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MODULATION OF PASSIVE PERMEABILITY BY EXTERNAL ATP AND CYTOSKELETON-ATTACKING AGENTS IN CULTURED MAMMALIAN CELLS

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External ATP causes a passive permeability change in several transformed cells, but not in untransformed cells. We previously demonstrated that in CHO-K1 cells, a transformed clone of Chinese hamster ovary cells, the external ATP-dependent permeability change was induced when the intracellular ATP concentration was reduced by a mitochondrial inhibitor (Kitagawa, T. and Akamatsu, Y. (1981) Biochim. Biophys. Acta 649, 76–82). A permeability change with similar characteristics was also observed when the CHO cells were treated with external ATP and a cytoskeleton-attacking agent such as vinblastine or cytochalasin B. Just like mitochondrial inhibitors, vinblastine could increase the sensitivity of transformed 3T3 cells to external ATP but showed no effect on passive permeability of normal 3T3 cells. However, in contrast with the effect of the mitochondrial inhibitors, the cytoskeleton drugs caused the permeability change with little reduction of intracellular ATP concentration, suggesting different actions of these two kinds of drug on the permeability change. The present results suggest an important role of cytoskeletal structures in controlling the external ATP-dependent permeability change in transformed cells. Possible effects of intracellular ATP on cytoskeletal structures are also discussed.

Introduction

The plasma membrane surrounding living cells serves as a selective permeability barrier against ions and charged molecules such as nucleotides and phosphate esters. The mechanism regulating this important function of mammalian cells is not well understood. Recently, it was reported that treatment of transformed cells such as 3T6, SV3T3 and B16 melanoma in cultures with external ATP caused a striking increase in passive permeability, allowing passage through the plasma membrane of nucleotides, glycolytic intermediates and ions [1–6]. Untransformed mouse fibroblasts, however, did not respond to the ATP. This permeability change in transformed cells was specific for external ATP and was also controlled by intracellular ATP; the sensitivity to external ATP increased several-fold when the intracellular ATP concentration was reduced [6,7]. Furthermore, we recently demonstrated that in CHO-K1 cells, a transformed clone of Chinese hamster ovary cells, this ATP-specific permeability change was observed only when the intracellular ATP concentration was reduced by a mitochondrial inhibitor [8].

There are several reports demonstrating the effects of intracellular ATP on various plasma membrane-associated functions such as control of the cell shape [9], cell agglutination [10] and receptor-mobility [11]. Since these membrane changes are known to be regulated by the assembly and function of the cytoskeleton which consists mainly of microtubules and microfilaments, these effects of cellular ATP can be implicated in the functions of the cytoskeleton [12,13]. The present experiments were undertaken to determine whether the per-

meability change controlled by external and intracellular ATP in transformed cells is modulated by drugs which affect microtubules and microfilaments. In this article, we report that an external ATP-dependent permeability change is also induced in CHO cells treated with vinblastine or cytochalasin B. These permeability changes were independent of changes in intracellular ATP concentration. The possible roles of the cytoskeleton and intracellular ATP in control of the permeability change are discussed.

Materials and Methods

Chemicals. 2-Deoxy[^{3}H]glucose (15 Ci/mmol) was obtained from Amersham International, U.K. Vinblastine sulfate was kindly supplied by Shionogi Co. Ltd., Tokyo, and was also obtained from Sigma, St. Louis, MO, USA. Vincristine sulfate, cytochalasin B, colchicine and all nucleotides used were purchased from Sigma. Other chemicals were of reagent grade.

Cell culture. Chinese hamster ovary cells, clone K1 (CHO-K1), originally obtained from Flow Laboratories Inc., Rockville, MD, U.S.A., were cultured as described [8] in Ham's F12 medium supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). 3T6 cells, spontaneously transformed mouse 3T3 fibroblasts, and Balb/C3T3 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and the antibiotics. The cells inoculated into 35 mm dishes at densities of $(1-2)\times 10^5$ cells/dish were cultured at 37°C for 2–3 days and then used for the present experiments.

