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Adenovirus-Dependent Changes in Cell Membrane Permeability: Role of Na^+,K^+-ATPase

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Adenovirus-dependent release of choline phosphate from KB cells at pH 6.0 was partially blocked by ouabain. In K^+-containing medium, maximum inhibition of release was obtained by 10^{-5} M ouabain and half-maximal inhibition was achieved by about 0.5×10^{-6} M ouabain. Ouabain did not block either the binding or the uptake of adenovirus by KB cells. Without K^+, about 25% of cell-associated choline phosphate was released by adenovirus, whereas with 1 mM K^+ about 50% was released. This activation by K^+ was blocked by 0.1 mM ouabain. HeLa cells behaved like KB cells, but a mutant of HeLa cells resistant to ouabain (D98-OR) released much lower amounts of choline phosphate in response to human adenovirus type 2 (Ad2). Wild-type D98-OR cells bound nearly the same amount of adenovirus as did normal HeLa cells. Ad2 also increased the activity of Na^+,K^+-ATPase in KB cells, with maximum activation at 50 μg of Ad2 per ml. In D98-OR cells, Ad2 failed to activate Na^+,K^+ ATPase activity. Ad2-dependent lysis of endocytic vesicles (receptosomes) was assayed by measuring Ad2-dependent enhancement of epidermal growth factor-*Pseudomonas* exotoxin toxicity. This action of adenovirus was increased when K^+ was present in the medium. Under the conditions used, K^+ had no effect on the amount of Ad2 or epidermal growth factor taken up by the cells. On the basis of these results, it is suggested that Ad2-dependent cellular efflux of choline phosphate and adenovirus-dependent lysis of receptosomes may require Na^+,K^+-ATPase activity.

Human adenovirus type 2 (Ad2) is a nonenveloped virus that enters the cell through a receptor-mediated process (4, 8, 25, 29). After initial binding to its cell surface receptor, Ad2 is sequestered in coated pits on the plasma membrane and then internalized into uncoated endocytic vesicles termed receptosomes (18) or endosomes (13). Ad2 appears to escape from receptosomes into the cytoplasm by rupturing the receptosome membrane (8). Lysis of a receptosome appears to be favored by its acidic pH (pH 5 to 5.5) (24). Fusion of enveloped viruses with the membrane of endocytic vesicles is also promoted by an acidic pH (16, 32). Ad2 also increases the permeability of the plasma membrane toward small molecules such as ^{51}Cr-labeled peptides, choline phosphate, deoxyglucose phosphate, and α-aminobutyric acid when the cells are exposed to an acidic environment (26, 27). This low-pH-dependent increase in cell membrane permeability allows one to study the mechanism by which Ad2 causes lysis of the receptosome membrane during its escape into the cytoplasm. We investigated here the role of a membrane-associated ion pump regulated by Na^+,K^+-ATPase, since it is likely that endocytic vesicles contain this ion pump (7, 10, 33). We studied this by using an inhibitor (ouabain) and an activator (K^+) of Na^+,K^+-ATPase. Ad2 effects were also studied in HeLa cells and in a mutant of HeLa cells (D98-OR) with altered Na^+,K^+-ATPase (30).

MATERIALS AND METHODS

Cells and virus. KB, HeLa, and HeLa D98-OR cells were maintained as monolayers in Dulbecco modified Eagle medium containing penicillin-streptomycin and 10% calf serum. KB cells were obtained from the American Type Culture Collection (Rockville, Md.). Ad2 was propagated in KB cells grown in suspension culture. Ad2 and ^{35}S-labeled Ad2 were prepared by the procedure described earlier (11, 28). All of the experiments were conducted by using UV-light-inactivated virus as described earlier (23).

Choline release assays. Ad2-dependent release of choline from cells was measured as described previously (26). (We have shown previously [26] that most of the choline released from the cells is in the form of choline phosphate.) In brief, KB, HeLa, or D98-OR cells were plated at a density of 0.5×10^6 cells per 35-mm (diameter) dish and were used 24 h later. Cells were labeled with [^3H]choline (10 μCi/ml) for 30 min in Dulbecco modified Eagle medium containing 2 mg of bovine serum albumin per ml (medium A). Cells were washed four times with medium A and once with basal Eagle medium, without bicarbonate, containing 2 mg of bovine serum albumin per ml (medium B) adjusted to pH 7.0. Cells were exposed to Ad2 (1 to 50 μg/ml) at 4°C for 60 min; 1 μg/ml corresponds to an average of 9×10^3 particles added per cell. Choline release was monitored at 37°C for 60 min, usually in medium B adjusted to pH 6.0 with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid). In certain experiments, isotonic sucrose-HEPES (225 mM sucrose, 5 mM glucose, 25 mM HEPES; pH 6.0) or isotonic saline (125 mM NaCl, 5 mM glucose, 25 mM HEPES; pH 6.0) was used. If required, various concentrations of other ions were also included in these buffers. The effect of ouabain was studied by adding various concentrations of ouabain (10^{-9} to 10^{-4} M) to the medium.

