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ATP analogues induce membrane permeabilization in transformed mouse fibroblasts

Ruth Arav [a,b] and Ilan Friedberg [a,*]

[a] *Department of Microbiology, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, and* [b] *Everyman's University, P.O. Box 39328, Tel Aviv 61392 (Israel)*

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The mechanism underlying ATP-induced permeabilization of transformed mouse fibroblasts was studied by using nonhydrolyzable analogues of ATP. Incubation of 3T6 cells with 0.6 mM of either ATP, 5'-adenylyl imidodiphosphate (p[NH]ppA) or adenosine 5'-[β,γ-methylene]triphosphate (p[CH_2]ppA) resulted in an increase of 17-, 8- or 5-times, respectively, in the cell membrane permeability, measured by the efflux of normally impermeant metabolites from the cells. The induced cell permeabilization was preceded by a reduction in the membrane potential ($\Delta\psi$), determined according to the distribution of the cation tetraphenylphosphonium (TPP^+) between the cells and the medium. Reduction of 26, 18 and 13 mV in $\Delta\psi$ was exerted by 0.6 mM of either ATP, p[NH]ppA or p[CH_2]ppA, respectively. In 3T3 cells the untransformed counterparts of 3T6 cells, neither reduction of $\Delta\psi$, nor alterations in membrane permeability were exerted by either ATP or by its analogues. The data indicate that the dissociation of the β,γ-phosphate bond is not essential for membrane permeabilization by external ATP, implying that the binding of ATP to the cell surface of transformed cells is sufficient to initiate the permeabilization process. The data also suggest that $\Delta\psi$ is involved in the control of membrane permeability.

Introduction

The cell membrane is normally impermeant to nucleotides and similar low molecular weight, charged molecules [1]. However, the cell membrane of transformed cells in culture can be rendered permeable by incubation with exogenous ATP in a buffered salt solution [2]. The increase in cell membrane permeability by exogenous ATP was found to be specific for ATP, selective for transformed cells, dependent on pH, temperature and ATP concentration, and occurs after a lag period of about 5 min [3]. The cells become more susceptible to external ATP when the internal ATP concentration is reduced [4,5]. The permeabilization process is reversible, and resealing of the cell membrane is achieved by addition of growth medium or some of its components to the cells [3,6].

Although the phenomenon of ATP-induced permeabilization is well documented, the mechanism underlying this process is still obscure. It was proposed that phosphorylation of membranal protein results in the formation of aqueous channels [7]. However, a later investigation indicates that the phosphorylation is unrelated to the permeabilization process [8]. More recently, a mechanism has

* To whom correspondence should be addressed.

Abbreviations: 3T6 cells, a cell line derived from 3T3 by spontaneous transformation; DMEM, Dulbecco's modified Eagle's medium; p[NH]ppA, 5'-adenylyl imidodiphosphate; p[CH_2]ppA, adenosine 5'-[β,γ-methylene]triphosphate; TTP^+, tetraphenylphosphonium ion; $\Delta\psi$, the electrical potential across the cell membrane.

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been suggested, in which exogenous ATP interacts with specific sites on the cell surface, to induce ion fluxes and reduction of membrane potential ($\Delta\psi$), which lead to topological and conformational changes of membranal components, followed by the formation of aqueous channels in the membrane [9,10]. Studies of the early events which occur during the lag period following the administration of ATP, have shown influx of Na^+, efflux of K^+, reduction of $\Delta\psi$, and a decrease in the intracellular level of ATP, prior to the increase in the membrane permeability [11]. An additional support for the suggested mechanism has been obtained from the effects of electrogenic ionophores, which exert dissipation of $\Delta\psi$ and cell permeabilization [12]. ATP-induced ion fluxes were detected in various cell types [13–26].

In the present paper the effects of non-hydrolyzable analogues of ATP were studied, to gain an additional sight into the mechanism underlying cell permeabilization. The data show, that the dissociation of the β,γ-phosphate bond of ATP is not essential for the permeabilization process, and support the hypothesis that the membrane potential plays a major role in the regulation of cell membrane permeability.

