Combined effects of ATP and its analogs on the membrane permeability in transformed mouse fibroblasts

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Abstract Extracellular ATP (0.6 mM) induces a marked decrease in the membrane potential, followed by an increase in cell membrane permeability in transformed mouse fibroblasts. The effects of the ATP analogs, p[CH_2]ppA and p[NH]ppA (0.6 mM), on the membrane potential and permeability are much less pronounced. ATP at 0.05 mM has no effect by itself, but markedly increases the analog-induced membrane potential dissipation and permeability. The data suggest that ATP-induced membrane permeation is composed of two processes: One is common to ATP and its analogs and appears to be a receptor-mediated process. The second is unique for ATP, effective even at low concentration (0.05 mM), and might be mediated by cell surface enzymes, for which ATP, but not its analogs, serves as a substrate.

Key words: Extracellular ATP; Membrane potential; Membrane permeability

1. Introduction

Incubation of transformed mouse fibroblasts with external ATP in a slightly alkaline medium, low in divalent cations, results in an increase in the cell membrane permeability for nucleotides and other small molecules. The membrane permeability of the non-transformed counterparts, however, is not affected [1]. The change of the permeability is rapid, but not immediate, temperature-dependent, and reversible [2]. Addition of growth medium results in re-sealing of the membrane, followed by cell proliferation [2]. The mechanisms underlying ATP-induced cell membrane permeabilization are only partly understood.

It has been shown that the interaction between ATP^{4-} and its receptor triggers the permeabilization [3]. Purinergic receptors, or purinoceptors, were found in many cells, and include two main types: P1 receptors for adenosine and related compounds, and P2 receptors for ATP, its analogs, and derivatives [4,5]. Five families of P2 receptors were defined and designated P2t, P2u, P2y, P2x and P2z. The receptors P2t, P2u and P2y are coupled to G-proteins, while P2x and P2z are receptors of ligand-gated channels. The P2z is also a pore-forming receptor in certain cells [4,6–8]. The P2t receptors were found in platelets, P2u and P2y were detected in many cell types, P2x in excitatory cells and P2z in certain cell types, like transformed and hemopoietic cells [8].

ATP-induced cell membrane permeabilization has been shown in many cells, including transformed mouse fibroblasts [2], mast cells [9], erythroleukemia cells [10], macrophages [11,12], Novikoff hepatoma cells [13], and certain other cells [8,14]. It is accepted by many investigators that permeabilization is mediated by the P2z receptor, but the mechanisms underlying this process are only partly understood [8]. It has been suggested that extracellular ATP induces the opening of hemi-gap junctions [13,15]. We have shown that the membrane permeation is preceded by ion fluxes and reduction of membrane potential, which can be mimicked by ionophores [16–19].

The data presented in this study suggest that ATP induces two effects, which lead to the increase in membrane permeability. One effect is related to the dissipation of the membrane potential and could be mimicked by non-hydrolyzable ATP analogs. The second effect is unique for ATP, unrelated to the membrane potential, and expressed at relatively low concentration of ATP.

2. Materials and methods

2.1. Materials

[^{3}H]Tetraphenylphosphonium bromide ([^{3}H]TPP^+) and $^{32}P_i$ were purchased from the Israel Atomic Energy Commission, Negev, Israel. ATP, 5′-adenylyl imidodiphosphate (p[NH]ppA), adenosine 5′-[β,γ-methylene]triphosphate (p[CH_2]ppA), and other chemicals and ionophores were purchased from Sigma (St. Louis, MO, USA), and were of highest purity available.

2.2. Cell cultures

3T6 cells, the transformed derivatives of Swiss mouse fibroblasts, 3T3 cells, were grown in plastic dishes, in Dulbecco's modified Eagle's Medium, containing 10% newborn calf serum, as described [16].

2.3. Measurement of TPP^+ level

Measurements of TPP^+ level and calculations of the membrane potential were performed as described [16]. Briefly, confluent cells, in 3.3 cm plastic dishes, were washed, incubated with medium A, composed of 100 mM Tris, 50 mM KCl, 0.05 mM $CaCl_2$, and 500 mg/ml dextran T-500, pH 7.8, at 37°C. [^{3}H]TPP^+ was added to a final concentration of 0.002 mM, and samples of buffer were withdrawn at the indicated time intervals for radioactivity measurements, to monitor the amount of TPP^+ taken up by the cells. The apparent membrane potential $\Delta\phi$ was calculated according to the Nernst equation:

$$\Delta\phi = RT/F \ln\{[TPP^+]_{in}/[TPP^+]_{out}\}$$

where R is the gas constant, T temperature (K), F the Faraday constant, and $[TPP^+]_{in}$ and $[TPP^+]_{out}$ are the concentrations of TPP^+ in the cells and surrounding medium, respectively. The apparent difference in the membrane potential ($\delta\Delta\phi$) was calculated as the difference in the membrane potential before ($\Delta\phi_1$) and after ($\Delta\phi_2$) additions were made, respectively:

$$\delta\Delta\phi = \Delta\phi_2 - \Delta\phi_1$$

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Abbreviations: p[NH]ppA, 5′-adenylyl imidodiphosphate; p[CH_2]ppA, adenosine 5′-[β,γ-methylene]triphosphate; TPP^+, tetraphenylphosphonium; $\Delta\phi$, the electrical potential across the cell membrane; $\delta\Delta\phi$, difference in $\Delta\phi$