Receptosome lysis assay. Lysis of the receptosome by Ad2 was monitored by measuring enhancement of the toxicity of a hybrid toxin of epidermal growth factor (EGF) and *Pseudomonas* exotoxin (PE) as described previously (23, 24). In brief, cells were incubated in medium B, pH 7.0, for 60 min with EGF-PE (0.5 μg/ml) and various concentrations of Ad2 (0.01 to 10 μg/ml). At the end of incubation, protein synthe-

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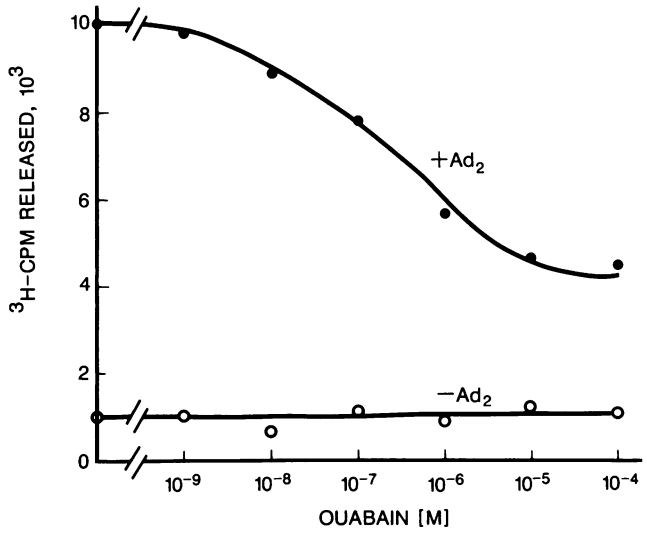

FIG. 1. Effect of ouabain on Ad2-dependent choline release. KB cells labeled with [^{3}H]choline were washed four times with medium A and once with medium B, pH 7.0. The cells were exposed to 10 μg of Ad2 per ml at 4°C for 60 min and then washed twice with medium B, pH 6.0. Choline release was monitored in medium B in the absence or presence of various concentrations of ouabain. The amount of choline released in the presence of Ad2 alone was considered maximum release (100%). The percentage of choline released in the presence of Ad2 and various concentrations of ouabain is represented by the symbol ●. The percentage of choline released in the presence of ouabain alone is shown by the symbol ○. The total cell-associated choline count was 20,260 cpm.

sis was estimated by monitoring incorporation of [^{3}H]leucine in trichloroacetic acid-precipitable material.

Binding and uptake of Ad2 by KB cells. Ad2 binding and uptake were measured as described previously (23, 24). For binding studies, cells grown in monolayer were incubated with the required concentration of Ad2 (usually 1 to 100 μg/ml) containing ^{35}S-labeled Ad2 as a carrier. Incubations were done at 4°C for 60 min with 2 ml of medium B, pH 7.0. At the end of incubation, the cells were washed five times with medium B and lysed in 2 ml of 0.1 N NaOH. Samples were counted to estimate the amounts of Ad2 associated with the cells. To study Ad2 uptake, cells were exposed to Ad2 for 60 min at 4°C and, after being washed five times with cold medium B, the cells were left at 37°C for 60 min in 2 ml of medium B, pH 6.0. At the end of incubation, the medium was collected and radioactivity was counted to estimate the amount of Ad2 dissociated from the cells. The amount of Ad2 bound to the cell surface was estimated by releasing the surface virus by trypsin. The amount of Ad2 internalized by the cells was estimated by lysing the cells in 2 ml of 0.1 N NaOH as described previously (23, 24).