Measurement of passive permeability change. The passive permeability change was measured as described previously [6,8] by monitoring efflux of the acid-soluble radioactive materials from the monolayer cells. The cells were labeled at 37°C for 3 h with 1 M deoxy[^{3}H]glucose (0.25 μCi/ml) in glucose-free F12 or Dulbecco's modified Eagle medium containing 10% dialyzed serum. The labeled cells were washed twice with 0.15 M NaCl and then incubated at 37°C for 10–15 min with 1 ml buffer A containing the indicated additions. Buffer A consisted of 0.1 M Tris-HCl, adjusted to pH 8.2 or 7.2, 0.05 M NaCl and 0.05 mM $CaCl_2$. After the incubation, the radioactivity released into the medium was measured with a liquid scintillation counter.

Reversibility of the permeability change was determined as described [2]. CHO cells permeabilized by ATP and the indicated drug in buffer A (pH 8.2) were incubated at 37°C for 2 h with [^{3}H]uridine (0.5 μCi/ml, 1 μM) in F12 medium containing 10% fetal calf serum, and the radioactivity incorporated into trichloroacetic acid-soluble fractions in the cells was measured.

Measurement of intracellular ATP. Intracellular ATP was extracted from the monolayer cells with ice-cold 0.4 M perchloric acid, and the ATP concentration was determined enzymatically with luciferin-luciferase, using a Packard Tri-Carb liquid scintillation spectrometer [14].

Results

Effects of anti-microtubule and anti-microfilament drugs on the ATP-dependent permeability change in CHO-K1 cells

To determine whether the drugs which alter cytoskeletal structures affect passive permeability of CHO-K1 cells, cells labeled with deoxy[^{3}H]glucose were treated at 37°C with Tris-buffered saline (buffer A, pH 8.2) containing colchicine, vinblastine or cytochalasin B in the presence or absence of ATP. A rapid increase in efflux of radioactive materials, which were mainly deoxy[^{3}H]glucose 6-phosphate, was observed by treatment of the cells with ATP and a microtubule drug, vinblastine (Fig. 1). The permeability change induced by external ATP and vinblastine was seen within 5 min incubation, and almost all the radioactive materials in the cells were released after 10–15 min incubation. ATP or the drug alone did not induce the efflux change.

In the presence of 0.5 mM external ATP, as little as 0.1 mM vinblastine was effective for induction of the permeability change (Fig. 2). A similar effect was also found with vincristine, a structural analog of vinblastine. However, another well-known anti-microtubule drug, colchicine, could not cause the permeability change. Cytochalasin B, an anti-microfilament drug, induced the permeability change at a concentration of more than 0.15 mM in the presence of the external ATP.

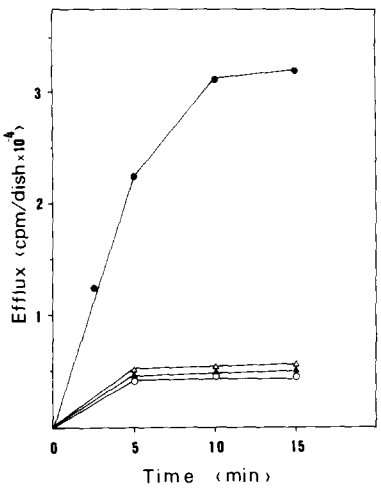

Fig. 1. Induction of a permeability change in CHO cells by addition of ATP and vinblastine. CHO-K1 cells labeled with deoxy[^{3}H]glucose were incubated at 37°C in 1 ml Buffer A (pH 8.2) containing the following additions: none, ○; 0.2 mM vinblastine, ▲; 0.5 mM ATP, △; 0.5 mM ATP+0.2 mM vinblastine, ●. The indicated additions were made at the start of the incubation. After the indicated period of incubation, radioactivity released into the medium was counted. The total radioactivity within the cells which could be extracted with 5% cold trichloroacetic acid was 32500 cpm/dish.