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2.4. Measurement of the membrane permeability

The membrane permeability was determined by following the efflux of normally impermeant nucleotides, as previously described [16]. Briefly, cells were propagated in 3.3 cm plastic dishes to confluency, incubated for 3 h with $^{32}P_i$ (1 μCi/ml), washed and incubated in medium A. Samples were withdrawn at the indicated time intervals for radioactivity measurements, from which the amount of soluble pool released was calculated. Total soluble pool was determined after extraction of the cells with trichloroacetic acid. The rate of efflux of the soluble metabolites from the cells was calculated as percent of soluble pool released per min (%/min).

3. Results

Fig. 1 shows that addition of ATP (0.6 mM) to cultures of transformed mouse fibroblasts, 3T6 cells, in the presence of a slightly alkaline medium, low in divalent ions, results in a marked increase in the permeability of the cell membrane, as shown by the efflux of normally impermeant nucleotides. ATP analogs, p[NH]ppA and p[CH_2]ppA (0.6 mM), also induce membrane permeabilization, but to a much lesser extent: while ATP induced the release of 95% of the soluble pool, only 45% and 25% of the pool were released by p[NH]ppA and p[CH_2]ppA, respectively.

At relatively low concentration (0.05 mM), ATP did not affect membrane permeability by itself, but markedly increased the permeability induced by the ATP analogs (Fig. 1). The combination of 0.6 mM p[NH]ppA plus 0.05 mM ATP, added at zero time, exerted membrane permeation equivalent to the permeation induced by 0.6 mM ATP. Addition of 0.05 mM ATP to culture preincubated with 0.6 mM p[CH_2]ppA for 15 min resulted in an increase of the efflux level from 20% to 90% during the following 15 min, as compared to the increase from 20% to 25% in the control without ATP.

Taken together, the data suggest that ATP exerts two effects on the cell membrane during the permeabilization process. One effect could be mimicked by the ATP analogs, but the second one could be fulfilled only by ATP. The first effect is expressed at relatively high concentrations of the nucleotides (e.g. 0.6 mM), whereas the second one is accomplished at a relatively low concentration of ATP (e.g. 0.05 mM).

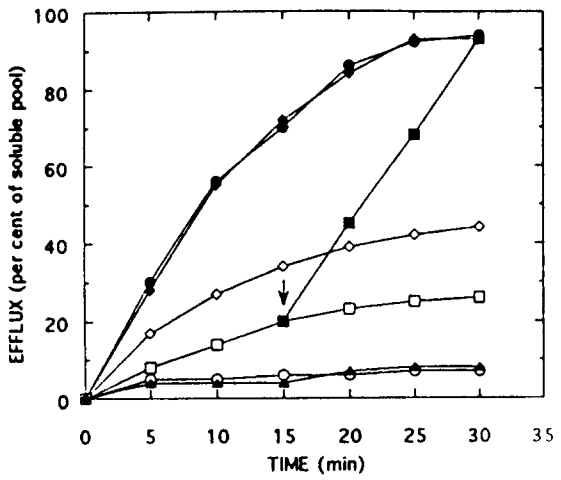

Fig. 1. Effects of ATP, and its analogs on the membrane permeability of 3T6 cells. Cell growth and efflux measurements were performed as described in section 2. Additions were made at zero time, except for addition of 0.05 mM ATP to 0.6 mM p[CH_2]ppA (arrow). (○) None; (▲) 0.05 mM ATP; (●) 0.6 mM ATP; (◇) 0.6 mM p[NH]ppA; (□) 0.6 mM p[CH_2]ppA; (◆) 0.6 mM p[NH]ppA plus 0.05 mM ATP; (■) 0.6 mM p[CH_2]ppA plus 0.05 mM ATP.

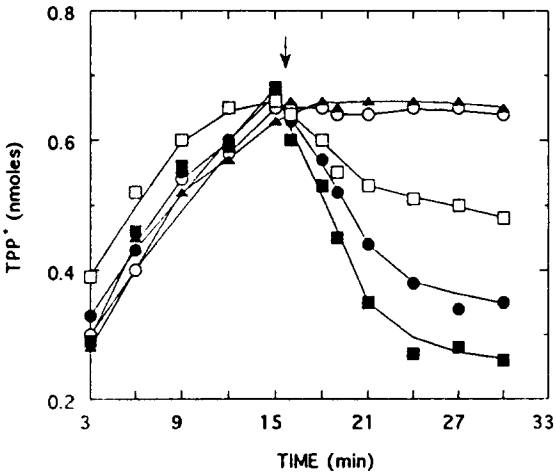

Fig. 2. Effects of ATP and its analogs on TPP^+ level in 3T6 cells. Cell growth and TPP^+ measurements were performed as described in section 2. TPP^+ was added at zero time and the nucleotides at 15 min. Additions: (○) control; (▲) 0.05 mM ATP; (●) 0.6 mM ATP; (□) 0.6 mM p[CH_2]ppA; (■) 0.6 mM p[CH_2]ppA plus 0.05 mM ATP.