[^{125}I]EGF uptake. Uptake of [^{125}I]EGF was measured by the procedure described earlier (24). In brief, cells were exposed to [^{125}I]EGF (50 ng/ml) for 60 min, usually in a buffer containing 120 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM KCl, 5 mM glucose, and 25 mM HEPES, pH 7.0. At the end of incubation, the cells were washed five times with the buffer. Surface-bound EGF was released by using an acidic buffer (0.2 N acetic acid in 0.5 M NaCl), and the amount of [^{125}I]EGF taken up by the cells was estimated after dissolving the cells in 0.1 N NaOH.

Enzyme assays. Plasma membranes were prepared from the cells by the previously published procedure (22). Na^+,K^+-ATPase activity was assayed in the crude plasma membrane preparation as described earlier (6). Adenylate cyclase activity was measured by the procedure described earlier (21). The enzyme activities were monitored for the required lengths of time in the absence or presence of 5×10^{-4} M ouabain.

EGF-PE. EGF-PE hybrid toxin was prepared as previously described (8).

Chemicals. [^{3}H]choline was obtained from New England Nuclear Corp. (Boston, Mass.). Mouse EGF was purchased from Bethesda Research Laboratories (Gaithersburg, Md.). Medium constituents were from GIBCO Laboratories (Grand Island, N.Y.). All other chemicals were from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Effect of ouabain on Ad2-dependent choline release. To investigate the role of Na^+,K^+-ATPase in Ad2-dependent release of choline from cells, we tested the effect of ouabain, an inhibitor of plasma membrane Na^+,K^+-ATPase. Increasing the concentration of ouabain blocked the Ad2-dependent choline released from cells (Fig. 1). Choline released by control cells was not affected. Maximum inhibition (40 to 50%) of Ad2-dependent release of choline was obtained by using 10^{-5} M ouabain and 50% inhibition by using ca. 0.5×10^{-6} M ouabain. Similar amounts of Ad2 were bound to or internalized by the cells whether or not ouabain was present (Table 1). This indicated that the presence of ouabain does not interfere with the association of Ad2 with cells. Since the concentration of ouabain required to inhibit Ad2-dependent choline release appears to be comparable to that required to inhibit Na^+,K^+-ATPase in these cells (30), it seems likely that Ad2-dependent choline release involves Na^+,K^+-ATPase activity.

Effect of K^+ on Ad2-dependent choline release. Since Na^+,K^+-ATPase activity requires the presence of K^+ ions in the external medium (1, 15), we tested the effect of varying the K^+ concentration on Ad2-dependent choline release. To do this, we placed the cells in a medium containing various concentrations of K^+. In the absence of K^+, Ad2 released about 25% of total cell-associated choline (Fig. 2). At 0.1 mM K^+, the amount of choline released was increased to 35%. At 0.5 mM K^+ or above, about 48% of cell-associated choline was released into the medium. Increasing the concentration of K^+ above 2 mM did not further increase the amount of choline released (data not shown). In the same experiment, addition of 0.1 mM ouabain prevented enhance-

TABLE 1. Effect of ouabain on binding and uptake of Ad2 by KB cells[a]

Ouabain concn (M)	Amt (cpm) of Ad2:		
	Dissociated from cells	Associated with cell surface	Internalized
0	1,107	2,275	2,562
10^{-6}	1,032	2,432	2,629
10^{-5}	1,116	2,315	2,718
10^{-4}	1,265	2,493	2,652
10^{-3}	1,416	2,102	2,490

[a] KB cells were exposed to Ad2 (5 μg/ml; specific activity, 7,260 cpm/μg of protein) for 60 min at 4°C in medium B, pH 7.0. At the end of incubation, the cells were washed and further incubated at 37°C for 60 min in the absence or presence of the concentrations of ouabain shown. Amounts of adenovirus dissociated from the cells, attached to the cell surface, and internalized by the cells were estimated as described in Materials and Methods.

ment of Ad2-dependent release of choline by K^+. However, Ad2-dependent choline release that occurred in the absence of K^+ was not blocked by ouabain. Basal choline release in the absence of Ad2 was not affected by either ouabain or K^+. These results indicate that the amount of choline released is significantly enhanced by K^+, although some Ad2-dependent release of choline does take place in the absence of K^+. Ouabain appears to block K^+-activatable Ad2-dependent choline release. Therefore, it seems that K^+ and ouabain compete on Ad2-dependent choline release. These results are expected from a reaction which requires Na^+,K^+-ATPase activity.