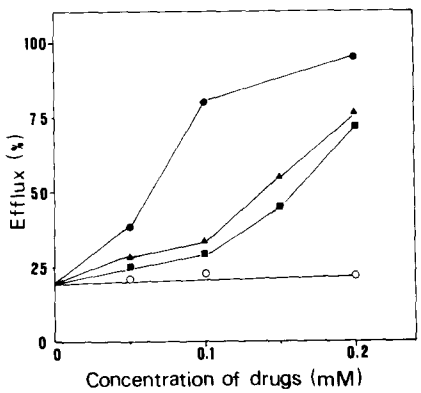

Fig. 2. Effect of various cytoskeleton-attacking agents on induction of the ATP-dependent permeability change. Deoxy[^{3}H]glucose-labeled CHO cells were incubated at 37°C for 15 min in buffer A (pH 8.2) containing 0.5 mM ATP and various concentrations of the indicated drugs. Symbols for each drug are: ●, vinblastine; ▲, vincristine; ○, colchicine; ■, cytochalasin B. Efflux (%) of the radioactivity was calculated on the basis of the total radioactivity within the cells extracted with 5% trichloroacetic acid (27600 cpm/dish). The control values of efflux (%) when the cells were incubated with the drug alone at 0.2 mM were as follows: vinblastine, 16.5; vincristine, 17.8; colchicine, 16.0; cytochalasin B, 16.5.

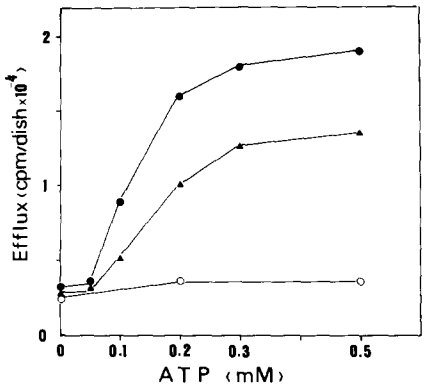

Fig. 3. ATP concentration required for the permeability change by vinblastine or cytochalasin B. The labeled CHO cells were treated at 37°C for 10 min with buffer A (pH 8.2) containing various concentrations of ATP together with the following drugs: 0.2 mM vinblastine, ●; 0.2 mM cytochalasin B, ▲; no addition, ○. After the incubation, the released radioactivity was measured. The total radioactivity in the cells was 20600 cpm/dish.

External ATP was required at more than 0.1 mM for induction of both the permeability change with vinblastine and that with cytochalasin B (Fig. 3). with F12 medium. Indeed, these cells in F12

Characteristics of the permeability change induced by ATP and cytoskeletal drugs

Some characteristics of the permeability change induced by external ATP and the cytoskeletal drugs were determined, and compared with those previously observed with ATP and a mitochondrial inhibitor [8]. The permeability change with vinblastine or cytochalasin B was specific for ATP, since Ado*PP*[NH]*P* ADP, AMP and other nucleotide triphosphates were all inactive (Table I). The permeability changes were also dependent on the pH of the reaction medium and were observed at pH 8.2 but not at pH 7.2. These characteristics of the permeability change induced by the ATP and the cytoskeleton-attacking drugs were quite similar to those observed for the permeability change induced by ATP and a mitochondrial inhibitor [8], suggesting that a common mechanism regulates these external ATP-dependent permeability changes. Furthermore, the ATP-dependent permeability change induced by either vinblastine, cytochalasin B or a mitochondrial inhibitor (KCN) was found to be reversible to the extent of 71–83% by incubating the permeabilized cells

TABLE I

NUCLEOTIDE AND pH DEPENDENCY ON THE PERMEABILITY CHANGE IN THE PRESENCE OF VINBLASTINE OR CYTOCHALASIN B

Efflux of deoxy[^{3}H]glucose-labelled materials from CHO cells was determined as described in Fig. 2 after incubation of the cells at 37°C for 10 min in buffer A containing each nucleotide indicated at 0.5 mM. Vinblastine or cytochalasin B was added at 0.2 mM.

