levels of Na^+ and K^+ in the acid-soluble pool, as assayed by flame-emision spectrometry, were expressed as μmol Na^+ or K^+ per mg protein. The efflux is expressed as a percentage of the initial trichloroacetic acid pool. TFP, trifluoperazine; CPZ, chlorpromazine.

Incubation condition	% Pool effluxed	μmol Na^+ per mg	μmol K^+ per mg
Medium I	3.0	0.32	1.3
+250 μM ATP	32	1.12	0.08
+ 50 μM ATP	4.8	0.32	1.0
+ 50 μM TFP	7.0	0.28	1.22
+ 50 μM ATP			
+ 50 μM TFP	34	0.74	0.6
+ 30 μM CPZ	4.4	0.36	1.14
+ 50 μM ATP			
+ 30 μM CPZ	35	0.7	0.3

intracellular K^+ levels. An increase in membrane permeability to Na^+ and K^+ did not occur with 50 μM ATP alone.

These results suggest that nucleotide permeability induced by calmodulin antagonists and low concentrations of external ATP or by higher concentrations of external ATP alone occurs by the same mechanism.

Increased permeability induced by trifluoperazine and ATP is preceded by a breakdown of intracellular ATP

It was shown previously that 250 μM external ATP induced hydrolysis of intracellular ATP prior

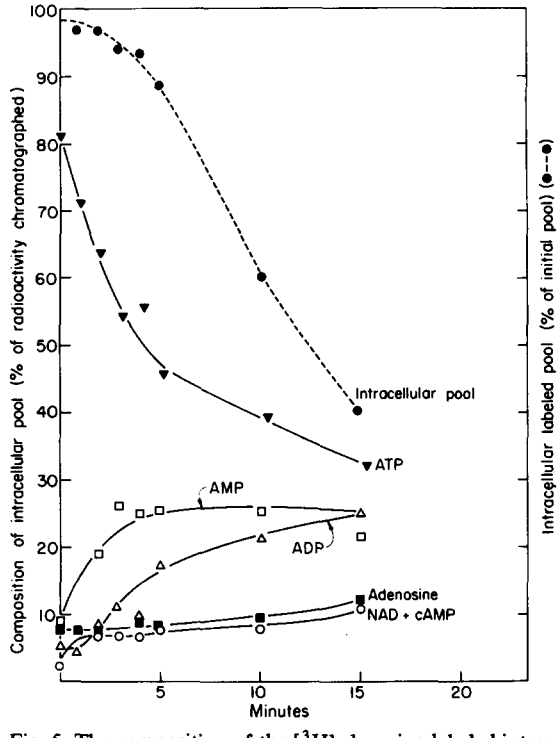

Fig. 5. The composition of the [^{3}H]adenosine-labeled intracellular pool of 3T6 cells treated with trifluoperazine and ATP. Cells labeled with [^{3}H]adenosine (1 Ci/mmol, 1 μCi/ml) were washed and incubated at 37°C in medium A (no $CaCl_2$) supplemented with 50 μM trifluoperazine and 25 μM ATP. The efflux medium was sampled at the times indicated, a trichloroacetic acid-soluble pool was obtained, and the radioactivity in each sample was determined. A portion (0.5 ml) of the trichloroacetic acid pool was neutralized with 1 M NaOH and buffered with 40 mM Tris-HCl (pH 8.0). Portions (0.1 ml) of these samples were chromatographed on polyethyleneimine-cellulose columns as described in Materials and Methods. ATP, ▼; ADP, △; AMP, □; adenosine, ■; NAD+cAMP, ○; intracellular pool, ●.

to the efflux of labeled nucleotides from 3T6 cells [9]. Similarly, 3T6 cells treated with 25 μM ATP and 50 μM trifluoperazine showed nearly a 2-fold reduction in the level of intracellular ^{3}H-labeled ATP prior to the efflux of the labeled pool (Fig. 5). Again, as was true for treatment with ATP alone, the bulk of the radioactivity effluxed from 3T6 cells treated with Ca^{2+}-calmodulin antagonists and low concentrations of external ATP consisted of nucleotides (Table II). Thus, permeabilization induced by Ca^{2+}-calmodulin antagonists and low concentrations of external ATP is not a result of massive hydrolysis of intracellular adenine nucleotides followed by carrier-mediated efflux of nucleosides.

Metal ions that inhibit ATP-dependent permeabilization also inhibit the breakdown of intracellular ATP

Since hydrolysis of intracellular ATP was found to occur prior to the external ATP-dependent efflux of nucleotides, it was of interest to investigate the effect of lanthanides and divalent cations on this breakdown. Table III indicates that 50 μM La^{3+} or Tb^{3+} and 200 μM Ca^{2+} inhibited the intracellular breakdown of ATP as well as the efflux of nucleotides induced by 200 μM external ATP. Lower concentrations of ions caused partial inhibition of both processes. Ruthenium red, which is known to inhibit Ca^{2+} transport [27] and permeabilization by external ATP [10], also inhibited the breakdown of intracellular ATP (Table III).

TABLE II

COMPOSITION OF THE EFFLUX FLUIDS AFTER TREATMENT OF 3T6 CELLS WITH ATP, TRIFLUOPERAZINE (TFP) AND CHLORPROMAZINE (CPZ)

Confluent cultures of 3T6 cells were labeled with [^{3}H]uridine or [^{3}H]adenosine (1 Ci/mmol, 1 μCi/ml) for 2 h. Then, cells were incubated at 37°C in medium A containing the supplements indicated. After 15 min, the efflux fluid was sampled and the composition of the radioactivity effluxed was determined by chromatography on polyethyleneimine-cellulose columns (see Materials and Methods).

