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External ATP-induced passive permeability change and cell lysis of cultured transformed cells: action in serum-containing growth media

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External ATP causes a marked increase in the passive permeability to phosphorylated metabolites in several types of transformed cells in alkaline medium containing low concentrations of Ca^{2+}, but not in untransformed cells. Such increased membrane permeability with external ATP was also observed in B16 melanoma cells at pH 7.4–7.5 in both Tris-buffered saline and a growth medium containing 10% calf serum and divalent ions at normal concentrations, although a higher concentration of ATP was required. The permeability change in the growth medium was significantly enhanced by calmodulin-interacting drugs, such as trifluoperazine (TFP), *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7) and chlorpromazine (CPZ). As expected, prolonged exposure of the cells to ATP in the serum-containing medium led to cell lysis. This ATP-dependent cell lysis was observed only in several transformed cell lines, and not in untransformed mouse fibroblasts. These results indicate that the effect of ATP on the membrane permeability in transformed cells is elicited under the physiological conditions and this would be useful in some limited way for cancer chemotherapy management.

Introduction

Passive permeability of the plasma membrane in mammalian cells plays an important role in the regulation of cellular growth as well as in the control of drug actions. It was recently demonstrated that the addition of ATP to the medium for several types of transformed cells in culture, such as transformed 3T3, B16 melanoma, HeLa and CHO-K1 cells, markedly increased the passive permeability, allowing passage through the membrane of phosphorylated metabolites and ions [1–10]. Untransformed cells including mouse 3T3 cells, mouse embryo fibroblasts and human lung fibroblasts, however, did not respond to external ATP under the same experimental conditions [2,4,5,8,10].

The ATP-dependent change in the permeability of these transformed cells is regulated by the cellular ATP concentration and cytoskeletal structures [3,9,11]. It is also stimulated by calmodulin antagonists [12,13], but suppressed by divalent ions and La^{3+} [1,4,7,11,13]. Furthermore, we recently isolated an ATP-resistant variant from CHO cells, constituting genetic evidence for this unique membrane alteration [14].

Although the molecular mechanism by which external ATP controls the permeability change is little understood, application of this phenomenon for the in vivo modulation of membrane permea-

Abbreviations: DMEM, Dulbecco's modified Eagle medium; TFP, trifluoperazine; CPZ, chlorpromazine; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; W5, *N*-(6-aminohexyl)-1-naphthalenesulfonamide.

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bility would be worthwhile examining: one possibility is its application to cancer chemotherapy to ensure selective toxicity of a drug toward transformed cells, since normally impermeant drugs were selectively incorporated into transformed 3T3 cells rendered permeable by external ATP [15].

However, most of these studies to modulate the membrane permeability with ATP at 0.1–0.5 mM have been made in mildly alkaline Tris- or Hepes-buffered saline containing 0.05 mM Ca^{2+} [1–15] and at neutral pH the permeability change did not occur, unless cellular ATP concentrations were reduced [2,3,8,10]. Therefore, it was interesting to determine if extracellular ATP would enhance the membrane permeability of transformed cells for nucleotides and ions under physiological conditions. In the present paper, we report that external ATP also induces a selective permeability change and cell lysis of B16 melanoma and transformed 3T3 cells in neutral serum-containing growth medium. Enhancement of the ATP effect by calmodulin-interacting agents is also described.

Materials and Methods

Chemicals. 2-Deoxy[1-^{3}H]glucose (15 Ci/mmol) and sodium [^{51}Cr]chromate (34 mCi/mg Cr) were obtained from Amersham International (Amersham, U.K.) and the Japan Radioisotope Association (Tokyo, Japan), respectively. Trifluoperazine was supplied by Yoshitomi Pharmaceutical Industries (Osaka, Japan). W7 and W5 were purchased from Riken Co., Ltd. (Tokyo, Japan). Chlorpromazine and all nucleotides used were obtained from Sigma (St. Louis, MO, U.S.A.). Calf serum and growth media (RPMI 1640, Ham's F12 and DMEM) were obtained from Flow Laboratories Inc. (McLean, VA, U.S.A.).