Nucleotide	Efflux (cpm/dish)($\times 10^{-3}$)	
	Vinblastine	Cytochalasin B
pH 8.2		
None	5.15	4.50
ATP	29.6	21.5
Ado*PP*[NH]*P*	8.30	6.30
ADP	6.98	5.48
AMP	4.80	5.20
GTP	4.70	5.64
CTP	4.90	5.35
UTP	4.80	5.60
pH 7.2		
None	4.43	4.20
ATP	7.96	4.90

TABLE II

REVERSIBILITY OF THE ATP-DEPENDENT PERMEABILITY CHANGE IN CHO CELLS

CHO cells were treated as indicated in buffer A at 37°C for 8 min to induce the permeability change. The cells were then incubated with F12 medium and 10% serum containing [^{3}H]uridine (0.5 μCi/ml, 1 μM) at 37°C for 2 h. After the incubation, the radioactivity incorporated into trichloroacetic acid-soluble fractions was measured. A small decrease in the radioactivity by treatment with ATP alone (line 2) was due to a small detachment of the cells from the dish.

Treatment	[^{3}H]Uridine incorporated	
	cpm/dish	%
Buffer A (pH 8.2)	38650	100
+ATP(0.5 mM)	33450	86
+ATP+vinblastine(0.2 mM)	28180	73
+vinblastine	31590	82
+ATP+cytochalasin B(0.2 mM)	27600	71
+cytochalasin B	30280	78
+ATP+KCN(1 mM)	32100	83
+KCN	40630	105
Untreated	41200	106

medium containing [^{3}H]uridine could accumulate the labeled acid-soluble nucleotides (Table II), possibly resulting from rapid sealing of the permeability barrier and restoration of the metabolic activities in the cells including intracellular ATP-generating systems as described [2,4]. This reversibility clearly indicated that the ATP-dependent permeability changes were not due to cell lysis.

Relationship between changes in cellular ATP concentration and the permeability change in CHO cells treated with vinblastine or cytochalasin B

Since mitochondrial inhibitors such as rotenone, oligomycin and KCN lowered the intracellular ATP concentration to induce the external ATP-dependent permeability change in CHO cells, we next determined the effect of the cytoskeletal drugs on the intracellular ATP concentration of CHO cells. The cellular ATP concentration was decreased only to 80–90% of that of the control culture by treatment with 0.2 mM cytochalasin B in buffer A (pH 8.2), under which conditions the permeability change was induced (Fig. 4). Judging from the previous results with mitochondrial in-

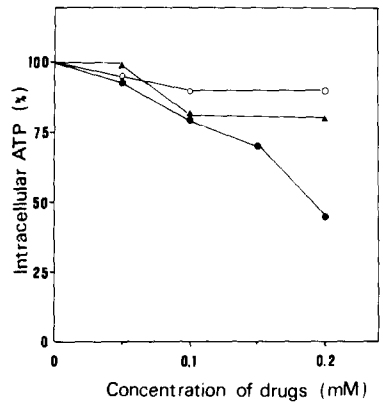

Fig. 4. Effect of vinblastine, colchicine and cytochalasin B on the intracellular ATP concentration of CHO cells. CHO-K1 cells which had been cultured in glucose-free medium containing dialyzed serum at 37°C for 3 h were treated with various concentrations of the indicated drugs for 10 min at 37°C in buffer A (pH 8.2). The amounts of intracellular ATP in these treated cells were determined. Colchicine, ○; cytochalasin B, ▲; vinblastine, ●. The 100% value of the cells which were incubated in buffer A alone was 2.20 nmol/10^6 cells. Similar results were obtained with the cells which had been cultured in glucose-containing medium and the control value of intracellular ATP concentration was 3.20 nmol/10^6 cells.