Ad2-dependent release of choline in ouabain-resistant cells. If Na^+,K^+ ATPase is involved in the Ad2 effect, then cells with a genetically altered Na^+,K^+-ATPase might have an altered response to Ad2. KB cells are thought to be closely related to HeLa cells, and the HeLa cell line D98-OR is about 100 times more resistant to ouabain than is its parental HeLa cell line. This cell line is known to have an altered Na^+,K^+-ATPase (30). A comparison of the dependence on Ad2 concentration of choline release from D98-OR cells with that of choline release from KB and HeLa cells is shown in Fig. 3. In wild-type KB or HeLa cells, there was a corresponding increase in the amount of choline released with increasing concentrations of Ad2. As expected, KB and HeLa cells were very similar. Up to 55% of cell-associated choline was released by 10 μg of Ad2 per ml in KB cells, and

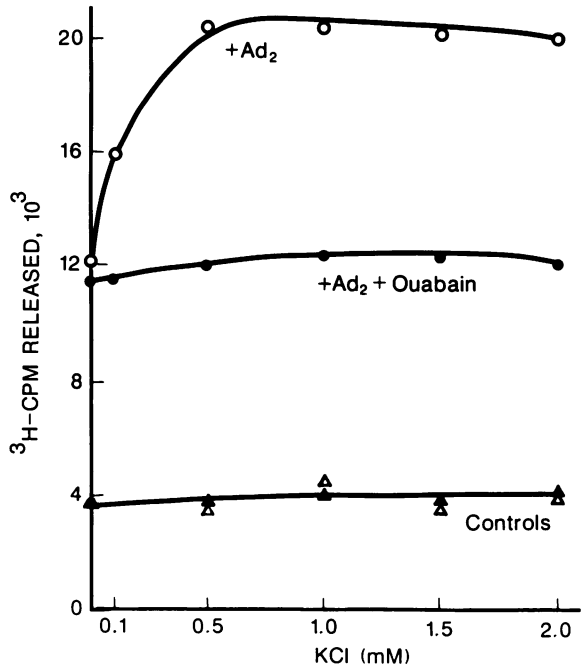

FIG. 2. Activation of Ad2-dependent choline release by K^+. KB cells were labeled with [^{3}H]choline, washed, and incubated with Ad2 described in the legend to Fig. 1. Choline release was monitored in a buffer containing 125 mM NaCl, 5 mM glucose, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM HEPES, pH 6.0, at 37°C fo 60 min. Different concentrations of KCl with and without ouabain (10^{-4} M) were also included in the buffer if required. Choline release from cells exposed to Ad2 and increasing concentrations of KCl with or without ouabain (10^{-4} M) is shown by the symbols ● and ○, respectively. Choline released from control cells which received buffer alone is shown by the symbol △, and that from the cells which received ouabain alone is indicated by the symbol ▲. The total cell-associated choline count was 44,626 cpm.

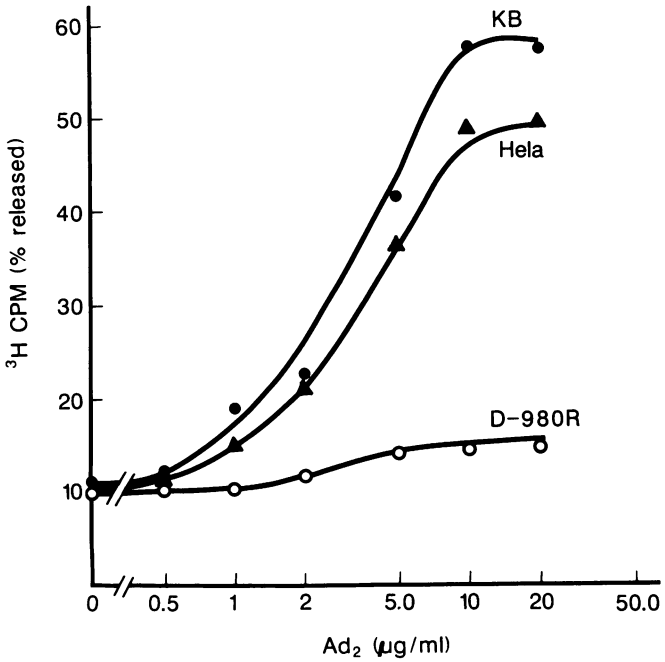

FIG. 3. Effect of increasing concentrations of Ad2 on choline release from KB, HeLa, and D98-OR cells. KB, HeLa, and D98-OR cells were labeled with choline and washed with buffer as described in the legend to Fig. 1. Cells were exposed to various concentrations of Ad2, and choline release was monitored in medium B, pH 6.0. The percentage of the total cell-associated choline released from KB cells is shown by the symbol ○, that from HeLa cells is shown by the symbol △, and that from D98-OR cells is shown by the symbol ○. Total cell-associated choline counts were 53,990, 51,572, and 46,285 for KB, D98OR, and HeLa cells, respectively.