Materials and Methods

Materials. ATP, adenylyl 5′-imidodiphosphate (p[NH]ppA), adenosine 5′-[β,γ-methylene]triphosphate (p[CH_2]ppA), SF-6847 and most other chemicals used were purchased from Sigma, St. Louis, MO, U.S.A. $^{32}P_i$ and tetra[^{3}H]phenylphosphonium bromide (TPP^+) were obtained from the Israel Atomic Energy Commission, Negev, Israel. ^{3}H-labelled water and [U-^{14}C]sorbitol were obtained from New England Nuclear, Boston, MA, U.S.A.

Cells. Swiss mouse 3T3 cells and their transformed derivative, 3T6 cells [2,27] were propagated in 33 mm plastic dishes (Nunc, Roskilde, Denmark) containing 2 ml of Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, U.S.A.) supplemented with 10% newborn calf serum (NBCS) (Sera-lab, Sussex, England), 100 units/ml penicillin, 100 μg/ml streptomycin (Biolab, Jerusalem, Israel) and 80 μg/ml miramycin (Teva, Jerusalem, Israel). Cells were maintained in a humidified atmosphere of 5% CO_2 and 95% air, at 37°C, and were used at confluence, 3–5 days, or 4–7 days after incubation, for 3T6 and 3T3 cells, respectively.

Measurement of the efflux of intracellular pools. 3T6 and 3T3 cells were labelled by incubation at 37°C with 1 ml DMEM supplemented with 10% newborn calf serum and containing 1.0 μCi of $^{32}P_i$ (1.1 Ci/mol), for 90 min. At the end of the incubation more than 80 percent of the intracellular label was in organic phosphates [11]. Then the medium was aspirated and the cells were washed three times at 37°C with 0.15 M NaCl, containing 50 μM $CaCl_2$, once with medium A, composed of 100 mM Tris-HCl (pH 7.8 at 37°C), 50 mM NaCl, 50 μM $CaCl_2$, 5 mg/ml Dextran T-500, and finally incubated with 1 ml of medium A. At indicated time intervals, samples of 100 μl were withdrawn from the supernatant, for radioactivity measurements. Then the rest of the supernatant was aspirated, and the radioactivity remaining in the acid-soluble intracellular pool was determined after extraction with 1 ml of 5% trichloroacetic acid, for 20–30 min at 0°C. The percent of the soluble pool released at each time point was calculated according to distribution of the radioactivity between the cells and the medium. The rate of efflux is designated as the percent of soluble pool released per min (%/min) when the efflux rate is linear (usually between 10 to 15 min after additions were made).

Measurement of TPP^+ level. Cells were prepared for TPP^+ uptake and efflux measurements by the same procedure as for the measurements of the intracellular pool, except that radioactive phosphate was not added. After 5 min equilibration with 1 ml medium A, [G-^{3}H]TPP^+, 100 Ci/mol, was added to a final concentration of 2 μM. Samples of 50 μl were withdrawn at indicated time intervals (usually 3 min), for radioactivity measurement. A steady-state level of TPP^+ within the cells was established after 12 to 15 min. Addition of an effector (e.g. ATP, its analogue or ionophore) was made at 16 min, and the changes in TPP^+ level were followed by sampling for additional 14 min. The amount of intracellular TPP^+ was calculated at each time point according to the alteration in the extracellular concentration of the probe, and expressed as nmoles TPP^+ taken up by the cells in the dish (usually about $2 \cdot 10^6$ or $1 \cdot 10^6$

cells per dish, for 3T6 or 3T3 cells, respectively). The advantage of this method is that the measurements are performed without manipulating the cells. Addition of the potent ionophore SF-6847 (5 μM) [28] dissipates $\Delta\psi$, resulting in rapid efflux of TPP^+, revealing the potential-sensitive and insensitive portions of $[^3H]TPP^+$ that was removed from the extracellular medium.

The apparent difference in the membrane potential ($\delta\Delta\psi$), before and after addition of an effector was calculated according to the Nernst equation:

$$\delta\Delta\psi = \Delta\psi_2 - \Delta\psi_1 = \frac{RT}{F} \ln \frac{[TPP^+]_{in,2}/[TPP^+]_{out,2}}{[TPP^+]_{in,1}/[TPP^+]_{out,1}} \quad (1)$$

$\Delta\psi_1$ is the membrane potential before, and $\Delta\psi_2$ is membrane potential after the addition was made; R, the gas constant; T, temperature (K); F, the Faraday constant. $[TPP^+]_{in}$ and $[TPP^+]_{out}$ are the concentrations of TPP^+ in the cells and in the surrounding medium, respectively, calculated according to the radioactivity of the samples and the cell volume. Subscripts 1 and 2 denote before and after addition, respectively. Corrections for TPP^+ accumulated in cell organelles were made [12,29]. By using Eqn. 1 the non-specific absorption of the TPP^+ is biased [30].

