Interactions between Diphtheria Toxin Entry and Anion Transport in Vero Cells

III. EFFECT ON TOXIN BINDING AND ANION TRANSPORT OF TUMOR-PROMOTING PHORBOL ESTERS, VANADATE, FLUORIDE, AND SALICYLATE*

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When Vero cells were incubated with TPA (12-O-tetradecanoylphorbol 13-acetate) and related tumor promoters, their ability to bind diphtheria toxin in a functional way was rapidly reduced to less than 1% of the normal value. Upon further incubation with TPA, the cells recovered their ability to bind the toxin, apparently because they became resistant to TPA. Treatment with Na3VO4 reduced the ability of the cells to bind diphtheria toxin to approximately the same extent as treatment with TPA, but the reduction required longer time to develop and it persisted upon prolonged incubation with Na3VO4. ATP depletion of the cells prevented the reduction in binding capability. Such treatment also prevented the reduction in toxin binding induced by treatment with salicylate or fluoride. Treatment with TPA, fluoride, vanadate, and salicylate altered the ability of the cells to carry out anion transport and interfered with their ability to regulate the transport. The results indicate that the binding sites for diphtheria toxin on Vero cells are modulated by TPA, Na3VO4, salicylate, and fluoride by a process which requires ATP. The possibility is discussed that the modulation consists in phosphorylation of the toxin binding sites, which may be identical with, or closely linked to, the anion antiporter in the cells.

The first step in intoxication of cells with diphtheria toxin consists of adsorptive endocytosis of the toxin (for review, see Ref. 1). Once the toxin-containing endosomes are sufficiently acidified, a normally hidden hydrophobic domain in the B-fragment of the toxin inserts itself into the membrane and somehow facilitates the transfer of the enzymatically active A-fragment into the cytosol. In the preceding paper we have shown that this insertion inhibits anion antiport in Vero cells (2).

Anion transport, possibly by the antiporter, appears to be necessary for entry of the A-fragment into the cytosol (3). In a previous paper we have shown that the ability of Vero cells to bind diphtheria toxin was strongly reduced by compounds and conditions which inhibit anion transport in the cells (4). This suggested to us that the anion antiporter, or a molecule linked to it, may be the receptor for diphtheria toxin.

Vero cells, and a number of other monkey kidney cells, possess a much higher number of binding sites for diphtheria toxin than other cells tested (5–7). We found that diphtheria toxin was only able to inhibit anion antiport to a measurable extent in Vero cells (2) although HeLa S3 cells, fetal hamster kidney cells, and L cells appear to have the same kind of anion antiporter (8). To test further the possibility that diphtheria toxin binding to Vero cells is associated with the anion antiporter, we have studied a number of compounds that decrease the ability of the cells to bind the toxin for their effect on anion transport.

We have previously found that the tumor-promoter 12-O-tetradecanoylphorbol 13-acetate (TPA1) protects cells against diphtheria toxin (9). Since TPA modulates the receptors for epidermal growth factor and other hormones (10, 11), apparently by inducing phosphorylation of the receptors (12), we decided to study in more detail the mechanism whereby TPA protects cells against diphtheria toxin. Furthermore, we have studied the protective effect of vanadate and fluoride, which have been reported to inhibit dephosphorylation of phosphoproteins (13, 14), and the protective effect of salicylate treatment. Finally, we have measured the effect of the same compounds on anion transport activity and on the ability of the cells to regulate the transport.

EXPERIMENTAL PROCEDURES

Materials—Diphtheria toxin was purified as earlier described (15) and labeled with 125I by the iodogen procedure (16). Phospholipase C from Clostridium perfringens, type XII, 4a-phorbol 12,13-didecanoate, 4a-phorbol 12,13-dibutyrate, 4b-phorbol 12,13-didecanoate, 4b-phorbol 12,13-dibutyrate, 4-O-methyl-TPA, phorbol 12-monomyristate, and mezerein were from C.C.R., Inc., Eden Prairie, MN, and TPA was from Consolidated Midland Corporation, Booster, NY. Na3VO4 was obtained from Ventron GmbH, Karlsruhe, West Germany, and VOSO4 from Merck, Darmstadt, West Germany. [PH]Phorbol 12,13-dibutyrate (specific activity 10.8 Ci/mmol) was purchased from New England Nuclear. H35Cl (specific activity 9.2 μCi/ mg of Cl), Na32SO4 (specific activity 876 Ci/mmol), and [PH]lescine (specific activity 131 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, United Kingdom.