hibitors, such a small decrease in cellular ATP concentration could be insufficient for the permeability change. Furthermore, a small change in the cellular ATP concentration was also seen upon treatment with colchicine. Vinblastine up to 0.1 mM, which was enough to induce the permeability change (Fig. 2), did not significantly reduce the cellular ATP concentration. However, on increasing the concentration of the drug, the cellular ATP concentration was reduced to about 40% of the control value. Therefore, we examined more directly the relationship between cellular ATP concentration and the permeability change with vinblastine. As described [8], addition of excess glucose to the medium containing rotenone or KCN restored the reduced cellular ATP concentration, resulting in complete suppression of the external ATP-dependent permeability change (Table III). In contrast to these results, the permeability change induced by external ATP and vinblastine remained in the presence of glucose, although the cellular ATP concentration was completely restored by glucose. All these facts clearly demonstrate that the permeability change with vinblastine or cytochlasin B did not require reduction of the cellular ATP concentration.

TABLE III

EFFECT OF GLUCOSE ON INTRACELLULAR ATP CONCENTRATION AND ATP-DEPENDENT PERMEABILITY CHANGE IN CHO-K1 CELLS

CHO cells were cultured at 37°C for 3 h in glucose-free medium plus dialyzed serum in the presence or absence of deoxy[^{3}H]glucose. The unlabeled cells were treated as indicated in buffer A at pH 8.2 for 10 min at 37°C to determine the change in intracellular ATP concentration. At the same time, the labeled cells were incubated at 37°C for 10 min with buffer A (pH 8.2) containing the indicated additions and 0.5 mM ATP to determine the efflux of the radioactivity.

Treatment	Cellular ATP (%)	Efflux change by ATP (cpm/dish) ($\times 10^{-3}$)
Buffer A (pH 8.2)	100	6.6
+ rotenone (3 μM)	5	31.7
+ rotenone + 10 mM glucose	75	7.5
+ KCN (1 mM)	5	32.5
+ KCN + 10 mM glucose	68	7.0
+ vinblastine (0.2 mM)	42	32.1
+ vinblastine + 10 mM glucose	100	30.2

Effect of divalent cations on the permeability change

On the cell surface of mammalian cells the existence of various ATP-requiring ectoenzymes such as ATPases [15–17] and protein kinases [18,19] has been reported. Since in most cases these ectoenzymes require Mg^{2+} and/or Ca^{2+}, it was of interest to determine whether these divalent cations modulated the external ATP-dependent permeability change. Buffer A used for the present experiments contained 0.05 mM $CaCl_2$ but this cation was not necessary for the induction of the permeability change by mitochondrial inhibitors or cytoskeletal drugs (data not shown). As shown previously in transformed 3T3 cells [1], in CHO cells the permeability change with rotenone, vinblastine or cytochalasin B was inhibited by addition of $MgCl_2$ (Fig. 5a). Different results were obtained with $CaCl_2$. The permeability change with rotenone was not affected by $CaCl_2$ up to 2 mM, but that with vinblastine or cytochalasin B was strongly inhibited by $CaCl_2$ (Fig. 5b). These results suggest that the ectoenzymes which may mediate the effect of external ATP on the permeability change do not require these cations exogenously.

Passive permeability change in normal and transformed mouse 3T3 cells

The effect of external ATP was limited to several

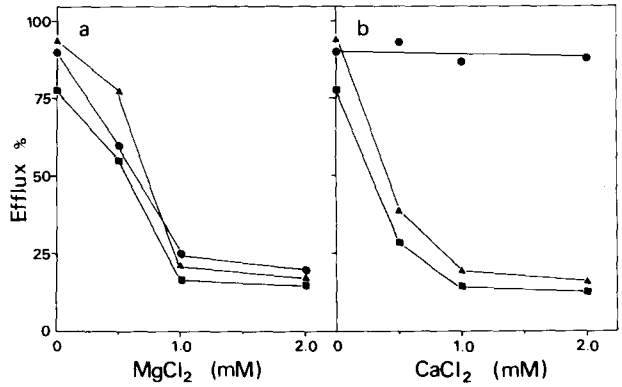

Fig. 5. Effect of divalent cations on the ATP-dependent permeability change. Effects of Ca^{2+} and Mg^{2+} on the permeability change in CHO cells were determined. Deoxy[^{3}H]glucose-labeled CHO cells were incubated in buffer A (pH 8.2) containing 0.5 mM ATP and either 3 μM rotenone (●), 0.2 mM vinblastine (▲) or 0.2 mM cytochalasin B (■) in the presence of various concentrations of (a) $MgCl_2$ or (b) $CaCl_2$. Efflux of the radioactivity was measured as described in Fig. 3.