Determination of intracellular water volume. The internal water volume was estimated according to Rottenberg [31], using ^3H-labelled water (50 nCi/ml) and [U-^{14}C]sorbitol (10 nCi/ml). The value obtained was 13 ± 0.6 μl water per mg of cell protein for 3T6 cells.

Protein determination. Cell protein was solubilized at the end of the experiment by incubation with 1 ml of 1 M NaOH for 30 min at 37°C; samples were taken for protein estimation according to Lowry et al. [32].

Radioactivity assay. The radioactivity was counted in TriCarb Model 3330 scintillation spectrometer (Packard Instrument Company, Inc., Downers Grove, IL, U.S.A.) and corrections were made for quenching.

Results

ATP analogues induce cell membrane permeabilization in transformed mouse fibroblasts

The effects of ATP and its analogues on the passive permeability of the cell membrane of 3T6 cells in culture were examined by following the efflux of intracellular, normally impermeant, metabolites, labeled with ^{32}P. Experiments were carried out with cultures incubated in medium A at 37°C, as described in Methods and in previous studies [2,3,11].

Fig. 1A shows that addition of either ATP, p[NH]ppA or p[CH_2]ppA, resulted in an increase

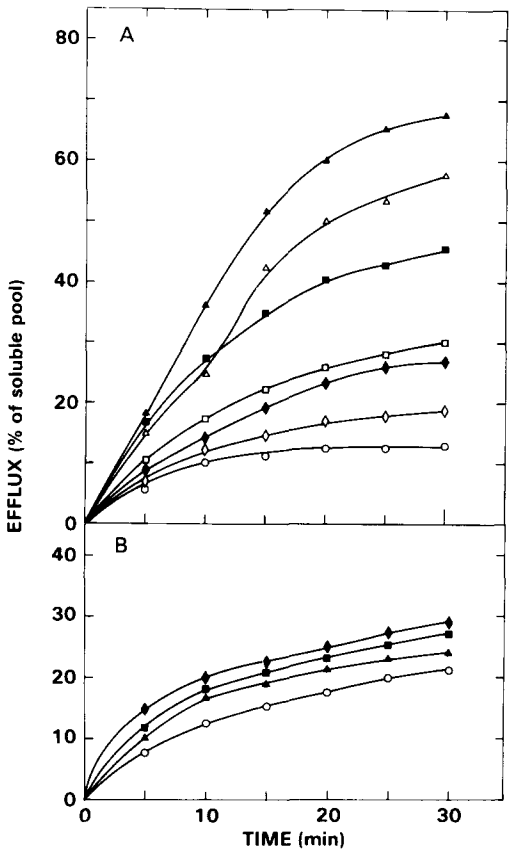

Fig. 1. Effect of ATP and its analogues on cell membrane permeability in 3T6 cells (A) and 3T3 cells (B). Confluent cultures of the cells were labeled with $^{32}P_i$ for 90 min, washed and incubated in 1 ml of medium A at 37°C. At the times indicated samples of 100 μl were withdrawn and the radioactivity in the extracellular medium was measured. Then the medium was removed, the remaining trichloroacetic acid-soluble pool was measured, and the percent of the soluble pool released at each time point was calculated. Additions were made at zero time as follows: (○), None; (△), 0.3 mM ATP; (▲), 0.6 mM ATP; (□), 0.3 mM p[NH]ppA; (■), 0.6 mM p[NH]ppA; (◇), 0.3 mM p[CH_2]ppA; (◆) 0.6 mM p[CH_2]ppA.

in the efflux of the intracellular acid soluble pools. The effect of ATP and its analogues was found to be concentration-dependent. The cell membrane permeabilization, exerted by ATP, was more pronounced than the analogues-induced permeability, and between the two analogues, p[NH]ppA was more effective than $p[CH_2]ppA$ (Table I). The efflux rates, exerted by either ATP, p[NH]ppA or $p[CH_2]ppA$ are 17-, 8- and 5-times higher, respectively, than the rate in the control cells.