Buffers—The following buffers were used. Mannitol buffer, 260 mM mannitol, 1 mM Ca(OH)2, 20 mM MES, adjusted to the indicated pH with Tris; and phosphate-buffered saline, 140 mM NaCl, 10 mM sodium phosphate, pH 7.4.

Cells—Vero cells, fetal hamster kidney cells, and human melanoma cells were propagated as monolayer cultures in minimal essential medium (Gibco, Glasgow, Scotland) as earlier described (17). The cells were seeded into 24-well disposable trays at a density of 5-10 cells/well.

Measurement of 125I-Diphtheria Toxin Binding—Labeled diphtheria toxin (specific activity 2,000-10,000 cpm/ng) was added, in 0.5 ml

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1 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol 13-acetate; SITS, 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; Hepes, N-2-hydroxyethylpiperazine-N' -2-ethanesulfonic acid.
of Hepes medium containing 1% fetal calf serum, to each well of 24-well disposable trays containing approximately 3 x 10^5 cells/well and kept at 24 °C for 30 min. The cells were then washed three times with medium to remove unbound toxin and finally the cells were dissolved in 0.1 M KOH and the radioactivity associated with the cells was measured.

Measurement of Protein Synthesis Inhibition—The experiments were carried out in Hepes medium as earlier described (17) when not otherwise indicated. After incubation with toxin for the indicated periods of time, the medium was removed and Hepes medium containing 1 pCi/ml [3H]leucine and no unlabeled leucine was added, and the incubation was continued for 15–30 min more. Then the medium was removed, the cells were treated twice with 5% (w/v) trichloroacetic acid, dissolved in 0.1 M KOH, and finally the radioactivity was measured as earlier described (17).

Measurement of Chloride and Sulfate Uptake and Efflux—To measure uptake of 35SO4 or 35Cl, the cells were washed twice with cold mannitol buffer, pH 5, and then the labeled anion, in buffers as given in the legends to the figures, was added. To measure efflux, the cells were first loaded with the labeled anions by incubation with 0.3 ml of mannitol buffer, pH 5, containing 0.17 µCi of H3Cl or 1.7 µCi of Na32SO4 for 20 min at 24 °C. The buffer was then removed and efflux buffer, as detailed in the legends to the figures, was added.

To measure the amount of radioactivity associated with the cells after different periods of time, the cells were washed twice with ice-cold phosphate-buffered saline and the trichloroacetic acid-extractable radioactivity was measured as described (8).

RESULTS

The findings in the accompanying papers (2, 3) suggested that the receptor for diphtheria toxin is identical with, or closely linked to, the anion antiporter in Vero cells. To study this relationship in more detail, we have studied a number of treatments that protect cells against diphtheria toxin with respect to their effect on toxin binding and anion transport.

Inhibition of Toxin Binding

Effect of Tumor-promoting Phorbol Esters on the Sensitivity of Vero Cells to Diphtheria Toxin and on Their Ability to Bind 125I-labeled Toxin—We have earlier reported that the tumor promoter, TPA, protects cells against diphtheria toxin when given to the cells together with the toxin (4, 18). We have now found that preincubation of the cells with TPA strongly increases the protective effect. The data in Fig. 1A show experiments where cells were preincubated with and without TPA and related compounds for 30 min, then exposed to toxin for 1 h, and finally the rate of protein synthesis during a 15-min interval was measured. It is clear that after pretreatment with 0.1 µM TPA, the cells became highly resistant to the toxin. No further protection was obtained with 10 µM TPA. Three other tumor promoters, 4α-phorbol 12,13-didecanoate, 4β-phorbol 12,13-dibutyrate (Fig. 1A), and mezerein (Fig. 1B) also protected against diphtheria toxin, whereas no protection was obtained with the phorbol esters 4α-phorbol 12,13-didecanoate (Fig. 1A), 4-O-methyl-TPA, and phorbol 12-dodecanoate (Fig. 1B), which are inactive as tumor promoters (10).

