Jpn. J. Cancer Res. 83, 121–126, January 1992

Modulation of Membrane Permeability, Cell Proliferation and Cytotoxicity of Antitumor Agents by External ATP in Mouse Tumor Cells

Tsutomu Mure,[1] Kaiso Sano[1] and Takayuki Kitagawa[2]

[1]*Division of Gastroenterology, Department of Surgery, Kawasaki Medical School, 577 Matsushima, Kurashiki 701-1 and* [2]*Department of Chemistry, National Institute of Health, 2-10-35 Kamiosaki, Shinagawa-ku, Tokyo 141*

External ATP causes a remarkable change in the passive permeability of the plasma membrane in several types of transformed cells. When mouse melanoma cells, Clone-M3, were exposed to ATP in Tris-buffered saline, a great increase in the passive permeability was induced within several minutes. Longer exposure of Clone-M3 cells to external ATP led to a decrease in cell viability. Similar results were obtained with Ehrlich ascites cells, but none of these ATP effects were noted in untransformed cells such as NIH 3T3 cells or BALB/c mouse embryonic fibroblasts. The *in vitro* cytotoxic effects of antitumor agents (5-fluorouracil, adriamycin, mitomicin C and nimustine hydrochloride) against Clone-M3 cells were additively potentiated by treatment with external ATP, which also synergistically enhanced the cytotoxicity of vincristine. However, the effects of these drugs on mouse embryonic fibroblasts were not modulated by ATP. These results suggest that ATP-treatment is a useful means of enhancing a selective toxicity for tumor cells.

Key words: External ATP — Membrane permeability — *In vitro* cytotoxicity — Antitumor agent — Mouse tumor cell line

Modulation of the membrane permeability for antitumor agents would be a useful method to achieve a selective chemotherapeutic effect against tumor cells. Recently, it has been demonstrated that brief exposure of several types of transformed cells in culture, such as transformed 3T3, B16 melanoma, HeLa and CHO-K1 cells, to external ATP markedly increases the passive permeability, allowing the passage through the plasma membrane of phosphorylated metabolites and ions.[1-8] Untransformed cells, including Swiss and BALB/c 3T3 cells, and mouse embryonic fibroblasts, did not respond to ATP under the same experimental conditions.[1-4, 6, 8] Although the molecular mechanism by which external ATP controls the permeability change remains unknown, application of this phenomenon for the *in vivo* modulation of the membrane permeability for antitumor agents would be worth examining. In the present study, we determined the effects of external ATP on the membrane permeability and proliferation of transformed and untransformed mouse cell lines, as well as on the cytotoxic effects of several antitumor agents on mouse melanoma cells in culture.

MATERIALS AND METHODS

Chemicals ATP (disodium salt, vanadium-free), ADP (sodium salt, vanadium-free), AMP (sodium salt) and adenosine were purchased from Sigma (St. Louis, MO). The pH of the solutions was adjusted to 7.4 with 1 *N* NaOH and they were kept at −20°C. [^{3}H]Uridine (23.3 Ci/mmol) was obtained from NEN (Boston, MA). The following five antitumor agents were used: 5-fluorouracil (5-FU; Kyowa Hakko Co., Tokyo), mitomycin C (MMC; Kyowa Hakko Co.), nimustine hydrochloride (ACNU; Sankyo Co., Tokyo) and vincristine (VCR, Shionogi, Co., Tokyo).

Cell culture Two types of transformed mouse cells (Clone-M3 cells and Ehrlich ascites cells) and two types of untransformed mouse cells (NIH 3T3 cells and BALB/c mouse embryonic fibroblasts) were used in the present study. Mouse embryonic fibroblasts were used at 2–10 passages from a freshly prepared primary culture. The cells were cultured in the following media: Clone-M3 cells, Ham's F10 medium containing 15% horse serum and 2.5% fetal calf serum; Ehrlich ascites cells and mouse embryonic fibroblasts, Eagle's MEM containing 10% fetal calf serum; and NIH 3T3 cells, Eagle's MEM containing 10% calf serum. For experiments, 1.5–2.0 × 10^5 cells were plated in 35 mm plastic dishes containing the above-mentioned media, and they were used after culture for 2–3 days at 37°C in a CO_2 incubator.

