J. Membrane Biol. 119, 179–186 (1991)
0022263191000175

The Journal of
Membrane Biology

Evidence for Involvement of Protein Kinase C in Regulation of Intracellular pH by Cl^-/HCO_3^- Antiport

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Summary. The activity of the main base-extruding mechanism in Vero cells, the Na^+-*in*dependent Cl^-/HCO_3^- antiport, increases 5- to 10-fold when the cytosolic pH (pH_i) is increased over a narrow range close to neutrality. We have studied the effect on this regulation of stimulation and inhibition of protein kinase C by short-term and long-term treatment with the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA). After short-term treatment with TPA to stimulate the kinase, the threshold value for activation of the antiport is shifted to a more acidic pH. After prolonged treatment with TPA to downregulate protein kinase C the sensitivity of the antiport to variation in proton concentration was lowered, possibly by reducing the number of essential proton-binding sites. Concomitantly, the steady state pH_i of the cells was increased. The data indicate that protein kinase C is involved in the regulation of the Na^+-*in*dependent Cl^-/HCO_3^- antiport.

Key Words anion antiport · pH regulation · protein kinase C · TPA · HCO_3^- · Vero cells

Introduction

Tumor-promoting phorbol esters, such as 12-O-tetradecanoylphorbol 13-acetate (TPA), induce a wide range of physiological and biochemical responses in cultured cells (for review, *see* Blumberg, 1981; Nishizuka, 1986; Berridge, 1987). These responses are mediated by specific binding of phorbol esters to protein kinase C (Castagna et al., Kraft et al., 1982; Niedel, Kuhn & Vandenbark, 1983). Shortly after addition of phorbol ester, the enzyme is translocated from the cytoplasm to the cell membrane and activated (Takai et al., 1979; Kraft et al., 1982; Kraft & Anderson, 1983). The natural initiator of this process is diacylglycerol formed by phosphatidyl inositol hydrolysis (Kishimoto et al., 1980; Bell, 1986). The activation is transient due to rapid degradation of the membrane-bound protein kinase C (Rodriguez-Pena & Rozengurt, 1984; Ballester & Rosen, 1985). Thus, prolonged incubation of the cells with tumor-promoting phorbol esters leads to downregulation of protein kinase C, which can be detected as a decrease in phorbol ester binding sites and as a decrease in protein kinase C activity (Rodriguez-Pena & Rozengurt, 1984; Ballester & Rosen, 1985).

In many cells TPA as well as a number of growth factors stimulate Na^+/H^+ exchange (Grinstein, Rotin & Mason, 1989). In the absence of bicarbonate this leads to alkalinization of the cytoplasm, an observation that has evoked extensive discussion as to whether or not pH_i increase is essential for mitogenic response. However, in the presence of bicarbonate, the alkalinization is prevented in several cell lines (Cassel et al., 1985; Bierman et al., 1988; Ganz et al., 1988), most likely due to the activity of bicarbonate-dependent pH-regulating mechanisms.

In Vero cells two bicarbonate-dependent mechanisms have been identified. The Na^+-linked Cl^-/HCO_3^- antiport utilizes the Na^+ gradient to bring $NaHCO_3$ into the cytoplasm and thereby extrudes acid. The Na^+-*in*dependent Cl^-/HCO_3^- antiport extrudes base under physiological conditions (Tønnessen et al., 1987). While the former antiport is constitutively active, the Na^+-*in*dependent Cl^-/HCO_3^- antiport is strictly regulated by the internal pH (pH_i) (Tønnessen et al., 1987; Frelin et al., 1988). Thus, the activity of the Na^+-*in*dependent Cl^-/HCO_3^- antiport increases several-fold over a narrow pH range around neutrality. Both Cl^- and HCO_3^- can be transported, and the direction of the transport is determined by the gradients of these anions (Tønnessen et al., 1987). Under normal conditions ($pH_o \approx 7.4$, $pH_i \approx 7.2$) the inward-directed Cl^- gradient is larger than the inward-directed HCO_3^- gradient, and the Na^+-*in*dependent Cl^-/HCO_3^- antiport therefore mediates net efflux of bicarbonate. When the internal pH is increased, the inward-directed bicarbonate gradient is reduced or reversed which further favors

efflux of HCO_3^-. Activation of the Na^+-*in*dependent Cl^-/HCO_3^- antiport therefore leads to reduction in pH_i.