up to 50% was released in HeLa cells. On the other hand, D98-OR cells released very little choline compared with HeLa or KB cells. Even 20 μg of Ad2 per ml released only about 15% of cell-associated choline from these cells. Increasing the length of incubation to up to 2 h or measuring choline release in a wide pH range (pH 5 to 7.5) did not result in the release of any more Ad2-dependent choline in these cells (data not shown). Although we concluded that inability of D98-OR cells to release choline is probably due to altered Na^+,K^+-ATPase, there is a possibility that it could be because D98-OR cells bind and internalize reduced amounts of Ad2 compared with KB cells. To investigate this, we measured the binding and uptake of Ad2 by D98-OR and KB cells. Both KB and D98-OR cells bound nearly the same amounts of Ad2 when the cells were exposed to Ad2 at 4°C for 60 min (Table 2). When bound Ad2 was allowed to be internalized at 37°C for 60 min, the percentages of Ad2 internalized by the cells or remaining on the cell surface were nearly equal. Therefore, it seems that the defect in the release of choline from D98-OR cells is not due to the inability of D98-OR cells to bind or internalize Ad2.

Activation of Na^+,K^+-ATPase by Ad2. If Na^+,K^+-ATPase activity is directly involved in Ad2-dependent membrane permeability changes, it is possible that Ad2 acts by altering Na^+,K^+-ATPase activity. To investigate this, we treated both wild-type KB and ouabain-resistant D98-OR cells with various amounts of Ad2. The activity of Na^+,K^+-ATPase was measured in crude membranes made from the cells in the absence or presence of ouabain. The enzyme activity obtained in control cells without ouabain was considered to

TABLE 2. Binding and uptake of Ad2 by KB and D98-OR cells[a]

Cell line	Amt (cpm) of Ad2 bound at 4°C	% of Ad2: Dissociated from cells	Associated with cell surface	Internalized
KB	6,255	22	31	46
D98-OR	6,492	27	29	44

[a] KB or D98-OR cells were exposed to Ad2 (5 μg/ml; specific activity, 5,965 cpm/μg of protein) for 60 min at 4°C, and the amount of Ad2 bound to the cells was determined as described in Materials and Methods. Duplicate dishes were washed and incubated further at 37°C for 60 min. The percentages of total virus dissociated from the cells, attached to the cell surface, and internalized were estimating as described in Materials and Methods.

be 100%. Na^+,K^+-ATPase activity observed in the absence of ouabain increased with the increasing concentration of Ad2 (Fig. 4). Up to 40% activation was observed at 50 μg of Ad2 per ml. On the other hand, Ad2 did not seem to activate the enzyme activity when assayed in the presence of ouabain, suggesting that Ad2 enhances specifically ouabain-sensitive Na^+,K^+-ATPase. In D98-OR cells there was insignificant activation of Na^+,K^+-ATPase when assayed either with or without ouabain. Activation of Na^+,K^+-ATPase by Ad2 in KB cells was also found to be time dependent, with maximum activation observed at around 30 min (Fig. 5B). As a control, another plasma membrane enzyme was measured. Adenylate cyclase activity was not affected by Ad2 (Fig. 5A). These results suggested that Ad2-dependent activation of Na^+,K^+-ATPase in KB cells was at least partially responsible for the changes in the membrane permeability observed.