Measurement of the passive permeability change The passive permeability change was determined as described previously,[1, 5] with minor modifications, by monitoring either the efflux of acid-soluble radioactive materials from labeled cells or hydrolysis of *p*-nitrophenyl phosphate (pNPP). The cultured cells were labeled with [^{3}H]uridine (0.5 μCi/ml, 1 μM) in the growth medium

[2] To whom all correspondence should be addressed.

at 37°C for 3 h. The cells were washed twice with 0.15 *M* NaCl and then incubated with various concentrations of ATP in 1 ml of Tris buffer (0.1 *M* Tris-HCl/pH 7.5 or 8.2, 50 m*M* NaCl, 50 m*M* $CaCl_2$ and 5 mg/ml of Dextran 500) at 37°C. After incubation for the indicated periods, the medium was removed and filtered through a GF/C glass fiber filter (Whatman) to remove the cells. The radioactivity of the filtrate was then measured with a liquid scintillation counter. Total cellular acid-soluble pools of the labeled cells were extracted with cold 5% trichloroacetic acid (TCA) at 4°C for 20 min. For studying the hydrolysis of pNPP, the cells treated in Tris-buffer as indicated were further incubated at 37°C for 10 min with the same medium in the presence of 5 m*M* pNPP. The supernatant was removed by centrifugation and then mixed with 0.1 ml of 1 *M* NaOH to measure the *p*-nitrophenol formed at 410 nm. The hydrolysis of pNPP was catalyzed by alkaline phosphatase that remained inside the ATP-treated cells.[5]

Determination of cell viability Clone-M3 cells treated with ATP as indicated were washed twice and then the cells were further incubated at 37°C for 72 h in Ham's F10 medium containing 15% horse serum and 2.5% fetal calf serum. The viable cells which excluded trypan blue were counted and the cell viability was calculated on the basis of the number of cells untreated with ATP. Changes in the viability of untransformed cells were determined similarly after incubation for 48 h of ATP-treated cells.

Combined effects of antitumor agents and ATP on cell growth Clone-M3 cells were treated with 0.5 m*M* ATP for 10 min and then washed twice, and 1×10^5 cells were suspended in 2 ml of F10 medium containing 15% horse serum, 2.5% fetal calf serum and various concentrations of the indicated drug. In the cases of mouse embryonic fibroblasts, the cells were cultured in 2 ml of MEM containing 10% fetal calf serum after similar ATP-treatment. Then, the cultures were further incubated at 37°C for 72 h and changes in the number of viable cells were determined as mentioned above.

RESULTS

Changes in the membrane permeability of the ATP-treated transformed cells When Clone-M3 cells labeled with [^{3}H]uridine were treated with 0.5 m*M* ATP in Tris-buffered saline at pH 8.2, a great increase in the efflux of radioactivity was induced within a few minutes after the addition of ATP, and was completed within 15 min (Fig. 1a). The released radioactivity was found to represent TCA-soluble components, indicating that it consisted mainly of the labeled nucleotides inside the cells, as described by Rozengurt *et al.*[1] The enhanced efflux was also induced at pH 7.5, but the rate was slower.