The regulation of Na^+/H^+ antiport has been under extensive investigation for years, and it is clear that several second messenger systems are involved, such as protein kinase C, cAMP and calcium (Owen & Villereal, 1985; Weinman, Shenolikar & Kahn, 1987; Frelin et al., 1988). Like that of the Na^+-*in*dependent Cl^-/HCO_3^- antiporter, the activity of the Na^+/H^+ antiporter is regulated by pH_i. The activity of the Na^+/H^+ antiporter decreases with increasing pH_i in a steeper manner than would be expected from the concentration gradients alone, and therefore H^+ is thought to have a role as an allosteric activator apart from the role as a substrate (Aronson, Nee & Suhm, 1982). Activation of Na^+/H^+ exchange by, e.g., growth-promoting agents apparently involves an alkaline shift in the pH_i dependence of the Na^+/H^+ exchange, with little change in other kinetic parameters (Grinstein & Rothstein, 1986; Moolenaar, 1986). This shift is thought to reflect an altered behavior of the modifier site, and according to this model, the set point of the modifier is adjusted upward by 0.15–0.3 pH units.

The regulation mechanism of the Na^+-*in*dependent Cl^-/HCO_3^- antiport is not known, but in smooth muscle cells, elevation of intracellular cAMP inhibits the Na^+-*in*dependent Cl^-/HCO_3^- exchange, apparently by shifting the pH_i dependency of the antiporter in the alkaline direction (Vigne et al., 1988). The unusually high pH sensitivity of the antiport suggests that strict control of its rate is essential for the cell. We have earlier made the observation that short-time incubation with TPA shifted the pH_i sensitivity of the antiporter in the acidic direction (Olsnes, Tønnessen & Sandvig, 1986). In the present paper we have extended this observation and measured the effect on anion antiport of prolonged treatment with phorbol ester, which we here show downregulates protein kinase C in Vero cells.

Materials and Methods

Cells

Vero cells (from African green monkey kidney) were grown in DMEM (pH 7.4) with 5% fetal calf serum in air containing 5% CO_2. Cells were seeded out into 24-well disposable trays (10^5 cells/well) in medium containing 10% fetal calf serum two days before use. For the purpose of protein kinase C downregulation, TPA or PDBu was added to this medium 18 hr before use.

Chemicals

TPA (12-O-tetradecanoylphorbol 13-acetate), MES (2-(N-morpholino)ethane sulfonic acid, Tris (tris(hydroxymethyl)aminomethane), potassium gluconate, nigericin, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonate), PDBu (phorbol-12,13-dibutyrate), 4α-phorbol-12,13-didecanoate, phorbol-12-myristate-13-acetate-4-O-methyl, and SDS (sodium dodecyl sulfate) were obtained from Sigma. $H^{36}Cl$ (specific activity 19.6 μCi/mg chloride), [^{3}H]PDBu (specific activity 20.8 Ci/mmol) and [^{14}C]DMO (dimethyloxoazolidine dione) were from the Radiochemical Center, Amersham, UK.

Buffers

Mannitol buffer contained (in mM): 260 mannitol, 1 $Ca(OH)_2$, and 20 MES, adjusted to the indicated pH with Tris. Potassium-gluconate buffer contained (in mM): 140 potassium gluconate, 1 $Ca(OH)_2$, and 20 HEPES, adjusted to the indicated pH with Tris. Phosphate buffered saline (PBS) contained (in mM): 140 NaCl and 10 sodium phosphate. KCl buffer contained (in mM) 140 potassium chloride, 1 $CaCl_2$, and 20 MES, adjusted to the indicated pH with Tris. HEPES medium contained: DMEM buffered with 20 mM HEPES instead of bicarbonate.

Measurement of $^{36}Cl^-$ Uptake

Cells in 24-well disposable trays were preincubated as indicated and then washed twice in ice-cold mannitol buffer (pH 7.0). Then 0.3 ml of mannitol buffer or potassium-gluconate buffer containing 0.17 μCi (0.5 mM) of $H^{36}Cl$ was added per well. The cells were incubated at 24°C for the indicated periods of time and then rapidly washed twice with ice-cold PBS, and finally 0.3 ml per well of 5% (wt/vol) trichloroacetic acid was added. After 10 min at room temperature the TCA was transferred to counting vials and the radioactivity was measured. In some cases the cells were subsequently dissolved in 0.2 M KOH, and the absorbance at 280 nm was measured. The number of cells was then estimated from a calibration curve.