Cell cultures. B16 melanoma, mouse 3T3 and transformed 3T3 (3T6, SV3T3) cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml), as described [8,10]. The cells, after inoculation into 35-mm plastic dishes at densities of $(1–3) \cdot 10^5$ cells/dish, were cultured in the same medium for 2–3 days at 37 °C and then used for the present experiments.

Measurement of the passive permeability change. The passive permeability change was determined as described previously [9–12] by monitoring the efflux of acid-soluble radioactivity from the cells. The cells were labeled for 3 h at 37 °C with deoxy[^{3}H]glucose (0.25 μCi/ml, 1 μM) in glucose-free Dulbecco's modified Eagle medium or F12 medium containing 10% dialyzed calf serum. The labeled cells were washed twice with 0.15 NaCl and then incubated for 10–30 min at 37 °C in 1 ml of buffer A or in the indicated growth medium containing 10% fetal calf serum. Buffer A consisted of 0.1 M Tris-HCl, adjusted to pH 7.4 or 8.2, 0.05 M NaCl and 0.05 mM $CaCl_2$. As growth media, RPMI 1640, Ham's F12 and DMEM were used. After the incubation, the radioactivity released into the buffer or the medium was determined with a liquid scintillation counter.

Measurement of cell toxicity. The ATP-induced cytolysis was determined by measuring ^{51}Cr released from the labeled cells. Cultured monolayer cells were trypsinized, and then $(0.5–1) \cdot 10^7$ of these cells were incubated with 1 ml of RPMI1640 medium containing 10% calf serum and sodium [^{51}Cr]chromate (50 μCi/ml) in a plastic tube for 1 h at 37 °C. The cells were washed three times with ice-cold RPMI medium. The viability of the labeled cells was higher than 95%, as judged from the trypan blue exclusion. Then the cells were inoculated into 96-well microtiter plates, at a density of $5 \cdot 10^4$ cells/well, containing 200 μl of medium, 10% calf serum and the indicated drug. The culture plates were incubated for 0.5–5 h at 37 °C and centrifuged for 5 min at 1000 rpm. Then, the radioactivity released into the supernatant fraction was measured. Specific ^{51}Cr release is defined as (experimental release − spontaneous release)/(total cellular radioactivity − spontaneous release). Spontaneous release means the amount of isotope released from the labeled cells during incubation with the medium alone.

Results

Permeabilization by external ATP of B16 melanoma cells in Tris-buffered saline

When [^{3}H]deoxyglucose-labeled B16 melanoma cells were treated in Tris-buffered saline (buffer A) at pH 8.2 with exogenous ATP at 0.1 mM or higher concentrations at 37 °C, markedly in-

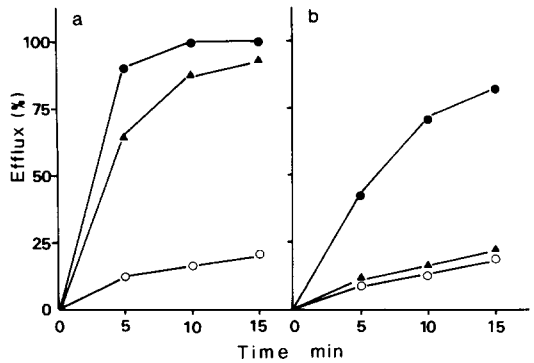

Fig. 1. External ATP-induced permeability change in B16 melanoma cells incubated in Tris-buffered saline. B16 melanoma cells labeled with deoxy[^{3}H]glucose were incubated at 37 °C in 1 ml of buffer A ((a) pH 8.2, (b) pH 7.4) containing the following additions: none, ○; 0.1 mM ATP, ▲; 0.5 mM ATP, ●. ATP was added at the start of the incubation. After incubation for the indicated periods, radioactivity released into the medium was counted. The total radioactivity within the cells which could be extracted with 5% cold trichloroacetic acid was $3.7 \cdot 10^3$ cpm/dish.