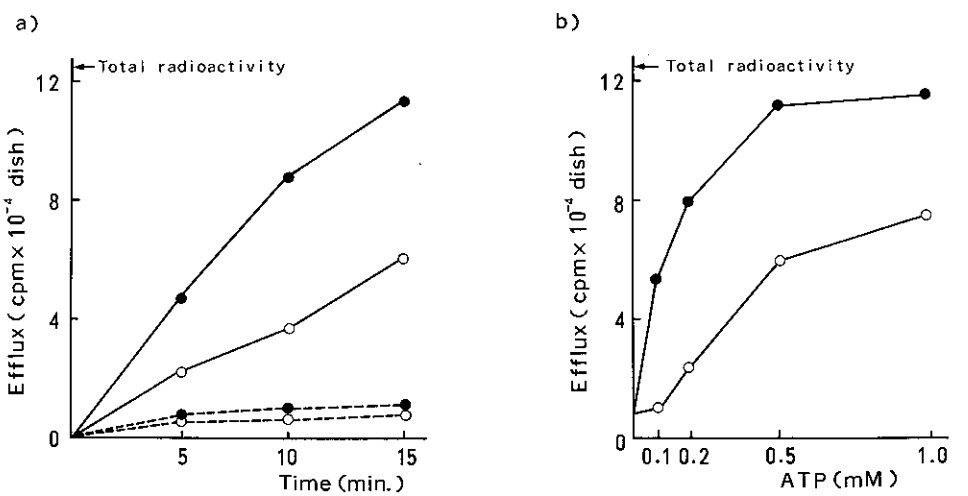

Fig. 1. Effect of exogenous ATP on the efflux of [^{3}H]uridine-labeled acid-soluble materials from Clone M3 cells. (a) Labeled cells were incubated at 37°C in 1 ml of Tris-buffer at either pH 8.2 (●) or 7.5 (○) in the absence (broken lines) or presence of 0.5 m*M* ATP (solid lines), and then the radioactivity released into the medium at the indicated time was determined as described in "Materials and Methods." (b) Labeled cells were similarly treated with various concentrations of ATP at pH 8.2 (●) or 7.5 (○) at 37°C for 15 min, and then the released radioactivity was measured. The amounts of total acid-soluble radioactivity within the cells which could be extracted with 5% cold trichloroacetic acid are indicated by the arrows.

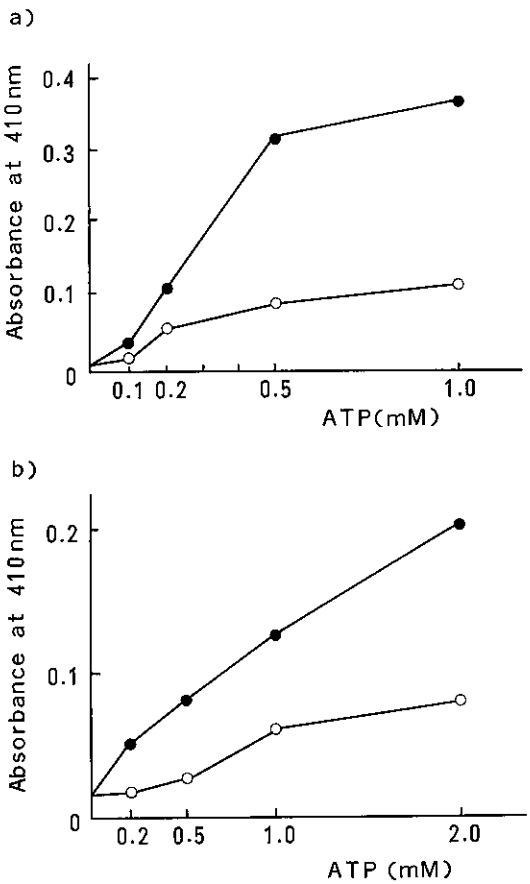

Fig. 2. Hydrolysis of *p*-nitrophenyl phosphate by transformed cells treated with external ATP. Clone M3 cells (a) or Ehrlich ascites cells (b) were treated with the indicated concentrations of ATP at pH 8.2 (●) or 7.5 (○) for 15 min and then the hydrolysis of *p*-nitrophenyl phosphate by the treated cells was determined as described under "Materials and Methods."

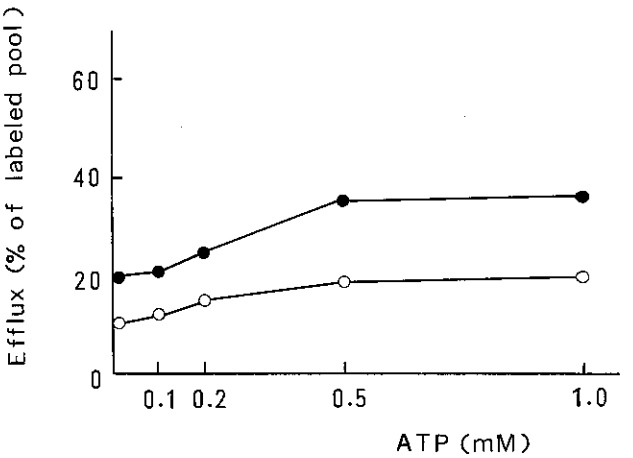

Fig. 3. Effect of ATP on the passive permeability of untransformed mouse fibroblasts. Untransformed NIH3T3 cells (●) or mouse embryonic fibroblasts (○) were labeled with [^{3}H]uridine and then changes in the efflux of radioactivity from the cells were determined after treatment at 37°C for 15 min at pH 8.2 with the indicated concentrations of ATP, as described in Fig. 1.

Table I. Effects of ATP and Its Metabolites on Acid-soluble Radioactivity Efflux from Clone-M3 Cells

Addition	Efflux of acid-soluble pools[a] (cpm/dish × 10^{-3})[b]	(%)
None	10.5	9.4
ATP	110	97.8
ADP	14.2	12.7
AMP	15.4	13.7
Adenosine	12.1	10.8
NaH_2PO_4	17.2	15.3

a) [^{3}H]Uridine-labeled Clone-M3 cells were incubated in Tris-buffer (pH 8.2) containing the indicated additions at 0.5 m*M* for 15 min, and the efflux was determined.
b) The total acid-soluble radioactivity within the cells was 112×10^3 cpm/dish.