Since cell membrane permeabilization by ATP was found to be selective for transformed cells [2,3], it was of interest to examine the effects of the ATP analogues on 3T3 cells, the non-transformed counterparts of 3T6 cells. As shown in Fig. 1B only slight increase in the 3T3 cell membrane permeability was exerted by 0.6 mM of either ATP or its analogues.

The similarity between the effects of the various nucleotides on normal and transformed cells suggest that both ATP and its analogues interact with the same membranal sites. The similarity is even more pronounced by the effect of Mg^{2+} which inhibits the permeabilization process induced by either ATP or by its analogues (data not shown).

Exogenous ATP and its analogues exert membrane depolarization

The effects of ATP and its analogues on $\Delta\psi$ were examined, using the lipophilic cation TPP^+ [11,12]. Fig. 2A shows the accumulation of $[^3H]TPP^+$ within 3T6 cells, up to a steady-state level, which was established during 15 min incubation, and was not changed for at least additional 15 min. The accumulation of TPP^+ within the cells implies a membrane potential, negative inside. At 16 min 0.6 mM of either ATP or an analogue was added, resulting in an immediated efflux of TPP^+. The apparent reduction of $\Delta\psi$ was calculated according to the intracellular and the external concentration of TPP^+, before and after addition of effector, using Eqn. 1. The internal concentration of TPP^+, after addition was made, was calculated according to its level 5 min after employing

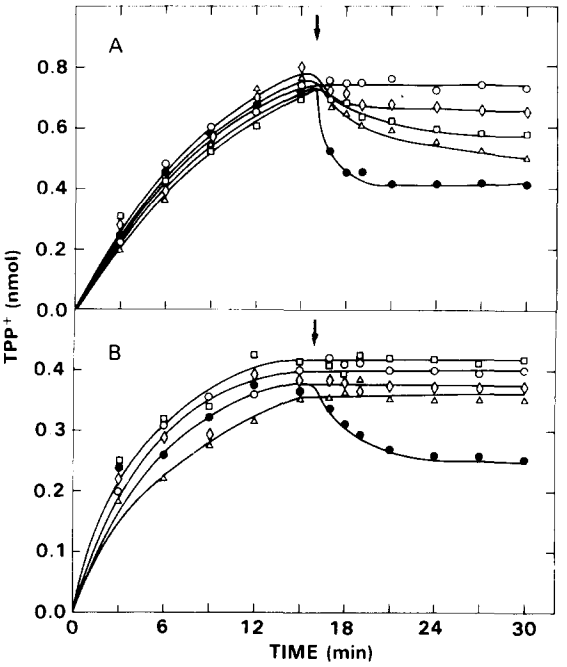

Fig. 2. Effect of ATP and its analogues on the intracellular level of TPP^+ in 3T6 cells (A) and 3T3 cells (B). Confluent cultures of the cells were washed and incubated with 1 ml of medium A at 37°C. $[^3H]TPP^+$ was added at zero time to a final concentration of 2 μM, and 50 μl samples were withdrawn at indicated time intervals to determine the radioactivity in the supernatant. At 16 min (arrow) additions were made as follows: (○), None: (△), 0.6 mM ATP; (□) 0.6 mM p[NH]ppA; (◇), 0.6 mM $p[CH_2]ppA$; (●), 5 μM SF-6847. The amount of intracellular TPP^+ at each time point is expressed as nmoles of TPP^+ per dish, taken up by the cells from the extracellular medium. The potent ionophore, SF-6847 enable to reveal the potential sensitive and insensitive portions of TPP^+ that was removed from the medium.

TABLE I

CELL MEMBRANE DEPOLARIZATION AND PERMEABILIZATION BY ATP ITS ANALOGUES AND IONOPHORE, IN 3T6 AND 3T3 CELLS

The reduction in the membrane potential ($\delta\Delta\psi$) was calculated as described in Fig. 2 and in Methods, and expressed in millivolts (mV). The rate of efflux is used as a parameter for cell membrane permeability. It was calculated as described in Fig. 1 and in Methods and expressed as the percent of cellular acid soluble pool released per min.