Label	Treatment	% Pool effluxed	% Nucleoside effluxed
Uridine	no ATP	5	64
	250 μM ATP	78	18
	50 μM CPZ	9	59
	50 μM ATP	8	65
	50 μM CPZ + 50 μM ATP	55	17
Adenosine	no ATP	6	69
	250 μM ATP	86	16
	50 μM TFP	10	47
	25 μM ATP	13	39
	50 μM TFP + 25 μM ATP	69	23

TABLE III

EFFECT OF DIFFERENT CATIONS ON THE ATP-DEPENDENT EFFLUX, INTRACELLULAR [^{3}H]ATP AND TOTAL ATP LEVELS IN 3T6 CELLS

Confluent cultures of 3T6 cells were labeled with [^{3}H]adenosine (1 Ci/mmol, 1 μCi/ml), washed and incubated at 37°C for 15 min in medium A containing the supplements indicated. Following incubation, the efflux medium was sampled, a trichloroacetic acid-soluble pool was obtained, and a portion of each sample was counted to determine the efflux as a percentage of the total ^{3}H-labeled pool. The percentage of [^{3}H]ATP in neutralized portions of the trichloroacetic acid pool, buffered with 40 mM Tris-HCl (pH 8.0), was determined by chromatography on polyethyleneimine-cellulose columns (see Materials and Methods). To measure total intracellular ATP levels, portions of the trichloroacetic acid extract, adjusted to pH 7.8, were assayed by the luciferase method.

Incubation conditions	% [^{3}H]ATP in intracellular pool	% Total ^{3}H pool effluxed per dish	Total intracellular ATP (nmol/dish)
Medium A	78.6	8	24
200 μM ATP	39.8	90	8
+ 10 μM La^{+3}	48	68	14
+ 50 μM La^{+3}	69.9	18	24
+ 10 μM Tb^{+3}	62	73	16
+ 50 μM Tb^{+3}	81.3	18	26
+ 100 μM Ca^{+2}	55	73	17
+ 200 μM Ca^{2+}	78.5	26	26.5
+ 10 μM Ruthenium red	71.5	36	20

Discussion

The present results provide evidence that calcium regulates the ATP-dependent permeabilization process in transformed cells. The data show that La^{3+} [10] and Tb^{3+} inhibited the permeabilization process at one-fifth the concentration of external ATP (Fig. 1A). La^{3+} and Tb^{3+} have

been reported to replace Ca^{2+} in modulating the activities of many proteins [11–14]. These trivalent cations bind to proteins with a much higher affinity than Ca^{2+}. They form a 1:1 complex with ATP over a wide range of ATP concentrations [28–29], and on this basis their inhibitory effect on permeabilization (Fig. 1A) cannot be explained simply by chelation of a sufficient fraction of the added ATP. Assignment of a specific role for Ca^{2+} in the permeabilization process is complicated by the fact that the concentration required is sufficient to chelate most of the ATP (Fig. 1B) and for a number of the external effects, the active agent is free ATP (literature reviewed in Refs. 2–6). In addition, Mg^{2+} can inhibit ATP-dependent permeabilization at concentrations sufficient to chelate the free ATP. However, it has been shown that Ca^{2+} or La^{3+} could seal 3T6 cells at an alkaline pH after permeabilization by external ATP, while Mg^{2+} was ineffective [10]. Also, Ruthenium red, which is known to bind to calcium-binding proteins and to inhibit calcium transport [27], is a potent inhibitor of permeabilization [10], confirmed in the present study (Table III).

Other evidence suggesting a role for the Ca^{2+}-calmodulin complex in the regulation of permeabilization by external ATP is that antagonists of this complex enhanced the effect of external ATP in 3T6 cells (Figs. 2–4) without lowering the concentration of intracellular ATP. This is in contrast to energy inhibitors which synergize with external ATP by lowering the intracellular ATP concentration [3]. The data also indicate that calmodulin antagonists and low concentrations of external ATP increase Na^+ and K^+ permeability (Table I) and induce hydrolysis of the intracellular adenine nucleotide pool prior to nucleotide efflux (Fig. 5). These results agree with earlier studies on nucleotide permeability induced by 250 μM ATP alone [9].

The concentration of trifluoperazine used in our studies was sufficient to bind calmodulin and inhibit calmodulin-dependent phosphodiesterase activity [22]. Hidaka et al. [26] used the antagonist, W-7, to inhibit the calmodulin-dependent proliferation of Chinese hamster ovary K_1 cells. Others have used the antagonists trifluoperazine and chlorpromazine to investigate the role of the Ca^{2+}-calmodulin complex in various cellular processes [30,31].

Although calmodulin antagonists have been widely used, effects of these drugs may be due to detergent-like properties that are independent of calmodulin antagonism. Some indirect evidence against this possibility was obtained with W-5, a compound of similar structure and hydrophobic properties as W-7. W-5 does not inhibit Ca^{2+}-calmodulin-requiring processes [26] and did not enhance the effect of external ATP (Fig. 4). More recently, we obtained direct evidence for an effect of the Ca^{2+}-calmodulin complex on ATP-dependent permeabilization. Exogenous calmodulin (20 μM) specifically inhibits the permeabilization process and acts synergistically with low concentrations (50 μM) of external Ca^{2+}. The mechanism of this interesting effect is currently under investigation.

Previous reports that ATP-dependent permeabilization in transformed cells is regulated by a specific plasma membrane protein kinase [10] have been convincingly ruled out [32]. More recent studies have shown that external ATP drastically increases membrane permeability to Na^+ and K^+ leading to a decrease in plasma membrane potential and an increase in nucleotide permeability [9]. The present studies provide new evidence that these events are regulated by calcium and calmodulin.

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