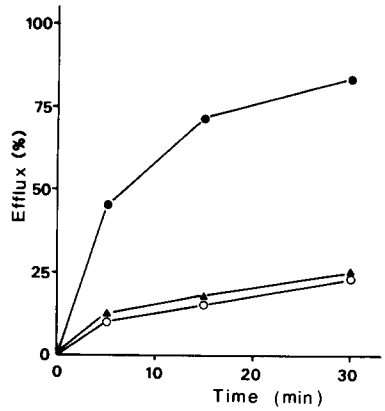

Fig. 2. Induction of a permeability change by ATP in B16 melanoma cells incubated in serum-containing growth medium. B16 melanoma cells labeled with deoxy[^{3}H]glucose were incubated at 37 °C in RPMI 1640 medium containing 10% calf serum and the following additions: none, ○; 0.5 mM ATP, ▲; 1.0 mM ATP, ●. After incubation for the indicated periods, radioactivity released into the medium was determined as described in Fig. 1.

creased efflux of radioactive materials, mainly deoxyglucose 6-phosphate [3,12], was observed (Fig. 1a), as described previously [9,10]. Such an increase in permeability caused by ATP was seen within 5 min incubation, most of the cellular radioactivity being released into the medium within 10–15 min incubation. It was also induced at pH 7.4 with 0.5 mM ATP (Fig. 1b).

Increase in membrane permeability in serum-containing growth medium

Since the ATP-dependent permeability change in B16 melanoma cells was induced in Tris-buffered saline at pH 7.4, it was of interest to see if it occurs in a complete medium suitable for cell growth. When [^{3}H]deoxyglucose-labeled cells were incubated with 1 mM ATP in RPMI 1640 medium, adjusted to pH 7.5, containing 10% calf serum, a rapid increase in the efflux of the radioactive materials was induced (Fig. 2). This phenomenon was specific for ATP, no other nucleotide being active (Table I), as in the case of Tris-buffered saline [1–5]. These results suggest that a similar mechanism is involved in the permeability change.

The effect of ATP on B16 melanoma cells was also examined in other growth media. The effect was seen in Ham's F12 medium, however, no effect was detected in DMEM containing up to 2 mM ATP (Table II). This may have been due to the higher concentrations of Mg^{2+} and Ca^{2+} in this medium than in RPMI 1640 and F12 medium (RPMI: 0.4 mM Ca^{2+}, 0.4 mM Mg^{2+}; F12: 0.3 mM Ca^{2+}, 0.6 mM Mg^{2+}; DMEM: 1.8 mM Ca^{2+}, 0.8 mM Mg^{2+}), since these divalent cations at millimolar concentrations in an alkaline Tris-

TABLE I

NUCLEOTIDE DEPENDENCY ON A PERMEABILITY CHANGE IN B16 MELANOMA CELLS INCUBATED IN GROWTH MEDIUM

The efflux of deoxy[^{3}H]glucose-labeled materials from B16 melanoma cells was determined as described in Fig. 2 after incubation of the cells in RPMI medium containing 10% calf serum at 37 °C for 30 min with the indicated nucleotide treatment. The total radioactivity within the cells was $4.8 \cdot 10^3$ cpm/dish.

Added nucleotide		Efflux (%)
Medium alone		21.2
ATP	1 mM	85.2
ADP	1 mM	24.4
AMP	1 mM	21.1
Ado*PP*[NH]*P*	1 mM	20.7
GTP	1 mM	20.1
CTP	1 mM	23.3
UTP	1 mM	21.5

TABLE II

COMPARISON OF VARIOUS GROWTH MEDIA AS TO THE PERMEABILITY CHANGE

The passive permeability change was determined as described in Table I after incubation of labeled B16 melanoma cells in the indicated medium containing 10% calf serum and ATP at 37 °C for 30 min.

Growth medium	ATP concn. (mM)	Efflux (%)
RPMI 1640	0.5	25.0
	1.0	90.6
F12	0.5	22.2
	1.0	85.0
DMEM	1.0	19.7
	2.0	23.0

buffer inhibit the effect of ATP [1,2,7,11,13]. Indeed, when either Mg^{2+} or Ca^{2} was added at 1 mM to the RPMI 1640 medium, the ATP-dependent increase in membrane permeability was completely inhibited (Table III). In contrast to this, a permeability change caused by external ATP in DMEM became observable when 1 mM EDTA was added with ATP to the medium.

These results agree with earlier works on the critical importance of these divalent ions in induction of the ATP-dependent permeability change in transformed fibroblasts [1,2,7], mast cells [16] and a macrophage-like tumor cell line [17], suggesting that ATP^{4-} is the effective agonist form of ATP [16,17].