Retinoic acid, which inhibits the effect of TPA in many systems (for review, see Ref. 19), had little effect on the sensitivity of cells to diphtheria toxin and did not counteract the protective effect of TPA.

To test whether the protective effect was due to reduced toxin binding, Vero cells were treated with TPA, and then their ability to bind 125I-labeled diphtheria toxin was measured. In the experiment shown in Fig. 2, somewhat more toxin was bound per cell than in the experiment shown in the preceding paper (2). This apparently reflects day-to-day variations in the cells. When cells were incubated for 30 min at 37 °C with 1 nM TPA, their ability to bind diphtheria toxin was reduced to approximately half, whereas 10 µM TPA and more reduced the binding to background values. If the preincubation at 37 °C was omitted and the binding was measured at 4 °C, 1 µM TPA did not interfere with the binding (data not shown). This indicates that TPA does not directly interfere with the toxin binding.

If the cells were incubated with 1 µM TPA for 30 min at 37 °C and then washed to remove free TPA, the cells were still unable to bind the toxin, showing that the effect of TPA is not rapidly reversible (see below). Furthermore, incubation with the analogue of TPA, 4α-phorbol 12,13-didecanoate had no effect on toxin binding. In addition, retinoic acid did not affect the binding (data not shown). The results indicate that TPA produces a process in the cells that is temperature- and time-dependent and which reduces the ability of the cells to bind diphtheria toxin.

Effect of Na3VO4 on the Sensitivity of Vero Cells to Diphtheria Toxin and on the Binding of the Toxin to the Cells—In some systems, vanadate stimulates cell growth in a similar fashion as TPA (20, 21). We therefore tested whether vanadate has a similar effect as TPA on the ability of cells to bind diphtheria toxin. The data in Fig. 2 show that this is indeed the case. Thus, preincubation of the cells for 30 min at 37 °C with 50 µM Na3VO4 strongly reduced the binding of 125I-diphtheria toxin to the cells. Such pretreatment also strongly protected the cells against intoxication. If the preincubation with Na3VO4 for 30 min was carried out at 0 °C rather than at 37 °C, there was no reduction in the ability of the cells to bind 125I-diphtheria toxin (data not shown). This indicates that the protective effect of Na3VO4 is due to a simple interference with the toxin binding, but that vanadate induces an alteration in the cells which results in reduced ability to bind the toxin.

It has been claimed that vanadate ions are rapidly reduced to vanadyl ions in the cytosol (20). In fact, in some cases, VOSO4 is as efficient as Na3VO4 to stimulate DNA synthesis in intact cells (20). In our system the two vanadium compounds acted differently. VOSO4 was much less efficient in protecting the cells than Na3VO4. The fact that some protective effect was obtained with VOSO4 is difficult to interpret, as some vanadyl ions could have been oxidized to vanadate ions in the culture medium.

Rate of Development of Protection by TPA and Na3VO4—To test the rate of desensitization of Vero cells to diphtheria toxin, cells were incubated at 37 °C with various concentrations of TPA and Na3VO4 for increasing periods of time and the toxin sensitivity of the cells was then measured. For this purpose increasing concentrations of toxin were added to the treated cells and dose-response curves were constructed as in Fig. 1. The sensitivity was taken as the ratio between the toxin concentration required to reduce protein synthesis to half in untreated cells and the corresponding value obtained in TPA- and Na3VO4-treated cells.

The data in Fig. 3 show that the kinetics for the development of the insensitive state by TPA and Na3VO4 were strikingly different. Thus, TPA induced a rapid reduction in sensitivity which, at 1 µM TPA, was maximal already when the first time point was taken after 10 min (Fig. 3A). Ten nM TPA induced almost the same extent of insensitivity as 1 µM TPA, but it developed somewhat more slowly. One nM TPA had little protective effect. Experiments with 125I-diphtheria toxin showed that in the presence of 1 µM TPA the binding was reduced to background values already after 10 min at 37 °C (data not shown).