Measurement of pH in the Cytosol

Cells in 24-well disposable trays were preincubated under the conditions indicated with 2 μCi [^{14}C]DMO at 37°C. When bicarbonate was present, a layer of liquid paraffin was placed on top of the solution to avoid escape of CO_2. After the indicated incubation periods, the medium and the paraffin were removed by suction, the cells were washed rapidly five times (within 15 sec) with ice-cold PBS, and then the radioactivity associated with the cells was extracted in 0.3 ml 5% (wt/vol) TCA and measured. pH_i was estimated from the distribution of the radioactivity as described (Grinstein & Rothstein, 1986).

Measurement of PDBu Binding

Cells in 24-well disposable trays were pretreated with and without PDBu as indicated in legends to figures for 18 hr in DMEM containing 10% fetal calf serum in air containing 5% CO_2. The

cells were then washed four times in DMEM and incubated for another 30 min at 37°C. The cells were washed in ice-cold HEPES medium and incubated for 90 min on a shaker at 4°C in 0.2 ml/well of HEPES medium, pH 7.4, with 20 nM [^{3}H]PDBu, 5 μg/ml 4α-phorbol-12,13-didecanoate (which does not inhibit PDBu binding) with or without 10 μM TPA. The cells were then washed four times with ice-cold PBS and dissolved in 0.2 ml of 0.1 M NaOH with 1% SDS. The cell-associated radioactivity was measured.

Abbreviations

Tris (tris(hydroxymethyl)aminomethane); HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid); DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonate); DMO ([^{14}C]dimethyloxoazolidine dione); TPA (12-O-tetradecanoylphorbol 13-acetate); PDBu (phorbol-12,13-dibutyrate); MES 2-(N-morpholino)ethane sulfonic acid; DMEM (Dulbecco's modified minimum essential medium); PBS (phosphate buffered saline); SDS (sodium dodecyl sulfate); pH_i (intracellular pH); and pH_o (extracellular pH).

Results

Different Effect on Chloride Uptake of Short-Term and Long-Term Treatment with TPA

When cells are transferred from medium containing 120 mM Cl^- to a Cl^--free buffer osmotically balanced with mannitol or gluconate and containing trace amounts of $^{36}Cl^-$, $^{36}Cl^-$ is accumulated in the cells by antiport energetically drived by Cl^- efflux (Olsnes et al., 1986, 1987*b*). We have earlier presented evidence that the majority of this Cl^-/Cl^- self-exchange is carried out by the Na^+-*in*dependent Cl^-/HCO_3^- antiporter (Olsnes et al., 1987*a*,*b*; Reinertsen et al., 1988). The activity of the Cl^-/HCO_3^- antiport can therefore be studied by measuring $^{36}Cl^-$ fluxes in the absence of other permeant anions.

To test the short-term effect of TPA on the rate of anion antiport, $^{36}Cl^-$ uptake was measured in cells after pretreatment with 1.6 μM TPA for 30 min. Intracellular pH was clamped at different values by incubating the cells at 0.14 M KCl with 2 μM nigericin for 15 min. When the extracellular concentration of K^+ equals the intracellular K^+ concentration, pH_i is equilibrated with the pH_o by the ionophore (Thomas et al., 1979). The $^{36}Cl^-$ uptake was subsequently measured in 0.14 M potassium-gluconate buffer with 2 μM nigericin. Figure 1*A* shows that the antiport was stimulated by TPA when pH_i was clamped at 7.0, whereas at pH 6.6 or 7.3 the rates of antiport was not altered by TPA. Short-time incubation of the cells with TPA did not induce loss of Cl^- from the cells (*data not shown*), and the driving force for the $^{36}Cl^-$ uptake should therefore be the same in the control as in the TPA-treated cells. It therefore appears that anion antiport *per se* is affected by short-term TPA treatment.