TABLE III

EFFECTS OF DIVALENT CATIONS ON THE ATP-DEPENDENT PERMEABILIZATION OF B16 MELANOMA CELLS IN GROWTH MEDIA

The ATP-dependent permeability change was determined, as described in Table I, in B16 melanoma cells treated as indicated at 37 °C for 30 min.

Treatment	Efflux (%)
RPMI medium	17.6
+1 mM ATP	61.6
+1 mM ATP+1 mM $CaCl_2$	18.6
+1 mM ATP+1 mM $MgCl_2$	17.9
DMEM	17.7
+1 mM ATP	16.8
+1 mM ATP+1 mM EDTA	42.7

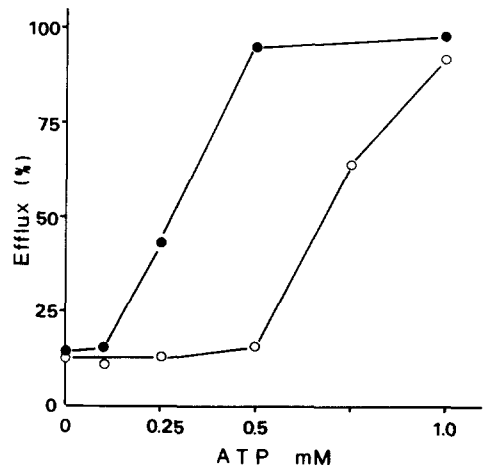

Fig. 3. Effect of TFP on the permeability change. B16 melanoma cells labeled with deoxy[^{3}H]glucose were incubated for 30 min at 37 °C in 10% serum containing RPMI medium with various concentrations of ATP in the absence (○) or presence of 60 μM TFP (●), and then the radioactivity released into the medium was determined as described in Fig. 1.

Enhancing effect of calmodulin antagonists on the permeability change

It has recently been reported that a calmodulin antagonist, trifluoperazine (TFP), potentiated the ATP-dependent increase in permeability of Chinese hamster ovary (CHO) cells [12] and mouse 3T6 cells [13]. This drug also stimulated the ATP-induced increase in permeability of B16 melanoma cells in serum-containing RPMI 1640 medium, and as little as 0.25 mM ATP was effective in the presence of TFP (Fig. 3). Similar results were obtained with other calmodulin antagonists, W7 and chlorpromazine (Table IV), but not with W5, which is an analogue of W7 having a lower affinity for calmodulin [18].

Cytotoxic effect of external ATP

We have reported that the ATP-induced permeability change in Tris-buffer leads to a concomitant loss of viability of the treated cells [14]. We therefore determined the cytotoxic effect of ATP on B16 melanoma cells in a growth medium. When the cells labeled with [^{51}Cr] were treated with ATP in serum-containing RPMI 1640 medium, a marked increase in the release of radioactivity was observed and the ATP concentration required for cell lysis was the same as that for the permeability

TABLE IV

ENHANCING EFFECTS OF CALMODULIN-INTERACTING DRUGS ON THE ATP-DEPENDENT PERMEABILITY CHANGE IN B16 MELANOMA CELLS

The efflux change of labeled B16 melanoma cells was determined as described in Table I, after incubation of the cells treated in RPMI medium containing 10% serum with the indicated drugs in the presence or absence of 0.5 mM ATP at 37 °C for 30 min.

Drugs	Efflux (%)	
	−ATP	+0.5 mM ATP
None	15.6	17.2
TFP 30 μM	17.2	33.4
60 μM	21.4	87.5
CPZ 60 μM	23.7	98.2
W7 60μM	23.2	79.2
W5 60 μM	21.0	25.1

change (Fig. 4). As expected, the rate of ^{51}Cr-release from the treated cells was lower than that of the efflux of [^{3}H]deoxyglucose 6-phosphate, as shown in Fig. 2. Furthermore, the ATP-induced cytolysis of B16 melanoma cells in the growth medium was also enhanced by TFP (Fig. 5). These results support the idea that the cytolysis of B16 melanoma cells is caused by the ATP-dependent permeability change.