The permeability change in Clone-M3 cells was induced by 0.1–0.5 m*M* ATP (Fig. 1b) and it was also apparent as an increase in hydrolysis by ATP-treated cells of pNPP added to the medium (Fig. 2a). With this procedure, the ATP-dependent permeability change was also observed with another type of mouse tumor cell line, Ehrlich ascites cells (Fig. 2b), although higher concentrations of ATP were required. In contrast to the ATP response in these transformed cells, little change in membrane permeability was observed in untransformed mouse cells, including mouse embryonic fibroblasts and NIH 3T3 cells, when they were treated with 0.5 m*M* ATP at pH 8.2 for 15 min (Fig. 3).

Specificity for ATP of the permeability change To examine the nucleotide specificity of the permeability change, the effects of various ATP metabolites were determined in [^{3}H]uridine-labeled Clone-M3 cells. The permeability change was highly specific for ATP, and ADP, AMP, adenosine and sodium phosphates were all inactive (Table I). These characteristics of the permeability change, including the time course, pH-dependency, required ATP concentration, and specificity for transformed cells and ATP, are quite similar to those previously observed in other transformed cells such as transformed 3T3 cells, B16 melanoma cells, and HeLa cells.[1-6]

Decrease in cell viability on ATP-treatment The effect of external ATP on cell viability was determined using conditions under which ATP induces the permeability

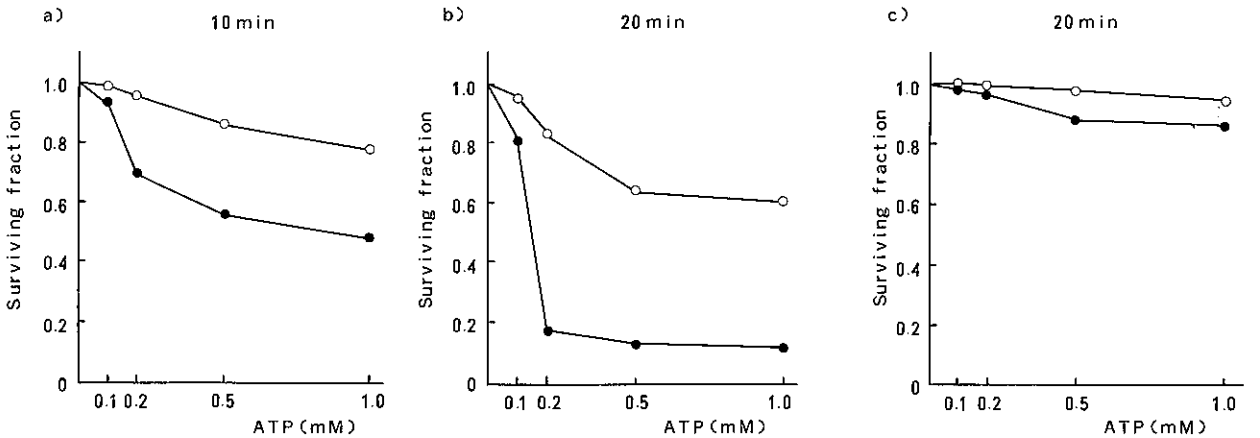

Fig. 4. Changes in the viability of Clone-M3 cells and mouse embryonic fibroblasts on treatment with external ATP. Clone-M3 cells were treated with the indicated concentrations of ATP at pH 8.2 (●) or 7.5 (○) for 10 min (a) or 20 min (b). Then the cells were washed and plated onto fresh dishes containing the growth media described in "Materials and Methods." After incubation at 37°C for 72 h, the number of viable cells was determined. A similar experiment was performed on mouse embryonic fibroblasts (○) or NIH3T3 cells (●) treated with ATP for 20 min (c).

change in transformed cells. The ATP-treatment of Clone-M3 cells at alkaline pH increased the number of trypan blue-stained cells in a time-dependent manner, most of the cells being stained with the dye within 30 min after the start incubation with ATP (data not shown).