The protective effect of Na3VO4 increased with the time of preincubation (Fig. 3B), whereas in the case of TPA, the sensitivity of the cells first rapidly decreased and then, after preincubation with TPA for more than 15–30 min, the toxin
FIG. 1. Ability of tumor promoters to protect cells against diphtheria toxin. To Vero cells in 24-well disposable trays in 0.5 ml of Hepes medium were added the compounds indicated below, and the cells were incubated for 30 min at 37 °C. Then increasing amounts of diphtheria toxin were added, the incubation was continued for 1 h more, and finally, the incorporation of [3H]leucine during 15 min was measured. The data are in each case expressed as per cent of a control sample treated with the same compound but without toxin. 4α-PDD, 4α-phorbol 12,13-didecanoate.

FIG. 2. Effect of phorbol esters, vanadate, fluoride, salicylate, and chloride deprivation on the ability of Vero cells to bind 125I-diphtheria toxin. Vero cells in 24-well disposable trays were incubated for 1 h at 37 °C in Hepes medium, pH 7, with additions as detailed below and then chilled to 24 °C. In one case (△) the incubation was in 260 mM mannitol, 1 mM Ca(OH)₂, 20 mM MES adjusted to pH 7 with Tris. Then increasing amounts of 125I-diphtheria toxin were added and binding was measured as described under “Experimental Procedures.” The additions were: △, none; ●, 1 nM TPA; △, 100 nM TPA; □, 30 mM sodium salicylate; ○, 50 μM Na₃VO₄; ▣, 10 mM NaF.

sensitivity started to increase again, and after 3 h it was 10–30% of that of untreated cells (Fig. 3A). After incubation with 1 μM TPA overnight, the cells had recovered almost full sensitivity to diphtheria toxin (Fig. 4). Further addition of TPA did not protect the cells under these conditions. This indicates that the gradual recovery of toxin sensitivity in TPA-treated cells is due to desensitization of the cells to TPA. Treatment with TPA overnight also abolished the protective effect of mezerein (Fig. 4) and treatment with 1 μM mezerein overnight induced desensitization to TPA (data not shown). On the other hand, treatment with TPA overnight did not induce desensitization to Na₃VO₄ (Fig. 4).

It has been shown by previous authors that cells treated with tumor-promoting phorbol esters lose their ability to bind the drugs (22, 23). To measure the binding capacity of the cells for phorbol esters, we used [3H]phorbol 12,13-dibutyrate, which is less lipophilic than TPA and can be removed from the cells by washing, in contrast to TPA, which binds very strongly to the cells (10). We found that after incubation with phorbol 12,13-dibutyrate overnight, the ability of the cells to bind [3H]phorbol 12,13-dibutyrate was indeed strongly reduced (data not shown). It therefore appears that the phorbol ester induces disappearance of the phorbol ester receptors in Vero cells as previously found in other cells (10, 22).

Ability of Metabolic Inhibitors to Prevent Desensitization of Cells to Diphtheria Toxin Induced by TPA, Na₃VO₄, NaF, and Sodium Salicylate—To test further the mechanism by which TPA and Na₃VO₄ reduce the ability of Vero cells to bind diphtheria toxin, we incubated ATP-deprived cells with the
Diphtheria Toxin Binding and Anion Transport

**Fig. 3.** Rate of desensitization of Vero cells to diphtheria toxin by TPA (A) and Na₃VO₄ (B). Vero cells in 24-well disposable trays were incubated with 0.3 ml of Hepes medium, pH 7.5, with the indicated concentrations of TPA and Na₃VO₄ for the time given at the abscissa. Then increasing concentrations of diphtheria toxin were added and the incubation was continued for 30 min at 0 °C. Then the cells were washed to remove unbound toxin and the incubation was continued for 3 h more at 37 °C. Finally, the ability of the cells to incorporate [³H]leucine during 15 min was measured. Dose-response curves as in Figs. 1 and 2 were made, and the toxin concentration required to reduce protein synthesis to half the control value (IID₅₀ test) was measured. Also, the toxin concentration required to reduce protein synthesis by 50% in cells not treated with TPA and Na₃VO₄ (IID₅₀ control) was measured. The sensitivity is expressed as IID₅₀ control/IID₅₀ test.