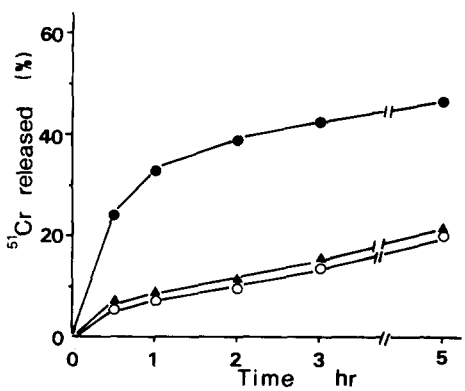

Fig. 4. Cytotoxic effect of external ATP on B16 melanoma cells. ^{51}Cr-labeled B16 melanoma cells were incubated for the indicated periods at 37 °C in RPMI medium containing 10% serum and the following additions: none, ○; 0.5 mM ATP, ▲; 1.0 mM ATP, ●. After the incubation, the radioactivity released into the medium was measured and it was expressed as percent of the total radioactivity incorporated into the cells.

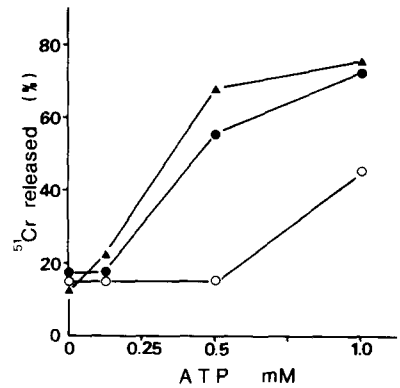

Fig. 5. Effects of calmodulin-interacting drugs on the ATP-induced cytotoxicity toward B16 melanoma cells. ^{51}Cr-Labeled B16 melanoma cells were incubated for 3 h at 37 °C in 10% serum-containing RPMI medium with the indicated concentrations of ATP and the following additions: none, ○; 20 μM TFP, ●; 20 μM CPZ, ▲. Then, the radioactivity released into the medium was determined as described in Fig. 4.

Cytotoxicity of external ATP toward normal and transformed 3T3 cells

The permeability change induced by external ATP in an alkaline buffer was limited to several types of transformed cells in culture, and untransformed cells did not respond to ATP [1–10]. The cytotoxic effect of ATP in the growth medium was further examined in both transformed and untransformed 3T3 cells. Apparent cytolysis was also induced by ATP in spontaneously transformed 3T3 cells (3T6) and this was enhanced in the presence of TFP (Table V). In another type of transformed 3T3 cell (SV3T3), cytolysis was not induced by ATP alone but it was seen with ATP

TABLE V

CYTOTOXIC EFFECT OF EXTERNAL ATP ON TRANSFORMED CELLS

^{51}Cr-Labeled 3T6, SV3T3 and 3T3 cells were treated as indicated in 10% calf serum-containing RPMI medium at 37 °C for 3 h, and then the specific ^{51}Cr release (%) from the cells was determined as described under Materials and Methods.

Treatment	Specific ^{51}Cr-released (%)		
	3T6	SV3T3	3T3
0.5 mM ATP	1.5	0.1	0.1
1.0 mM ATP	14.9	0.1	2.7
20 μM TFP	2.8	3.5	8.4
+0.5 mM ATP	23.6	15.2	11.3
+1.0 mM ATP	57.8	28.0	13.0

in combination with TFP. However, no ATP-dependent cytolysis was observed in normal 3T3 cells, even in the presence of TFP. These results further suggest that the effect of external ATP on the membrane permeability is different between normal and transformed cells under physiological conditions.

Discussion

It has been demonstrated that various types of transformed cells in culture are rapidly rendered permeable by exogenously added ATP at 0.1–0.5 mM to phosphorylated metabolites and ions in an alkaline medium [1–7]. This phenomenon is highly specific for ATP and transformed cells. In the present study, we observed a change in membrane permeability of B16 melanoma cells on treatment with 1 mM of external ATP in growth medium at pH 7.5 containing 10% calf serum and divalent ions. The characteristics of the permeability change were similar to those of that seen in an alkaline Tris buffer as to the time course (Figs. 1 and 2), nucleotide specificity (Table I) and enhancement by calmodulin-interacting drugs (Fig. 3). A higher concentration of ATP was required for induction of the permeability change in the serum-containing medium than in the Tris buffer (Fig. 2). This is mainly due to the high concentrations of divalent ions in these media, since the permeability change caused by ATP in RPMI medium is inhibited by the addition of 1 mM Mg^{2+} or Ca^{2+} (Table III), and the permeability change in DMEM, which contains the highest concentrations of divalent cations, occurred only when 1 mM EDTA was added to the medium (Table III). These results strongly suggest a common mechanism by which the ATP-dependent permeability change is regulated in both an alkaline buffer and a serum-containing growth medium.