To study further the effect of pH_i on the rate of $^{36}Cl^-$ uptake in the absence and presence of TPA, pH_i was clamped at a number of pH values by the K^+/nigericin method for 15 min and then uptake of $^{36}Cl^-$ was measured for 2.5 min. As shown in Fig. 1*B*, the uptake of $^{36}Cl^-$ increased strongly with increasing pH. In a number of similar experiments (n = 17) the activity in the absence of TPA was half-maximal at pH 7.19 ± 0.03. On the other hand, after a 30-min preincubation with TPA the activity of the antiport was half-maximal at pH 7.06 ± 0.04 (n = 18). In both cases the uptake was strongly reduced in the presence of 0.1 mM DIDS (Fig. 1*B*), which is a potent inhibitor of anion antiport (Cabantchik, Knauf & Rothstein, 1978). Similar results were obtained with 10 nM TPA, whereas 1.6 μM of two structural analogues of TPA, phorbol-12-myristate-13-acetate-4-O-methyl ether and 4α-phorbol-12,13-didecanoate, which have no demonstrable effect on protein kinase C (Castagna, 1987) had no effect on the $^{36}Cl^-$ uptake (*data not shown*).

Long-term treatment with tumor-promoting phorbol esters like TPA and PDBu is known to strongly reduce the content of protein kinase C in the cells (Rodriguez-Pena & Rozengurt, 1984; Ballester & Rosen, 1985). If the effect of TPA shown in Fig. 1*B* were due to activation of protein kinase C, the opposite effect on anion antiport might be expected in cells depleted of the enzyme. We therefore incubated cells with TPA for 18 hr, which is sufficient to downregulate protein kinase C in a number of cells (Adams & Gullick, 1989), and we then measured $^{36}Cl^-/Cl^-$ exchange.

The cells were transferred to HEPES medium, pH 7.4, with TPA to adjust them to bicarbonate-free conditions. After 30 min pH_i was clamped as in Fig. 1*B*, and $^{36}Cl^-$ uptake during 2.5 min was measured. As shown in Fig. 1*C*, after TPA treatment the antiport failed to become fully activated, even at the highest pH values tested. After incubation with 600 nM TPA the increase in activity over the pH range tested was about twofold, whereas it was at least sevenfold in the control cells. At the low pH values the uptake was slightly stimulated by TPA as compared to the control cells. These effects were only observed in cells incubated overnight with 50 nM or more TPA, while 10 nM had no effect. Similar results were obtained in the presence of 7 mM Cl^- during the uptake measurements, indicating that the effect was not due to an alteration in the K_m value (~7 mM) of the antiport to Cl^-. After subsequent incubation