**Fig. 4.** Ability of prolonged treatment with TPA to desensitize cells to TPA and mezerein. Vero cells in 24-well disposable trays were incubated overnight with and without 1 μM TPA. The next day the cells were transferred to Hepes medium, additions were made as indicated and, after incubation at 37 °C for 30 min, increasing amounts of diphtheria toxin were added. One h later the medium was removed, new medium with [³H]leucine was added, and the ability of the cells to incorporate [³H]leucine during 15 min was measured. O, control, no tumor promoter added; Δ, 1 μM TPA overnight, then 1 μM more TPA for 90 min; X, 1 μM TPA overnight, then 1 μM mezerein for 90 min; □, 1 μM mezerein for 90 min; ▲, 1 μM TPA overnight, then 1 μM mezerein for 90 min; ▼, 1 μM TPA overnight, then 0.1 μM Na₃VO₄ for 90 min.

Due to the difficulty in preventing nonspecific binding of compounds at 37 °C and then measured the ability of the cells to bind ¹²⁵I-diphtheria toxin. Under these conditions, there was little reduction in diphtheria toxin binding (data not shown).

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In order to obtain a more accurate measure of the ability of TPA and vanadate to inhibit functional binding of diphtheria toxin, we adopted a binding assay where we used the toxic effect of the bound toxin as a measure of the amount of toxin bound to the cells in a functional way under the different conditions. Control experiments showed that the compounds and conditions used inhibited only the binding and not the ability of bound toxin to intoxicate cells.

Cells were preincubated in the absence and presence of metabolic inhibitors and then treated with 0.1 μM TPA for 15 min at 37 °C (Fig. 5A), or with 0.1 mM Na₃VO₄ for 60 min at 37 °C (Fig. 5B). The cells were then chilled to 0 °C, and increasing concentrations of diphtheria toxin were added. After the toxin had been allowed to bind for 30 min, unbound toxin was washed away, and the cells were incubated with Hepes medium without inhibitors for 3 h. Finally, the ability of the cells to incorporate [¹H]leucine during 10 min was measured. It is clear that the combination of NaN₃ and 2-deoxyglucose prevented the reduction in toxin-binding ability otherwise induced in the cells by TPA and Na₃VO₄.

The combination of the two inhibitors reduced the level of ATP in Vero cells to approximately 3% of that in untreated cells. NaN₃ alone reduced it only slightly (to 93%), whereas 2-deoxyglucose alone reduced the level of ATP to 19% of that in untreated cells (data not shown). The data in Fig. 6, A and B show that neither of the metabolic inhibitors alone prevented the desensitization to diphtheria toxin induced by TPA, indicating that the effect of the combined drugs is due to the reduction in the ATP level. 2-Deoxyglucose had some inhibitory effect on the ability of Na₃VO₄ to desensitize to the toxin, but it was much more efficient when combined with NaN₃. Altogether, the data indicate that ATP is necessary for TPA and Na₃VO₄ to desensitize cells to diphtheria toxin.
Fig. 5. Effect of metabolic inhibitors on the ability of TPA and Na$_3$VO$_4$ to reduce the sensitivity of Vero cells to diphtheria toxin. Vero cells in 0.3 ml of Hepes medium in 24-well disposable trays were incubated for 15 min at 37°C in the absence and presence of 10 mM Na$_3$ and 50 mM 2-deoxyglucose (2-dG) as indicated. In A, the medium was then made up to contain 0.1 µM TPA where indicated, and the incubation was continued for 10 min more and then the tray was chilled in an ice bath. In B, 0.1 mM vanadate was added and the incubation at 37°C was continued for 60 min before the tray was chilled. Increasing amounts of diphtheria toxin were then added to all plates, and the toxin was allowed to bind on ice for 30 min. The cells were then washed twice with cold medium and new medium was added. The cells were incubated for 3 h at 37°C to allow the bound toxin time to inhibit protein synthesis, and then the ability of the cells to incorporate [³H]leucine during 15 min was measured.