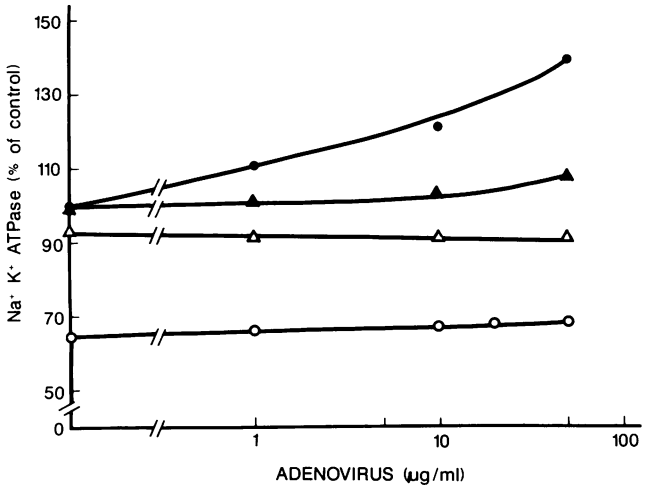

FIG. 4. Effects of various concentration of Ad2 on Na^+,K^+-ATPase activity in KB and D98-OR cells. One-day-old KB or D98-OR cells grown in 100-mm dishes at an initial cell density of 10^6 cells per dish were exposed to various concentrations of Ad2 for 60 min at 4°C in medium B, pH 7.0. The cells were then washed twice with medium B, pH 6.5, and incubated further in this medium for 60 min at 37°C. The cells were scraped and homogenized, and crude membrane preparations were made. Portions (100 μg) of the samples were used for assaying Na^+,K^+-ATPase activity in the absence or presence of 0.5×10^{-3} M ouabain. The enzyme activity obtained in the absence of ouabain in untreated cells was considered to be 100%. Shown is the Na^+,K^+-ATPase activity obtained in each experiment relative to control cells. The enzyme activities obtained in KB and D98-OR cells in the absence of ouabain are represented by the symbols ● and ▲, respectively, and those obtained in the presence of ouabain are represented by the symbols ○ and △, respectively.

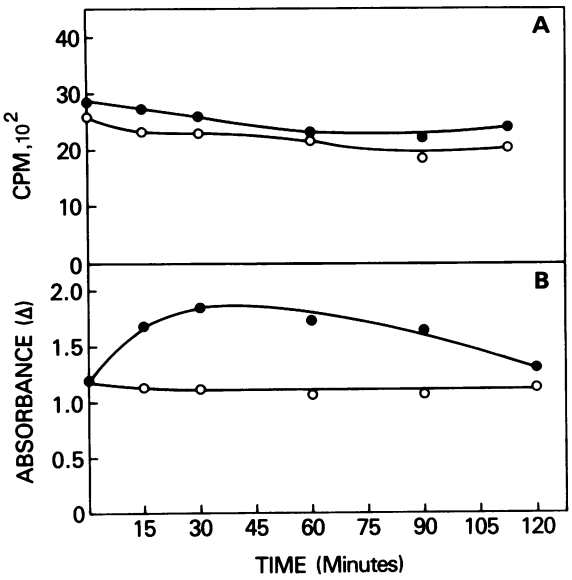

FIG. 5. Effect of Ad2 on membrane-bound adenylate cyclase and Na^+,K^+-ATPase. KB cells were exposed to 10 μg of Ad2 per ml at 4°C. After being washed, cells were further incubated at 37°C for the indicated periods of time. At the end of incubation, the cells were homogenized, and plasma membranes were prepared as described in Materials and Methods. Portions (100 μg) of the protein samples were used to assay adenylate cyclase and Na^+,K^+-ATPase activities as described in Materials and Methods. (A) Counts per minute produced, which represent the amounts of cAMP produced by the membranes prepared in the presence (●) or absence (○) of virus. (B) Difference in A_{340} between the samples incubated with and without membranes, which represents the Na^+,K^+-ATPase activity. Na^+,K^+-ATPase activities produced in the presence or absence of Ad2 are represented by the symbols ● and ○, respectively.

Ion requirement of Ad2-dependent membrane permeability changes. We also investigated the effect of varying the concentrations of Na^+, K^+, and Ca^{2+} on Ad2-dependent release of choline (Table 3). When isotonic sucrose-HEPES

TABLE 3. Effect of Na^+, K^+, and Ca^{2+} on Ad2-dependent choline release[a]

Buffer	Choline released: Without Ad2 cpm	Without Ad2 %	With Ad2 cpm	With Ad2 %
Sucrose-HEPES	752	4	3,561	19
+ 0.1 mM $CaCl_2$	746	4	1,529	8
+ 1.0 mM $CaCl_2$	685	4	1,429	8
+ 1 mM $CaCl_2$–1 mM KCl	1,326	7	1,509	8
NaCl-HEPES	1,021	5	6,693	33
+ 0.1 mM $CaCl_2$	1,006	5	6,406	31
+ 1 mM $CaCl_2$	1,329	6	4,862	24
+ 1 mM $CaCl_2$–1 mM KCl	1,617	8	9,529	47