Changes in cell viability on ATP-treatment were further studied by examining the growth of the cells. When Clone-M3 cells were treated with various concentrations of ATP for 10 min, a decrease in the number of viable cells was observed at more than 0.2 m*M* ATP at an alkaline pH, but at pH 7.5 the extent of the decrease was smaller (Fig. 4a), in accordance with the pH-dependence of the permeability change shown in Fig. 1. This decrease in cell viability in the ATP-treated cells at pH 8.2 was more dramatic when the cells were treated for 20 min (Fig. 4b). In contrast, the viability of untransformed fibroblasts (NIH3T3 and mouse embryo), whose permeability was not increased by ATP, was unchanged by the ATP-treatment at pH 8.2 (Fig. 4c).

Potentiation by ATP of the effects of antitumor agents on Clone-M3 cells To examine how external ATP modulates the cytotoxic effects of antitumor agents in Clone-M3 cells as well as in untransformed mouse embryonic fibroblasts, cells were treated with 0.5 m*M* ATP at pH 8.2 for 10 min, and then the treated cells were washed and further incubated for 72 h with the indicated concentrations of an antitumor agent (5-FU, ADM, MMC, ACNU, VCR). Each drug alone decreased the number of viable Clone-M3 cells due to the cytotoxic effect (Table II). The ATP-treatment caused an about 50% decrease in the viability, as described above, and it additively decreased the growth of the cells in combination with either 5-FU, ADM, MMC or ACNU. These additive effects of antitumor drugs and external ATP on the growth of tumor cells were also noted when the cells were treated with ATP at pH 7.5 (data not shown). In the case of VCR, however, the ATP-treatment enhanced its cytotoxicity synergistically in the range of 0.1–1 ng/ml. By this ATP-treatment, the effective concentrations of these antitumor agents for the killing of Clone-M3 cells were lowered 10- to 50-fold. In contrast, external ATP did not alter the cytotoxicity of any of these antitumor agents towards mouse embryonic fibroblasts, although these cells were more resistant to these agents.

DISCUSSION

It has been demonstrated that various types of transformed animal cells in culture are rapidly rendered permeable to nucleotides, sugar phosphates and ions by exogenous ATP at 0.1–1 m*M*.[1-10] This passive permeability change is highly specific for ATP and transformed cells. Alkaline conditions with low concentrations of divalent cations are also preferable, but it can be induced at neutral pH (Fig. 1 and ref. 8). In the present study, we observed an external ATP-dependent permeability change in two types of transformed mouse cells, Clone-M3 melanoma cells and Ehrlich ascites cells. The characteristics of the permeability changes, such as pH-dependency and nucleotide specificity, are quite similar to those previously observed in other transformed cells.[1-6] These

Table II. Effect of External ATP on the Growth Inhibition of Normal and Transformed Cells by Various Antitumor Drugs

Cells	Drug[a]	Dose (ng/ml)	Survival rate (%)[b] ATP (−)	ATP (+)
Clone-M3	5-FU	0	100.0	51.0
		100	93.4	50.4
		1,000	69.7	34.7
		10,000	25.1	13.7
		100,000	24.9	12.1
	ADM	0	100.0	48.1
		1	70.9	35.9
		10	32.6	16.0
		100	27.0	11.6
		500	2.5	0.5
	MMC	0	100.0	52.1
		10	66.4	35.8
		100	24.7	13.5
		1,000	19.7	10.6
	ACNU	0	100.0	47.5
		10	94.6	46.3
		100	89.0	40.8
		1,000	20.7	10.2
		10,000	16.9	8.8
	VCR	0	100.0	45.7
		0.1	96.2	33.4
		1	77.2	26.9
		5	25.6	9.8
		10	18.0	7.8
Mouse fibroblasts	ADM	0	100.0	98.0
		1	95.1	90.0
		10	—[c]	—
		100	75.1	75.2
		500	55.2	50.0
	VCR	0	100.0	—
		1	95.2	95.2
		5	82.0	85.1
		10	75.1	60.0

a) 5-FU, 5-fluorouracil; MMC, mitomycin C; ACNU, nimustine hydrochloride; VCR, vincristine.
b) Clone-M3 cells or mouse embryonic fibroblasts were treated with 0.5 m*M* ATP for 10 min at pH 8.2, and the number of viable cells was determined after incubation for 72 h with the indicated drugs.
c) Not determined.

results suggest that a common ATP-responsive element is present on the surface of a variety of transformed cells.