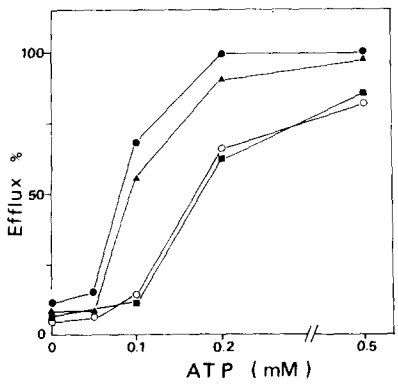

Fig. 6. Effect of rotenone, vinblastine and cytochalasin B on the ATP-dependent permeability change in 3T6 cells. 3T6 cells labeled with deoxy[^{3}H]glucose were incubated in buffer A (pH 8.2) containing various concentrations of external ATP and either 3 μM rotenone (●), 0.2 mM vinblastine (▲), 0.2 mM cytochalasin B (■) or no addition (○) for 10 min at 37°C. The total radioactivity in the cells was 31 700 cpm/dish.

transformed cells and untransformed mouse fibroblasts did not respond to the ATP [1–8]. We determined the effect of the cytoskeletal drugs on passive permeability of normal (3T3) and transformed (3T6) mouse fibroblasts. The response of 3T6 cells to external ATP increased about 2-fold when the cells were treated with either rotenone or vinblastine (Fig. 6). Cytochalasin B had little effect on the ATP-dependent permeability change in 3T6 cells. In 3T3 cells, the external ATP-dependent permeability change was not induced in the presence of rotenone, vinblastine or cytochalasin B, demonstrating further a differential effect of external ATP on transformed and normal cells (Table IV).

TABLE IV

EFFLUX CHANGE IN 3T3 CELLS TREATED WITH EXTERNAL ATP AND DRUGS

Balb/C 3T3 cells were labeled with deoxy[^{3}H]glucose in glucose-free Dulbecco's modified Eagle medium and serum. The labeled cells were treated with buffer A as indicated at 37°C for 10 min and the efflux of the radioactivity was measured. Total radioactivity within the cells was 34000 cpm/dish.

Treatment	Efflux	
	cpm/dish ($\times 10^{-3}$)	%
Buffer A (pH 8.2)	2.70	8.0
+0.5 mM ATP	2.95	8.5
+3 μM rotenone	4.40	12.9
+rotenone+ATP	4.90	14.4
+0.2 mM vinblastine	4.31	12.7
+vinblastine+ATP	7.58	22.2
+0.2 mM cytochalasin B	3.32	9.8
+cytochalasin B+ATP	3.20	9.3

Discussion

The present results demonstrate that a passive permeability change is induced in CHO-K1 cells when the cells are treated with external ATP in the presence of vinblastine, vincristine or cytochalasin B, which could affect the structure and functions of the cytoskeleton. These permeability changes are specific for added ATP and are observed at pH 8.2 but not at pH 7.2. These characteristics of the permeability change are quite similar to those of that induced by ATP and a mitochondrial inhibitor [8]. In addition, the ATP-dependent permeability change with a mitochondrial inhibitor or the cytoskeletal drugs in CHO cells are shown to be reversible as described in the case of transformed mouse fibroblasts which was permeabilized by ATP alone [2]. These facts strongly suggest that the cytoskeleton-attacking drugs and the mitochondrial inhibitors play a common role in inducing the external ATP-dependent permeability change. However, a significant difference in the action of these drugs is that the mitochondrial inhibitors alter the intracellular ATP concentration to achieve the effect of external ATP, whereas vinblastine or cytochalasin B can induce the permeability change without a drastic change in the intracellular ATP concentration (Fig. 4 and Table III). Furthermore, the sensitivity to Ca^{2+} of these two kinds of permeability change is also very different (Fig. 5). According to these facts, it is possible to speculate that the depletion of cellular ATP causes some specific alterations in the structures of the cytoskeleton and plasma membrane which are induced by vinblastine or cytochalasin B.