[a] KB cells were labeled with [^{3}H]choline, washed, and exposed to 5 μg of Ad2 per ml. Choline release was monitored in either 225 mM sucrose–25 mM HEPES–5 mM glucose, pH 6.0 (sucrose-HEPES) or 125 mM NaCl–25 mM HEPES–5 mM glucose, pH 6.0 (NaCl-HEPES). If required, $CaCl_2$ (0.1 or 1 mM) and KCl (1 mM) were also included in the buffers. The actual counts released in each case are shown. The percentages of total cell-associated choline calculated are shown.

medium was used as the basal medium, about 19% of the cell-associated choline was released by the cells. The addition of 0.1 or 1 mM $CaCl_2$ reduced the amount of choline released to about 8%. Further addition of 1 mM KCl did not increase the amount of choline released by the cells, suggesting that choline released in this medium does not require Na^+,K^+-ATPase activity. When NaCl-HEPES buffer was used alone, adenovirus caused about 33% of cell-associated choline to be released. Addition of 0.1 mM $CaCl_2$ did not block the Ad2-dependent release of choline, but 1 mM $CaCl_2$ reduced the amount released to about 25%. Further addition of 1 mM KCl increased the amount of choline released to about 47%. These data are consistent with the notion that choline released in a medium containing Na^+ involves Na^+,K^+-ATPase activity.

Activation of Ad2-dependent lysis of receptosomes by K^+. Since the Na^+,K^+-ATPase of receptosomes is probably derived from the plasma membrane (7, 33), we anticipated that this enzyme activity also plays a role during the lysis of receptosomes by Ad2. We therefore hypothesized that the enhancement of EGF-PE toxicity by adenovirus, which appears to be due to vesicle lysis, would also be dependent on the K^+ concentration. K^+ effects were studied in cells which were either not preincubated or incubated for 30 or 60 min in K^+-free buffer. Preincubation in K^+-free buffer is necessary to bring down the cellular content of K^+ to a low level. In cells preincubated with K^+-free buffer for 60 min, there was no Ad2-dependent enhancement of the inhibition of protein synthesis by EGF-PE (Fig. 6A). However, with increasing concentrations of K^+, there was an increase in the Ad2-dependent enhancement of EGF-PE toxicity. For example, additions of 0.1, 1, 2, and 5 mM K^+ to the incubation buffer resulted in about 25, 60, 65, and 75% inhibition of protein synthesis, respectively. In cells preincubated in K^+-free buffer for 30 min, about 35% inhibition of protein synthesis was observed in the absence of added K^+. There was a further increase in the inhibition of protein synthesis with increasing concentrations of K^+; about 80% inhibition was observed at 5 mM K^+. Cells not preincubated with K^+-free buffer showed about 80% inhibition of protein synthesis. In these cells, there was no further increase in the inhibition of protein synthesis by Ad2. Cells which are not extensively depleted of their cellular K^+ probably have enough K^+ present to allow Ad2 to lyse endocytic vesicles.

The most plausible explanation for K^+ activation of Ad2-dependent lysis of receptosomes is that K^+ is required for the normal functioning of Na^+,K^+-ATPase, and thus this enzyme participates in the lytic reaction. However, there was a possibility that the removal of K^+ from the medium reduced the amounts of Ad2 or EGF-PE internalized by the cells. To investigate this possibility, we tested the uptake of Ad2 and EGF (which goes through the same route as EGF-PE) under the conditions used to study the effect of K^+ on Ad2-dependent receptosome lysis as discussed above. The uptake of Ad2 and EGF was not affected by the absence or presence of K^+ in the medium, even in cells preincubated with K^+-free buffer for 60 min (Fig. 6B and C).