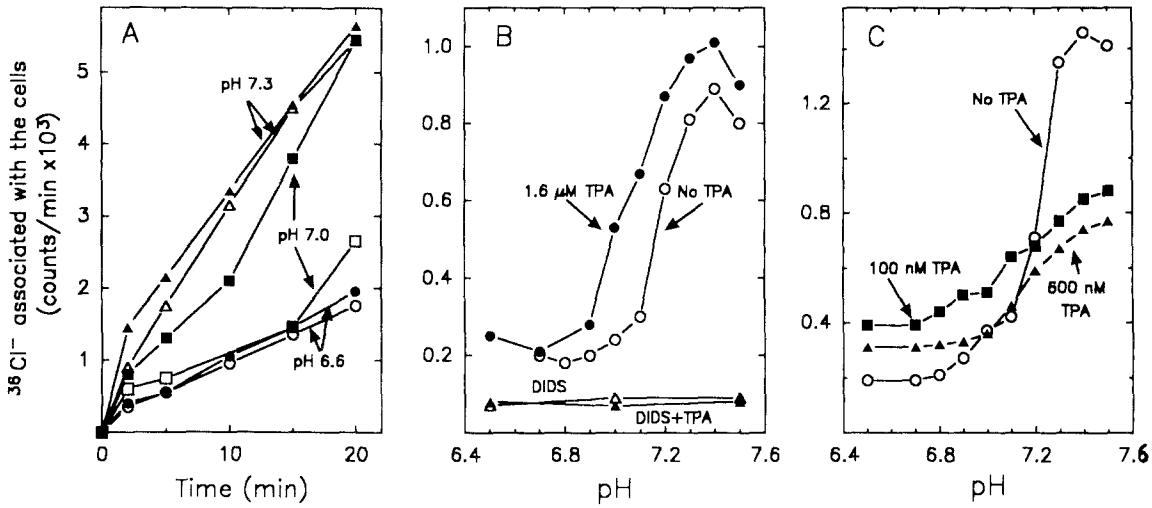

Fig. 1. Effect of TPA on $^{36}Cl^-/Cl^-$ exchange. (*A*) Cells were incubated for 30 min in HEPES medium, pH 7.4, with 1.6 μM TPA (closed symbols) or without TPA (open symbols) and then transferred to KCl buffer, pH 6.6 (○, ●), pH 7.0 (□, ■) or pH 7.3 (△, ▲), with 2 μM nigericin, with and without 1.6 μM TPA. After 15 min, the cells were transferred to potassium-gluconate buffer, adjusted in each case to the same pH as the KCl buffer and containing 2 μM nigericin, 0.5 mM $H^{36}Cl$, with and without 1.6 μM TPA. Uptake of $^{36}Cl^-$ was measured after increasing time periods. (*B*) Cells were transferred to HEPES medium, pH 7.8, and incubated for 30 min. They were incubated without (○) or with (●) 1.6 μM TPA for 30 min more and then transferred to KCl buffer containing 2 μM nigericin and, when indicated, 1.6 μM TPA. After 15 min, the uptake of $^{36}Cl^-$ during 2.5 min was measured in mannitol buffer containing 0.17 μCi/ml (0.5 mM) $H^{36}Cl$. The pH values for half-maximal activation of the uptake was 7.19 ± 0.03 (n = 17) in the absence of TPA and 7.06 ± 0.04 (n = 18). In some cases (△, ▲) 0.1 mM DIDS was present in the KCl buffer and during the $^{36}Cl^-$ uptake. (*C*) Cells incubated overnight without (○) or with TPA (■, ▲) were transferred to HEPES medium, pH 7.4, without or with TPA and incubated for 30 min to adjust them to bicarbonate-free conditions. pH_i was then clamped for 15 min by the KCl/nigericin method as in Fig. 1*B*, and $^{36}Cl^-$ uptake during 2.5 min was measured. There was a certain loss of cells (approximately 17%) after prolonged treatment with TPA which is corrected for in the figure.

for one day in the absence of TPA, the normal regulation of the antiport resumed (*data not shown*).

Since the efflux of Cl^- is the driving force for the uptake of $^{36}Cl^-$ (Deutsch et al., 1979; Olsnes et al., 1987*b*) the possibility existed that the inhibitory effect of long-term TPA treatment could be due to loss of chloride from the cells. We have earlier found that when Vero cells are incubated in buffers deprived of permeant anions (to prevent antiport) cells lose chloride. The rate of chloride loss strongly increases above pH_i = 7.0 (Olsnes et al., 1987*b*). The rate of $^{36}Cl^-$ loss from preloaded cells was therefore measured.

Cells were incubated overnight with or without 600 nM TPA and then transferred to HEPES medium, pH 7.4, containing 1.7 μCi/ml $^{36}Cl^-$ and 600 nM TPA when indicated. The cells were incubated for 3 hr to equilibrate $^{36}Cl^-$ over the cell membrane and then transferred to K- gluconate buffer, pH 6.7 or 7.5, with 7 μM nigericin to ensure rapid equilibration of pH_i. After incubation for the indicated periods of time the cell-associated radioactivity was measured. It is clear from Fig. 2 that long-term treatment with TPA did not strongly alter the rate of $^{36}Cl^-$ loss at either pH. The $^{36}Cl^-$ content of the cells at the start of the experiment (time zero in Fig. 2) was the same (~40 mM) in the TPA-pretreated and in the control cells.

To test if downregulation of protein kinase C occurred in the same concentration range of phorbol ester as that required to exhibit an effect on anion transport, phorbol-ester binding was measured with and without pretreatment of the cells with the same compound. Due to the high affinity of TPA to protein kinase C, it was necessary to use the TPA analogue PDBu which exerts the same effect as TPA and binds to protein kinase C at the same binding site, but with a lower affinity constant. The lower affinity allows removal of the compound from the cells after preincubation, before the binding assay is carried out.