Our previous studies have shown that the increase in the membrane permeability is preceded by ion fluxes across the cell membrane and a reduction of the membrane potential [16,18,19]. The membrane potential was measured by the accumulation of the hydrophobic cation TPP^+ in the cells. As shown in Fig. 2, TPP^+ accumulated within the cells to steady state, the level of which is dependent on the membrane potential. Addition of ATP at 0.05 mM did not affect the membrane potential, but at 0.6 mM ATP resulted in a decrease in the membrane potential, expressed as a reduction of cellular TPP^+ level. p[CH_2]ppA (0.6 mM) induces a moderate reduction of the membrane potential, but when ATP was added (0.05 mM) a marked reduction in the membrane potential was obtained.

The ratio: efflux/$\delta\Delta\phi$ is increased by 60% upon addition of ATP (0.05 mM) to an analog (Fig. 3). The increase in the ratio efflux/$\delta\Delta\phi$ indicates that ATP, at relatively low concentration, increases the efficiency of the permeabilization, induced by the analog.

This is additional support to the suggestion that two effects are exerted by ATP to obtain high membrane permeability: dissipation of the membrane potential, which could be mimicked by ATP analogs, and an additional effect, unique for ATP, that could be expressed at a low ATP concentration. The first effect induces partial permeation of the cell membrane, whereas the second effect does not influence the membrane permeability by itself, but markedly enhances the first effect.

4. Discussion

The phenomenon of ATP-induced cell membrane permeabilization has been shown, and partly characterized, in a variety of cell types [1,2,8,14,20]. Although it is generally accepted that the pore-forming P2z receptor, for which ATP^{4-} serves as a ligand, plays a major role in the permeabilization

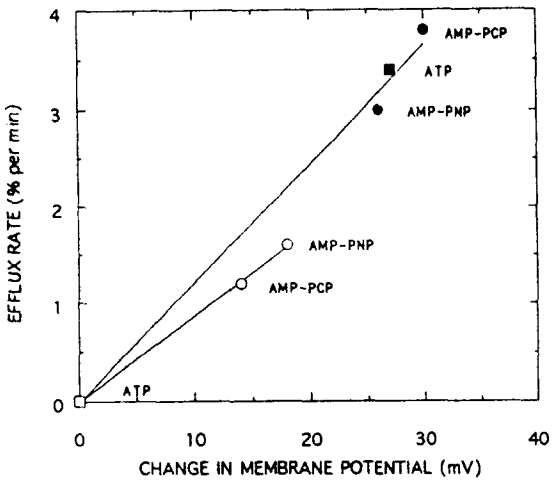

Fig. 3. Alterations of membrane potential ($\delta\Delta\phi$), and the rate of efflux, induced by ATP, its analogs, and their combinations. (□) 0.05 mM ATP; (△) 0.6 mM p[CH_2]ppA; (◇) 0.6 mM p[NH]ppA; (■) 0.6 mM ATP; (▲) 0.6 mM p[CH_2]ppA+0.05 mM ATP; (◆) 0.6 mM p[NH]ppA+0.05 mM ATP.

process, the mechanisms underlying this process are only partly understood [3,12,21–23].

The data presented in this study reveal that extracellular ATP exerts two effects on the cell membrane that lead to maximal permeabilization. One effect is initiated by the interaction of ATP with its receptor, followed by a reduction of the membrane potential and formation of non-selective pores. This effect could be mimicked by poorly hydrolyzable ATP analogs, like p[CH_2]ppA and p[NH]ppA. The second activity induced by ATP, even in its low concentration, does not affect the membrane permeability by itself, but markedly enhances the first effect.

It is suggested that the enhancement of the induced permeabilization by ATP at low concentration is mediated by cell surface enzyme, for the following reasons: (i) ATP at low concentration does not induce membrane permeabilization, and thus its effects are probably not mediated by the pore forming receptor. (ii) The ligand for the pore-forming receptor is ATP^{4-}, and relatively high concentration of total ATP is needed under the experimental conditions used to obtain an effective concentration of ATP^{4-} [3]. The substrate for cell surface protein kinases (ecto-PK) and nucleotidases is $(M^{2+}ATP^{4-})^{2-}$, and the K_m for the enzymatic reaction is in the micromolar range [24-26]. (iii) Ecto-PK was found in all cells examined, and it mediates the phosphorylation of membrane and extracellular proteins [24,27–29], and alters the response of the cell to extracellular ATP [26].

Other possible effects that might be exerted by ATP at low concentration, in addition to enzymatic reactions, are (i) the involvement of an additional receptor for ATP, like the P2x receptor, that reduces membrane potential [30,31] and (ii) activation of phospholipase D by the P2z receptor [32].

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