Additions	Concn.	$\delta\Delta\psi$ (mV)		Efflux rate (%/min)	
		3T6	3T3	3T6	3T3
None	–	0	0	0.2	0.4
ATP	0.6 mM	26	0	3.4	0.4
p[NH]ppA	0.6 mM	18	0	1.6	0.4
$p[CH_2]ppA$	0.6 mM	13	0	1.0	0.4
SF-6847	5.0 μM	66	61	4.6	4.6

either ATP or its analogue; namely, before the permeabilization to acid soluble pools took place. The effect of the potent ionophore, SF-6847 (5 μM), which exerts complete dissipation of $\Delta\psi$, was measured as a control. The data summarized in Table I show that the membrane depolarization induced by ATP is higher than that of its analogues, and between the two analogues p[NH]ppA is more effective. Thus, there is a correlation between the degree of the initial membrane depolarization and the following increase in cell membrane permeability. This correlation is supported by the effects of SF-6847 (Table I). Addition of the ionophore to a final concentration of 5 μM induced rapid efflux from the cells.

Fig. 2B shows the effect of ATP and its analogues on the TPP^+ level in 3T3 cells. In these cells, neither ATP nor its analogues affected $\Delta\psi$, whereas the addition of the ionophore resulted in a rapid dissipation of $\Delta\psi$. The decrease of $\Delta\psi$ correlates with an increase in the rate of efflux from the cells, as was found in 3T6 cells (Table I).

Discussion

The data presented in this study suggest, that the binding of ATP, by itself, is sufficient to initiate the early events leading to membrane permeabilization. This assumption is supported by the similarity of the effects induced by either ATP, or by its non-hydrolyzable analogues on the cell membrane: (a) Increase in cell membrane permeability, selective for transformed cells. (b) Exertion of selective cell membrane depolarization. The degree of depolarization is correlated with the subsequent degree of permeabilization. (c) The effects exerted by the analogues are concentration-dependent and inhibited by Mg^{2+}, as was shown for ATP [3,7]. Since the analogues are non-dissociable at the β,γ-phosphate bond [33,34] we ruled out the possibility that this bond is essential for the initiation of the permeabilization process. Neither is it likely that dissociation of the α,β-phosphate bond is involved, as the resultant AMP is not effective [3].

The lower degree of membrane depolarization and permeabilization, induced by the analogues, as compared to the effect produced by ATP, might be due to the differences in the molecular configuration of these compounds. Since the membranal alterations exerted by ATP are highly specific for this nucleotide [3], even minor differences in the molecular structure of ATP, p[NH]ppA and $p[CH_2]ppA$, could attribute to the different activities. An alternative possibility is that the permeabilization process, initiated by the binding of nucleotide to specific site, is further enhanced by ATP hydrolysis. Between the two analogues, $p[CH_2]ppA$ is less effective, and cell permeabilization induced by this nucleotide was detected only after 15 min of incubation. This delayed effect could explain previous observations, in which the activity of $p[CH_2]ppA$ was not detected during 10 min incubation [3].

Indications for purinergic receptors in various cells have been recently reviewed [35], and interaction of ATP with specific receptors has been suggested for ATP-induced permeabilization in mast cells [25,26]. It has been further suggested, that the active species is the free acid ATP^{4-}, the concentration of which could be much lower than the total ATP concentration, since it is dependent on the pH and the levels of bivalent ions [36]. In 3T6 cells, permeabilization by ATP has been induced by low concentrations (< 50 μM) of total ATP, under certain conditions [5,6,11]. Taken together with the specificity for ATP and the selectivity for 3T6 cells (as compared to 3T3 cells), it is warrantable to examine whether or not specific sites for ATP binding are present on the surface of 3T6 and other transformed cells.

The data presented in this study, as well as in previous ones [9–12] have shown a correlation between membrane depolarization and membrane permeabilization, when transformed cells were treated with either ATP, its non-hydrolyzable analogues, electrogenic ionophores, or high K^+ concentration. This correlation is even more pronounced in the non-transformed counterparts, 3T3 cells. In these cells, ATP, or its analogues, neither dissipates $\Delta\psi$ nor permeabilize the cells, whereas electrogenic ionophores exert both depolarization and permeabilization (Table I) [12]. Thus, it seems that $\Delta\psi$ plays a significant role in the maintenance of the cell permeability barrier. We propose that the binding of external ATP activates membranal channels, enables passive ion fluxes and membrane depolarization, followed by efflux of

intracellular, acid-soluble pools, which implicate the formation of aqueous channels in the membrane. Such channels might be produced by $\Delta\psi$-dependent conformational and topological alterations of membranal components.

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