Cells were preincubated for 18 hr with different concentrations of unlabeled PDBu, and then the binding of [^{3}H]PDBu was measured. The binding was measured in the presence of 5 μg/ml of 4α-phorbol-12,13-didecanoate to inhibit binding to the plastic, and in the presence and absence of 10 μM TPA. As Fig. 3*A* shows, the specific binding of [^{3}H]PDBu started to decrease after preincubation

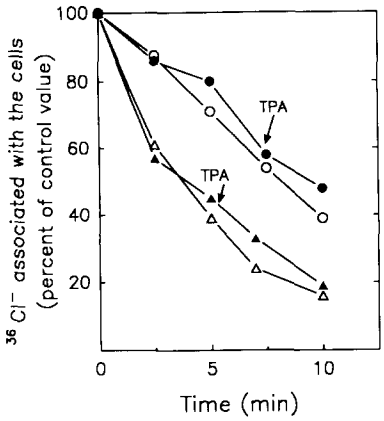

Fig. 2. Effect of long-term treatment with TPA on the rate of $^{36}Cl^-$ efflux from Vero cells. Cells were preincubated overnight in medium without and with 60 nM TPA, and then transferred to HEPES medium, pH 7.1, with the same additions and containing 0.85 μCi/ml $H^{36}Cl$. After 3 hr the cells were washed twice in K-gluconate buffer and incubated in the same buffer (pH 6.7 (circles) or pH 7.5 (triangles)) with 7 μM nigericin. The remaining radioactivity associated with the cells after the indicated periods of time was measured. The initial (control) level of cell-associated radioactivity (100%) was the same (536 ± 5 cpm) in the two cases after correction for cell loss

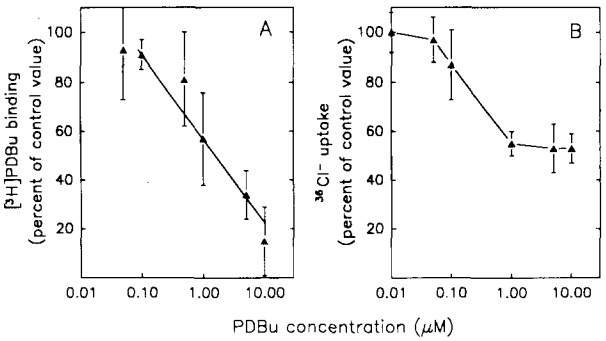

Fig. 3. Effect of long-term preincubation with PDBu on [^{3}H] PDBu binding and on $^{36}Cl^-$ uptake. Cells were preincubated for 18 hr with the indicated concentrations of PDBu. The cells were then washed four times in DMEM (*A*) or HEPES medium (*B*) and incubated in the same medium at 37°C in the presence (*A*) or absence (*B*) of 5% CO_2. (*A*) After 30 min ice-cold HEPES medium with 20 nM [^{3}H]PDBu was added, and the amount bound during 90 min at 4°C was measured. The amount of [^{3}H]PDBu bound in the presence of 10 μM TPA was subtracted, and the specific binding as percent of control values (no PDBu during the preincubation) is demonstrated. (*B*) After 15 min the cells were transferred to K-gluconate buffer, pH 7.6, with 2 μM nigericin and incubated at 37°C for 15 min more. Uptake of $^{36}Cl^-$ during 2.5 min was then measured and expressed as percent of the control values (no PDBu during the preincubation). The data are expressed as mean ± SD in 15–18 measurements

with 50–100 nM PDBu, and it was reduced to half after pretreatment with 1 μM PDBu.

PDBu had a similar effect on anion antiport as TPA. In both cases the rate of antiport at alkaline pH was reduced to approximately half after treatment overnight with high concentrations of the phorbol ester. In the experiment shown in Fig. 3*B* cells were incubated overnight with increasing concentrations of PDBu, and then anion antiport was measured as in Fig. 1*C*, but at only one pH value (pH 7.6). To achieve analogous conditions to Fig. 3*A*, the PDBu was removed 30 min before the $^{36}Cl^-$ uptake was measured. The results showed that while $^{36}Cl^-$ uptake was close to the control value after preincubation with 50 nM PDBu, it was reduced to 50% (i.e., maximal reduction) at 1 μM PDBu. Clearly, the effect on anion antiport was obtained in the same concentration range as that required to reduce protein kinase C in the cells.

Effect of TPA on Intracellular pH

We also measured if the TPA-induced alterations in activity of the Na^+-*in*dependent antiport affected the resting pH_i. We first tested the effect of short-term treatment with TPA. Cells were preincubated for 30 min in the absence and presence of 1.6 μM TPA with or without 28 mM HCO_3^-, and pH_i was then measured by the DMO method. With this approach we were unable to demonstrate a difference in resting pH_i with and without treatment with TPA (*data not shown*). Possibly, the low effect on pH_i is due to compensatory pH-regulating mechanisms. It should be noted that although the Na^+/H^+ antiporter is activated in Vero cells under these conditions (Tønnessen et al., 1990) the presence of the Na^+/H^+-antiport inhibitor, amiloride, did not alter the results.