Fig. 6. Ability of metabolic inhibitors to prevent the fluoride- and salicylate-induced reduction in diphtheria toxin binding capability. The conditions were as described in the legend to Fig. 5, except that the cells were incubated for 1 h with NaF (A) and sodium salicylate (B) as indicated.

Middlebrook (24) reported that treatment with fluoride and salicylate rapidly reduced the ability of Vero cells to bind diphtheria toxin. The data in Figs. 2 and 6 confirm this. It is also shown that a reduction in binding capability did not occur in ATP-depleted cells (Fig. 6).

When cells were treated with 1 µM phorbol 12,13-dibutyrate, 0.1 mM Na$_3$VO$_4$, 10 mM NaF, or 30 mM sodium salicylate in the presence of 3.5 µM cycloheximide, their ability to bind diphtheria toxin was reduced in a similar way as in cells with active protein synthesis (data not shown).

Phospholipase C strongly reduces the ability of cells to bind diphtheria toxin (25). This is also shown in Fig. 2. The reduction could either be due to a direct enzymatic effect on the toxin binding sites, or to a process involving protein kinase C, which can be activated by diacyl glycerol released by the phospholipase treatment. The data in Fig. 7A show that the desensitization to the toxin takes place even in ATP-depleted cells, indicating that the reduced binding is due to an enzymatic effect at the toxin binding sites.

Also, SITS strongly reduces the ability of Vero cells to bind diphtheria toxin (Fig. 2). Since this agent forms covalent bonds with protein, its action would not be expected to be dependent on metabolic energy. The data in Fig. 7B show that treatment with SITS was equally efficient in the absence and presence of 2-deoxyglucose and Na$_3$ and that it occurred even at 0°C. Clearly, the protective effect of TPA, Na$_3$VO$_4$, and Na$_3$...
FIG. 7. Inability of metabolic inhibitors to prevent desensitization by phospholipase C and SITS. Vero cells in 0.3 ml of Hepes medium in 24-well disposable trays were incubated for 15 min in the absence and presence of 10 mM NaPi and 50 mM 2-deoxyglucose (2-dG) as indicated. In A, the cells were incubated for 30 min the absence and presence of 10 units/ml phospholipase C (from C. perfringens) and then chilled on ice. In B, the cells were incubated in the absence and presence of 1 mM SITS for 30 min at 37 °C or for 10 min at 0 °C as indicated. The plates were then placed on ice, increasing amounts of diphtheria toxin were added, and the ability of bound toxin to intoxicate the cells was measured as described in the legend to Fig. 5.

NaF, and sodium salicylate is of a different kind than that of phospholipase C and SITS.

Rate of Recovery of the Ability of Cells to Bind Diphtheria Toxin—When cells were treated with vanadate, fluoride, and salicylate and then the different compounds were washed away and the cells were transferred to normal medium, their ability to bind diphtheria toxin reappeared comparatively slowly. Thus, only after approximately 2 h had the cells recovered the same sensitivity as that found in untreated cells. Similar results were obtained with phorbol 12,13-dibutyrate, which, in contrast to TPA, is water-soluble and can therefore be washed away from the cells. It should be noted that the recovery in binding after removal of phorbol 12,13-dibutyrate is different from the adaptation to the phorbol esters which takes place upon prolonged incubation in the presence of the compound as shown in Fig. 3A.

Desensitization in Other Cells—The ability of TPA and NaF,VO4 to desensitize cells to diphtheria toxin was also tested on two cell lines which are less sensitive than Vero cells, viz. fetal hamster kidney cells and human melanoma cells. In both cases, the two compounds reduced the sensitivity of the cells to diphtheria toxin, but the reduction (3-10-fold) was less than that found with Vero cells. Possibly, the reason for the low number of toxin binding sites in these cells is due to a regulatory state analogous to that induced by low concentrations of TPA or vanadate.

Requirement of Anions for Toxin Binding—We have earlier shown that when Vero cells were preincubated in the absence of isotonic concentrations of a number of anions with low permeability, the ability of the cells to bind diphtheria toxin is strongly reduced (4). The data in Fig. 2 show that, even without preincubation, the binding of diphtheria toxin is strongly reduced when the assay was carried out in buffer osmotically balanced with mannitol. It is not clear if the anions alter the toxin or the receptor to ensure binding.