Extracellular ATP has also been shown to affect ion fluxes in a variety of tumor cell lines, such as Ehrlich ascites cells [19,20], erythroleukemia cells [21] and a macrophage-like cell line [17,22]. Rapaport [23] reported that extracellular ATP as well as ADP at micromolar concentrations had a selective inhibitory effect on the growth of transformed human cell lines. However, such concentrations of ATP had little effect on the growth of B16 melanoma cells (unpublished results). External ATP also affects the membrane permeability for small ions and phosphorylated compounds of normal mast cells [16,24], lymphocytes [25], macrophages [22] and erythrocytes [26], although the mechanisms and physiological role and the correlation to the present results remains unknown.

It was recently demonstrated that the modulating effects of external ATP on membrane permeabilities of mast cells [16] and macrophages [17] were induced by micromolar concentrations of ATP^{4-} as an active agonist in the medium, and neither by Mg^{2+}-ATP^{2-} nor Ca^{2+}-ATP^{2-}. In the present study on B16 melanoma cells, a higher concentration of ATP (0.75–1 mM) was required for the permeability change in serum-containing media compared to that in an alkaline Tris-buffer containing 0.05 mM Ca^{2+} (Figs. 1,2). The ATP^{4-} concentration present in RPMI medium containing 1 mM ATP, 0.4 mM Mg^{2+} and 0.4 mM Ca^{2+} was roughly estimated to be about 10^{-4} M by the use of known values and equations [17,27], and it was nearly identical with the estimated concentration of ATP^{4-} in the alkaline buffer when the permeability change was induced. These facts as well as the evidences presented previously [16,17] strongly suggest that ATP^{4-} is an effective form. However, other factors might be also involved in this permeabilization, since at neutral pH it did not occur in several transformed cells even when the effective concentrations of ATP^{4-} might be present [2,5,9]. Further assays with various concentrations of Mg^{2+} and Ca^{2+}, and pH values make it clearer a critical role of ATP^{4-} in the control of membrane permeability by external ATP.

It has been reported that the ATP-dependent permeability change in 3T6 cells is reversible [2,5,6]. We recently observed that the permeability change in CHO cells was also reversible [11,12], but prolonged exposure to ATP reduced the reversibility, markedly decreasing the cell viability [14]. By this procedure, an ATP-resistant variant was isolated from the CHO cells [14]. More recently, an ATP-resistant cell was also isolated from a macrophage-like cell [17]. These studies on the resistant cells clearly demonstrated that Mg^{2+}-ATPase activity in the cell surface was not

associated with the ATP-dependent permeabilization. In the present study, we demonstrated that treatment with ATP of B16 melanoma and 3T6 cells in serum-containing medium induced cell lysis, as indicated by the ^{51}Cr release from the cells (Fig. 4 and Table V). Like the permeabilization due to ATP, the ATP-induced cell lysis of transformed cells was also enhanced by calmodulin antagonists (Fig. 5 and Table V). In contrast, untransformed 3T3 cells did not respond to ATP, even in the presence of TFP, showing the selective effect of ATP on these transformed cells, including B16 melanoma, 3T6 and SV3T3 cells.

In this connection, it is of interest to note that calmodulin inhibitors like TFP and W7 are very useful for increasing the cellular accumulation of anti-cancer agents, resulting in enhancement of the cytotoxicity and in a partial reversal of the drug resistance of cancer cells both in vitro and in vivo [28,29]. The present results and the above-mentioned facts taken together suggest the great usefulness of exogenously added ATP in chemotherapeutic management against transformed cells. However, it should be also noted that external ATP has some pronounced effects on normal cells [16,22,24–26]. The experiments on ATP effects both in vitro and in vivo as well as studies on the mechanism of the permeability change are now in progress in this laboratory.

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