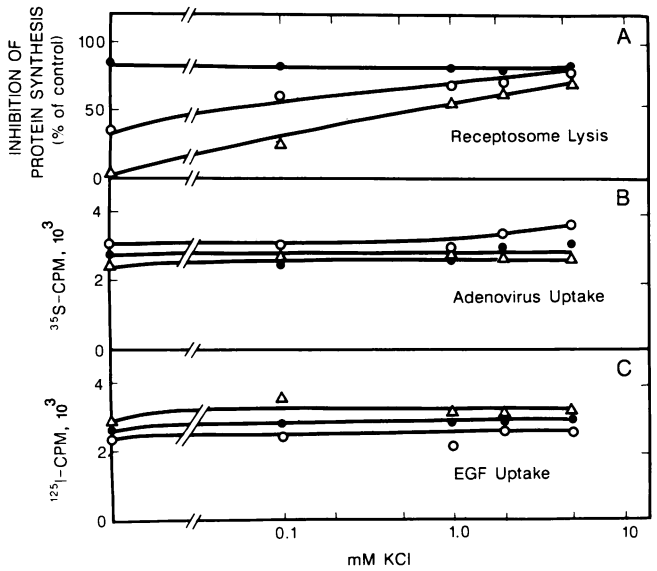

FIG. 6. Effect of K^+ on Ad2-dependent enhancement of the toxicity of EGF-PE and on Ad2 and EGF uptake in KB cells. One-day-old KB cells grown in 35-mm dishes plated at an initial cell density of 3×10^5 cells per dish were preincubated in a K^+-free buffer containing 120 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, and 25 mM HEPES, pH 7.0, for 0, 30, or 60 min. These cells were then used to monitor (i) Ad2-dependent enhancement of EGF-PE toxicity measured by monitoring the inhibition of protein synthesis with 1 μg of Ad2 per ml and 0.5 μg of EGF-PE per ml (A), (ii) Ad2 uptake with 5 μg of Ad2 per ml (B), and (iii) [^{125}I]EGF uptake with 50 ng of EGF per ml (C). Experiments were done in the presence of various concentrations of KCl, as described in Materials and Methods. In all of the panels, results obtained in the cells preincubated in K^+-free buffer for 0, 30, or 60 min are represented by the symbols ●, ○, and △, respectively.

DISCUSSION

The major new finding reported here is that Ad2-mediated release of choline from intact cells is partially blocked by ouabain. The concentration of ouabain required to inhibit choline release parallels the concentration required to block Na^+,K^+-ATPase in these cells (30). These results suggest that Ad2-mediated increase in cell membrane permeability is at least partly due to Na^+,K^+-ATPase activity. As predicted from a reaction involving Na^+,K^+-ATPase, Ad2-dependent release of choline is enhanced when K^+ ions are present in the medium, and this activation by K^+ is prevented by ouabain (Fig. 2). Moreover, HeLa cells resistant to ca. 100-fold concentrations of ouabain (D98-OR cell line) release significantly lower amounts of choline as compared with wild-type cells. We have shown in this study that Ad2 activates Na^+,K^+-ATPase present in KB cell membranes, and this activation seems to be absent in ouabain-resistant D98-OR cells. The inability of Ad2 to release choline or activate Na^+,K^+-ATPase in D98-OR cells, along with the fact that these cells have an altered Na^+,K^+-ATPase (30), strongly suggests a role of this enzyme in Ad2-dependent membrane permeability changes.

We also found that lysis of endocytic vesicles by Ad2 was enhanced by the presence of physiological concentrations of K^+ (Fig. 6). Since, in KB cells, K^+ depletion did not affect the amount of Ad2 taken up by the cells, we favor the hypothesis that K^+ functions via the activation of Na^+,K^+-ATPase of the endocytic vesicles. This activation would be required for lysis of endocytic vesicles by Ad2, as we have recently postulated (Fig. 1; 25).

Results presented here also show that, under conditions in which Na^+,K^+-ATPase activity is considerably suppressed (e.g., in the presence of 10^{-4} M ouabain or in the absence of K^+ in the medium), there is still a significant amount of choline released from the cells by Ad2. It is difficult to know the exact nature of this alternate mechanism of choline

efflux, but there are several possibilities which deserve further investigation. For example, choline could passively diffuse from the cells as a result of increased membrane fluidity; an increase in membrane fluidity has been observed in cells infected with a few enveloped viruses (9, 14, 17). Addition of 100 μM $CaCl_2$ blocks the Ad2-dependent choline release in the absence of K^+. This result may indicate a passive mode of choline release, as Ca^{2+} can bind with the negatively charged components of the membrane (12, 20) and make them impermeable for passive diffusion (5, 19, 31). Other possible ways by which Ad2 could release choline is by opening new channels (3) or by depolarizing the cell membrane (2), as suggested for certain enveloped viruses and toxins.

The biochemical mechanism by which adenovirus increases the activity of Na^+,K^+-ATPase has not yet been studied. It could be a direct effect on the enzyme or an indirect effect. If it is an indirect effect, it is not a general change in the plasma membrane, because adenylate cyclase activity was unaffected.

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