Effects on Anion Transport

Table 1

Effect of TPA on the uptake of 125I- and 35SO4 by Vero cells

Cells growing in 24-well disposable trays were preincubated at the pH indicated and then incubated with 125I- and 35SO4 and increasing concentrations of the corresponding unlabeled anion, in 260 mM mannitol, 1 mM Ca(OH)2, 20 mM MES adjusted to the pH indicated with Tris. When more than 20 mM salt was added, the concentration of mannitol was reduced sufficiently to ensure isotonicity. The cells were kept at 24 °C and, after increasing periods of time, the acid-soluble radioactivity associated with the cells was measured. The initial rate of uptake at each concentration was calculated, the data were transformed into Lineweaver-Burk plots, and Km and Jmax were calculated. The values given represent the average ± 1 standard deviation.

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<th>Ion</th>
<th>pH during preincubation</th>
<th>pH during measurement</th>
<th>TPA, 1 μM</th>
<th>Km (mM)</th>
<th>Jmax (ions x cell−1 x s−1 x 108)</th>
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<td></td>
<td>7</td>
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<td></td>
<td>8</td>
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<td>4.7 ± 0.4</td>
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were preincubated with 1 μM TPA for 30 min and then their ability to take up 125I- and 35SO4 was measured in the presence of various concentrations of the corresponding unlabeled anion, as described in one of the preceding papers (8). The data were transformed into Lineweaver-Burk plots and the Km and Jmax were measured. The data in Table I show that under a number of conditions where we varied the pH during the preincubation, as well as during the uptake measurement, pretreatment with TPA increased the affinity for

Uptake of 125I- and 35SO4 in Cells Treated with TPA—To test whether TPA treatment affects anion transport, cells

Effects on Anion Transport
Cl⁻ (i.e. reduced the $K_m$) whereas the uptake capacity ($J_{\text{max}}$) was less effected.

The comparatively high variation in the data presented in Table I reflects primarily day-to-day variations in the cells, which influenced the anion transport capacity both in untreated and TPA-treated cells. In those cases where the experiments with and without TPA were carried out on the same day, the relative effect of TPA was highly reproducible.

Pretreatment with TPA did not alter the $K_m$ for sulfate (Table I). Altogether, the data indicate that treatment of cells with TPA alters the functional state of the anion antiporter.

Effects of Vanadate, Fluoride, and Salicylate—In one of the preceding papers (8) we showed that when cells were preincubated at pH 6.5, the rate of Cl⁻ efflux, measured at pH 7 in mannitol buffer containing no permeant anions, was much lower than when the preincubation had been carried out at pH 8. Furthermore, we showed that induction of the state characterized by the high efflux rate did not occur in ATP-depleted cells (8). We found here that ATP depletions prevented the pH 8-induced transition from the state of low efflux rate to that of high efflux rate (Fig. 8B), but not the converse transition from high to low efflux rate (Fig. 8D). It should also be noted that although the loading of the cells with $^{36}\text{Cl}^-$ occurred in mannitol buffer, pH 6, the loading time (20 min) was not sufficiently long to induce transition from high to low efflux rate in the cells preincubated at pH 8 in the absence of metabolic inhibitors.

A possible reason for the ATP requirement is that phosphorylation, possibly of the anion antiporter, is required for the transition from low to high efflux rate, whereas dephosphorylation induces the state of low efflux rate. If this is the case, fluoride and vanadate, which inhibit dephosphorylation, should prevent the transition from high to low efflux rate. The data in Fig. 8D show that this was indeed the case. Also, salicylate inhibited the transition. On the other hand, neither of the compounds inhibited the transition from low to high efflux rate (Fig. 8B). We also found that the presence of salicylate or fluoride during the preincubation at pH 6.5 increased the efflux of Cl⁻, but these compounds did not alter the efflux rate in cells preincubated at pH 8 (Fig. 8, A and C). The data are compatible with a model where alkaline pH induces phosphorylation of the antiporter, whereas at low pH the antiporter is dephosphorylated. Altogether, the data show that those treatments that reduce the ability of Vero cells to bind diphtheria toxin also interfere with anion transport in the cells.