Although the mechanism of the ATP-dependent permeability change remains largely unknown, it has been postulated that the responses to extracellular ATP are mediated by cell surface receptors (P_2 purinoceptors).[11] The ATP binding to a P_2 purinoceptor may lead to the formation of membrane channels available to normally impermeant molecules.[10, 11]

The external ATP-treatment of Clone-M3 cells also decreased the viability of the cells. This change could be due to the ATP-induced permeability change, because these two changes were well correlated, depending on the pH of the medium and the ATP concentration (Figs. 1 and 4). Furthermore, the viability of mouse embryonic fibroblasts, whose permeability was not increased by ATP, was unchanged by the ATP-treatment. We also reported previously that longer exposure of permeabilized CHO cells to external ATP markedly decreased the cell viability, and by using this procedure, an ATP-resistant variant was isolated from CHO cells.[9] These results indicate that the difference in the cytotoxicity of exogenous ATP between normal and transformed cells is caused by the selective permeability change.

These findings led us to consider the possible application of this procedure to cancer chemotherapy. As an initial experiment, ATP-sensitive Clone-M3 cells were permeabilized with ATP in an alkaline buffer and then the cells were further cultured with an antitumor agent. The combined treatment with a drug and ATP decreased the *in vitro* growth of the tumor cells additively (Table II). Similar results, but to lesser extents, were observed when the ATP-treatment was performed at pH 7.5. VCR and ATP showed a synergistic cytotoxicity to Clone-M3 cells, especially at low deses of VCR. This might be linked in part to the fact that the ATP-dependent permeability change is enhanced by VCR or vinblastine.[7]

In conclusion, the ATP-treatment of Clone-M3 cells resulted in about 10- to 50-fold decreases in the effective concentrations of these antitumor agents for the killing of the cells. In contrast, the same ATP-treatment did not alter the sensitivities of untransformed cells to the antitumor drugs. We demonstrated previously that normally impermeant drugs could be incorporated into ATP-treated 3T6 cells.[4] The present results may be more significant, because the ATP-treatment potentiated the effects of several antitumor agents which are used clinically. These results warrant further *in vivo* experimentation on cancer chemotherapy by using chemotherapeutic agents in combination with ATP.

ACKNOWLEDGMENTS

This research was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan, and a research grant from the Human Science Foundation of Japan.

(Received August 29, 1991/Accepted October 25, 1991)

REFERENCES

1) Rozengurt, E., Heppel, L. A. and Friedberg, I. Effect of exogenous ATP on the permeability properties of transformed cultures of mouse cell lines. *J. Biol. Chem.*, **252,** 4584–4590 (1977).
2) Makan, N. R. Induction of permeability changes and restoration of membrane permeability barrier in transformed cell cultures. *Exp Cell Res.*, **114,** 417–427 (1978).
3) Kitagawa, T. Protein synthesis in transformed cells permeabilized by exogenous ATP. *J. Cell. Physiol.*, **102,** 37–43 (1980).
4) Kitagawa, T. Selective inhibition of macromolecular synthesis in transformed cells by means of ATP-treatment. *Biochem. Biophys. Res. Commun.*, **94,** 167–173 (1980).
5) Kitagawa, T. and Akamatsu, Y. Control of passive permeability of Chinese hamster ovary cells by external and intracellular ATP. *Biochim. Biophys. Acta,* **649,** 76–82 (1981).
6) Kitagawa, T. and Akamatsu, Y. Different sensitivities to external ATP in passive permeability of transformed cells. *Jpn. J. Med. Sci. Biol.*, **35,** 213–219 (1982).
7) Kitagawa, T. and Akamatsu, Y. Modulation of passive permeability by external ATP and cytoskeleton-attacking agents in cultured mammalian cells. *Biochim. Biophys. Acta,* **734,** 25–32 (1983).
8) Kitagawa, T., Amano, F. and Akamatsu, Y. External ATP induces passive permeability changes and cell lysis in cultured transformed cells: action in serum-containing growth media. *Biochim. Biophys. Acta,* **941,** 257–263 (1988).
9) Kitagawa, T. and Akamatsu, Y. Control of membrane permeability by external ATP in mammalian cells: isolation of a ATP-resistant variant from Chinese hamster ovary cells. *Biochim. Biophys. Acta,* **860,** 185–193 (1986).
10) Weisman, G. A., Lustig, K. D., Friedberg, I. and Heppel, L. A. Permeabilizing mammalian cells to macromolecules. *Methods Enzymol.*, **171,** 857–869 (1989).
11) Gordon, J. L. Extracellular ATP: effects, sources and fate. *Biochem. J.*, **233,** 309–319 (1986).