Since long-term treatment with TPA to down-regulate protein kinase C strongly inhibited activation of $^{36}Cl^-/Cl^-$ exchange by the Na^+-*in*dependent Cl^-/HCO_3^- antiporter, we measured the resting pH_i after such treatment. The cells were treated with 600 nM TPA for 18 hr and then transferred to HEPES medium with or without bicarbonate and TPA. After 45 min the distribution of [^{14}C]DMO was measured and pH_i was estimated. The data in the Table show that in the presence of 28 mM HCO_3^- pH_i was 0.18 pH units higher in TPA-treated cells than in the control cells. In the absence of added HCO_3^- TPA raised pH_i only slightly. The slight increase in the absence of added HCO_3^-, could at least partly be due to Cl^-/HCO_3^- exchange, since ~0.2 mM HCO_3^-

Table. Effect of long-term treatment with TPA on pH_i

HCO_3^- added	Control	TPA
	pH_i	pH_i
28 mM	7.22 ± 0.06 (n = 18)	7.40 ± 0.08 (n = 15)
0	7.41 ± 0.04 (n = 15)	7.47 ± 0.04 (n = 15)

Vero cells were incubated for 18 hr in the absence and presence of 600 μM TPA, and then transferred to HEPES medium with 2 μCi/ml [^{14}C]DMO. HCO_3^- (28 mM) and TPA (600 μM) were added when indicated. After 45 min the medium was removed, and the cells were rapidly washed five times in ice-cold PBS. Then 0.3 ml/well of 5% TCA was added and, after 10 min at room temperature, the extracted radioactivity was measured. The data represent mean ± SD in the number of experiments given in parenthesis.

is present in HEPES medium, pH 7.4, kept in a normal atmosphere. The results indicate that lowering the ability of the Na^+-*in*dependent Cl^-/HCO_3^- antiport to respond to increased pH_i reduced the ability of the cells to extrude base.

Discussion

The main findings in the present paper are that both stimulation and downregulation of protein kinase C affected the regulation of anion antiport in Vero cells. After short-term treatment with TPA to stimulate protein kinase C the pH_i set point for activation of the Na^+-*in*dependent Cl^-/HCO_3^- antiporter was lowered, but the antiport was still strongly activated over a narrow pH range. On the other hand, after prolonged TPA treatment to downregulate protein kinase C the ability of the antiporter to be regulated by pH_i was reduced. Thus, while in control cells the activity of the antiport was increased at least seven times over a 0.5–0.6 pH-unit interval, it was only doubled over a pH_i interval of 0.8 units after pretreatment with 100 nM TPA overnight.

In attempts to elucidate the relation between H^+ concentration and antiport activity, the data from Fig. 1*C* were replotted on a linear scale (Fig. 4). If the activation of the antiport were dependent on deprotonation of a single titratable group, one would expect the initial $^{36}Cl^-$ uptake rate to decrease linearly with increasing internal H^+ concentration (first-order kinetics) and then to level off as the internal H^+ site became saturated (zero-order kinetics). However, in the pH range 7.6–6.9 the $^{36}Cl^-$ uptake rate showed a greater than first-order dependence on the H^+ concentration, suggesting that more than one titratable group are essential for activation.

If the binding of protons is considered as a positive cooperative process, the Hill coefficient would be at least 5 in the absence of TPA. However, cooperation in this context implies that binding of one proton affects the subsequent binding of protons. This does not necessarily have to be the case, as required protonation of all out of several titratable groups with approximately the same pK value could give a similarly shaped curve. The Hill coefficient may still give an estimate of the number of titratable groups essential for regulation of antiport activity. For comparison, the published values of Hill coefficients for the Na^+/H^+ antiporter vary between 1.2 and 3 in different cell types (Kinsella & Aronson, 1982; Grillo & Aronson, 1986).