The cellular cytoskeleton, which is mainly composed of microtubules and microfilaments, is associated with the plasma membrane and is implicated in the control of various plasma membrane-associated phenomena including changes in cell shape, membrane movement, receptor mo-

bility and cell agglutination [20]. These membrane changes are also reported to be influenced by intracellular ATP [9–11,13]. Several studies have shown the effects of cellular ATP on the cytoskeleton. First, cellular ATP can directly stabilize the cytoskeletal structures [21]. Second, the cellular ATP can control the concentrations of other important nucleotides like GTP through the transphosphorylation reaction [22,23]. Another attractive possibility is that cellular ATP modulates cytoskeletal structures through phosphorylation-dephosphorylation of the cytoskeletal proteins [24–26]. At the moment, however, we have no direct evidence as to how intracellular ATP modulates changes of cytoskeletal structures and functions that may lead to the permeability change.

Vinblastine is known to bind to tubulin and to disassemble microtubules [27,28]. This drug also induces some aggregation of microtubules at higher concentrations (10^{-4}–10^{-3} M; [28,29]). It should be noted that the concentration for the formation of these aggregates agrees well with that for the permeability change described here (Fig. 2). Furthermore, colchicine, which was ineffective for the permeability change, cannot cause the aggregation of microtubules and binds to them at different sites from those for vinblastine [28,29]. We have recently observed by electron microscopic examination that vinblastine, but not colchicine, induces aggregation of microtubules stained with fluorescent tubulin antibody in CHO cells under the condition for the permeability change (unpublished results). A different action of vinblastine from that of colchicine on membrane permeability is also reported in avian erythrocytes [30]. These facts may indicate that vinblastine induces some specific alterations in microtubule structures associated with the permeability change.

Cytochalasin B is known to act on actin microfilaments, but the effective concentration for the permeability change is somewhat higher than that for the reported effects on microfilaments [11,13,20]. Therefore, the permeability change with this drug might be due to some nonspecific effects on the cytoskeleton and plasma membrane. It is also possible that cytochalasin B primarily acts on actin microfilaments to modulate the interactions between the cytoskeleton and the plasma membrane, since they are closely associated with each other [11,13,20,31,32]. Alternatively, this drug might alter the cytoskeletal structures by modulating protein phosphorylations of the cytoskeleton [33]. Recently, several mutants resistant to vinca alkaloids or to colchicine have been isolated [34,35]. Such a genetic approach, as well as electron microscopic study, is expected to be useful for clarifying the complex actions of these drugs on the structures and functions of the cytoskeleton and the plasma membrane.

On the basis of the characteristics observed in the present and previous studies, involvement of protein phosphorylations in the plasma membrane in the ATP-dependent permeability change is suggested [2,7,8,36]. If this is the case, alteration in the cytoskeletal structures could modulate the external ATP-dependent protein phosphorylations in the plasma membrane. In recent years there have been a number of reports concerning the effects of external ATP on membrane permeabilities. For example, ATP affects Ca^{2+}, Na^{+} and nucleotide permeabilities in mammalian cells [37–40]. In addition, external ATP is reported to inhibit insulin-mediated glucose transport in fat cells [41]. This inhibitory effect of ATP is associated with some protein phosphorylations in the plasma membrane. In another report, drug-permeability in CHO cells is associated with phosphorylation of a glycoprotein in the membrane [42] and this drug-permeability is also modulated by intracellular ATP or treatment with vinca alkaloids [43]. Further biochemical and genetic studies on the ATP effect will provide useful information on regulation of passive permeability in mammalian cells.

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