DISCUSSION

The data presented here show that treatment of Vero cells with tumor-promoting phorbol esters, vanadate, fluoride, and salicylate strongly reduces the ability of the cells to bind diphtheria toxin and alter the rate of anion transport in the cells. The data are compatible with a model where the dephosphorylated state of the diphtheria toxin binding sites on Vero cells is required for toxin binding. Treatment with TPA and related tumor promoters could induce a phosphorylation of the binding sites that changes their conformation and thereby either strongly reduce the affinity for the toxin, as in

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**Fig. 8.** Effects of NaF, sodium vanadate and sodium salicylate on the rate of $^{36}\text{Cl}^-$ efflux from cells. Vero cells were preincubated in Hepes medium adjusted to pH 6.5 (A, B) or in Hepes medium containing 5 mM NaHCO₃ adjusted to pH 8 (C, D) for 30 min at 37°C. Then the cells were transferred to Hepes medium, pH 6.5 (A, D), or pH 8.0 containing 5 mM NaHCO₃ (B, C) with additions as indicated, and the cells were incubated at 37°C for 15 min more. The cells were then loaded with $^{36}\text{Cl}^-$ as described under "Experimental Procedures," and subsequently transferred to mannitol buffer, pH 7, and kept at 24°C. The amount of radioactivity associated with the cells was measured after increasing periods of time. The additions were: (○), none; (□), 10 mM NaF; (△), 0.1 mM sodium vanadate; (▲), 30 mM sodium salicylate; (●), 10 mM NaN₃ and 50 mM 2-deoxyglucose.
the case of the epidermal growth factor receptor (26, 27), or induce endocytosis of the binding sites without diminishing their affinity, as in the case of the transferrin receptor (28). If the anion antiporter is the toxin receptor or part of it, it is unlikely that it is removed from the cell surface by endocytosis, since the capacity of anion antiport in TPA-treated cells was only slightly reduced.

Vanadate and fluoride could have the same end effect as TPA, because, as inhibitors of dephosphorylation, they may cause accumulation in the binding sites of phosphate groups introduced by endogenous phosphorylation activity. The observation that vanadate acted more slowly than TPA is consistent with this model, and so is the finding that the disappearance of binding capability is prevented when the cells are depleted for ATP. It is interesting that band 3, the anion channel in erythrocytes, is phosphorylated on several sites (29).

High intracellular pH could induce phosphorylation in one site which increases the uncoupling ("slippage") of the antiporter. Pretreatment with alkaline pH reduces the ability of the cells to bind diphtheria toxin to some extent, but much less than TPA treatment. TPA, on the other hand, does not increase the rate of anion efflux, but induces a reduction in the Kᵦ. Possibly, therefore, TPA treatment mimics another regulatory process than that responding to alkaline pH. This process could consist in phosphorylation of a different site of the antiporter, resulting in an allosteric alteration of the exterior part of the antiporter which then is unable to bind the toxin.

TPA and vanadate have previously been found to interfere with other ion transport systems in cells. Thus, several authors have presented evidence that vanadate and a variety of growth factors induce alkalinization of cells by stimulating the amiloride-sensitive Na⁺/H⁺ exchange system (30–32). Tumor-promoting phorbol esters were found to counteract this stimulation and thus prevent the rise in pH induced by the mitogens (33). In the absence of mitogens, TPA induced a slight alkalinization by stimulating the Na⁺/H⁺ antiporter (34–37), possibly by increasing its affinity for H⁺ (32). TPA has been found to inhibit the Na⁺,K⁺,Cl⁻ cotransport system in BALB/c 3T3 cells (38), whereas in smooth muscle cells, the system was stimulated (39). The ability of vanadate to inhibit water transport in renal cells (40) may also be due to an effect on this cotransport system. The present findings that also the anion antiporter in Vero cells appears to be regulated in response to pH and by treatment with TPA, vanadate, and other compounds, opens up the possibility that also this system is involved in the regulation of cell proliferation.

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REFERENCES

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