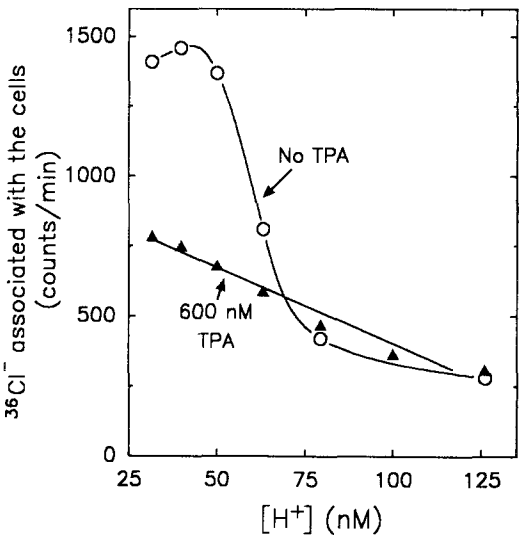

Fig. 4. Replot of data from Fig. 1*C* with the proton concentration on a linear scale. (○) no TPA; (▲) 600 nM TPA

After treatment with TPA overnight, a linear curve was obtained in the same pH interval (Fig. 4), suggesting that in this case the activity of the antiporter was dependent on titration of a single group. It is possible, therefore, that prolonged treatment with TPA reduces the pH_i dependency of the antiporter by reducing the number of proton-binding sites involved in regulation of the activity. If this were the case, the activity of the antiport should continue to increase for at least one more pH unit before reaching the maximal value. This is difficult to test because of toxic effects of high pH_i as well as increased chloride leakage at alkaline pH values, which may be the reason for the plateau reached in Fig. 1*C*.

Possibly, phosphorylation of the antiporter by protein kinase C stabilizes a conformation where deprotonation of all out of a number of titratable groups induces a strong activation by increased pH_i. In the dephosphorylated antiporter the activity

could be regulated to a limited extent by a single or a few titratable groups. Different states of phosphorylation of the antiporter could explain why Cl^-/HCO_3^- antiport is regulated by pH_i to different extents in different cell lines (Olsnes et al., 1987*b*; Reinertsen et al., 1988; Vigne et al., 1988).

Stimulation of protein kinase C by short-time incubation with phorbol ester did not have the opposite, but rather a different effect on anion transport as compared to that obtained by downregulation of the enzyme. Possibly, the shift in triggering pH_i to more acidic values after stimulation of protein kinase C with TPA reflects phosphorylation at an additional site on the antiporter.

PDBu is a phorbol ester known to have the same effect on protein kinase C as TPA, and after prolonged treatment with PDBu anion antiport was altered in the same way as by TPA. We found that downregulation of protein kinase C occurred in the same concentration range of PDBu as that required to affect anion antiport. The data indicate that the amount of active protein kinase C is the limiting factor in the regulation, which is expected since the level of active protein kinase C is carefully regulated in the cells. Apparently, the low levels of protein kinase C normally present in the membrane of metabolically active cells is sufficient to ensure normal pH_i regulation of the antiporter.

In accordance with the findings in other systems, TPA was more efficient than PDBu in altering anion antiport. Thus, while 100 nM TPA reduced the $^{36}Cl^-$ uptake almost maximally at pH_i 7.6, 500 nM–1 μM PDBu had to be added to obtain the same effect. This difference is most likely due to the different affinities of the two compounds for protein kinase C.

pH_i appears to be regulated at several levels. When pH_i deviates from the correct value to the acidic or alkaline side, the Na^+/H^+ antiporter and the Na^+-*in*dependent Cl^-/HCO_3^- antiporter are activated, respectively, possibly by a direct effect of the H^+ concentration on the antiporters. In addition to this, the set points of the two antiports with respect to triggering pH are also regulated, in the case of the Na^+/H^+ antiport by a number of growth factors and phorbol esters, and in the case of the Na^+-*in*dependent Cl^-/HCO_3^- antiport by TPA, nonsteroidal anti-inflammatory drugs (Tønnessen et al., 1989) and vasopressin (Ganz et al., 1989). It should be noted that in Vero cells also a Na^+-linked Cl^-/HCO_3^- antiport exists, which appears not to be regulated and which corrects minor pH_i deviations at the acidic side (Reinertsen et al., 1988).

We are grateful to Mrs. Jorunn Jacobsen for her skillful handling of the cell cultures. This work was supported by the Norwegian Cancer Society and by the Norwegian Research Council for Science and Humanities.

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Received 30 April 1990