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# Regulation of $Na^+/H^+$ exchange in opossum kidney cells by parathyroid hormone, cyclic AMP and phorbol esters

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**Abstract.** Parathyroid hormone (PTH) controls two proximal tubular brush border membrane transport systems, $Na^+$/phosphate co-transport and $Na^+/H^+$ exchange. In OK cells, a cell line with proximal tubular transport characteristics, PTH acts via kinase C and kinase A activation to inhibit $Na^+$/phosphate co-transport [6, 8, 9, 19, 22]. In the present study, we show that PTH inhibits $Na^+/H^+$ exchange and that this effect can be mimicked by pharmacological activation of kinase A and kinase C. Ionomycin-dependent increases in cytoplasmic $Ca^{2+}$ concentration do not induce inhibition of $Na^+/H^+$ exchange; PTH-dependent inhibition of $Na^+/H^+$ exchange is not prevented by ionomycin or by the intracellular $Ca^{2+}$ chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid ($Ca^{2+}$ clamping). Detailed dose-response curves for the different agonists, given either alone or in combination, suggest that the two regulatory cascades (kinase A and kinase C) are operating independent of each other and reach a common final target, resulting in 40–50% inhibition of $Na^+/H^+$ exchange. An analysis of intracellular pH sensitivity of $Na^+/H^+$ exchange suggests that inhibition is not related to a shift in set point, but is rather explained by a reduced $V_{max}$ of $Na^+/H^+$ exchange and/or reduced affinity for protons at the internal membrane surface. It is suggested that kinase A as well as kinase C can mediate PTH inhibition of renal proximal tubular $Na^+/H^+$ exchange and that the relative importance of a particular regulatory cascade is determined by the PTH-concentration-dependent rates in the liberation of diacylglycerol (phospholipase C/kinase C) and cAMP (adenylate cyclase/kinase A).

**Key words:** $Na^+/H^+$ exchange, proximal tubule – Tissue culture – Hormonal control – Parathyroid hormone

## Introduction

Parathyroid hormone (PTH) inhibits proximal tubular phosphate reabsorption and proximal tubular bicarbonate reabsorption, leading also to inhibition of proximal tubular volume reabsorption (for review see [2, 12]). It has been generally accepted that biological effects of PTH are mediated through the second messenger cAMP via activation of kinase A. In addition to cAMP/kinase A, however, it has been suggested that phospholipase C/kinase C may also be involved in the hormone action on proximal tubular transport functions. In OK cells and in primary cultures of proximal tubular cells it was shown that PTH stimulated a rise in both 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate ($IP_3$) and in parallel caused an increase in cytosolic free $Ca^{2+}$ ($Ca_i$). The question related to a second messenger role of phospholipase C/kinase C in the action of PTH on renal cells is of particular interest in view of the discrepancies observed for physiological concentrations of the hormone (of about $10^{-12}$ M) and PTH concentrations required for half maximal stimulation of adenylate cyclase (AC) ($EC_{50} = 10^{-8}$ M PTH) [4, 33].

In the past few years several laboratories have used the OK cells, derived from opossum kidney [17], to study PTH control of proximal tubular transport functions [6, 8, 9, 19, 22]. This cell line shows typical proximal tubular properties, including PTH-dependent inhibition of apically located $Na^+/H^+$ exchange and apically located $Na^+$/phosphate co-transport (Reshkin and Murer, to be published; Montrose and Murer, to be published;

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*Abbreviations:* HEPES, 4-(2-hydroxyethyl)-1-piperazineethane-sulphonic acid; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; PTH, parathyroid hormone; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; BCECF, 2′,7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein; $P_i$, inorganic phosphate; $pH_i$, intracellular pH; $Ca_i$ cytosolic free $Ca^{2+}$; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $IP_3$, inositol 1,4,5-trisphosphate; DAG, 1,2-diacylglycerol

[6, 8, 9, 19–22, 24, 27, 29]). Detailed dose-response curves on mechanisms of OK cell activation by PTH have indicated that phospholipase C, generating the second messengers DAG and $IP_3$, is activated at hormone concentrations lower than those required to produce measurable increases in cAMP [8, 9, 30]. In addition to these findings, it was shown that hormone concentrations required for half maximal stimulation of phospholipase C correlated well with concentrations required for half maximal inhibition of $Na^+$/phosphate transport [8, 9, 30]. Additional evidence that phospholipase C/kinase C may be an important signalling mechanism involved in regulation of $Na^+$/phosphate transport by PTH in OK cells was provided from studies on pharmacological activation of kinase C followed by inhibition of $Na^+$/phosphate co-transport [8, 9, 22].

With respect to regulation of renal proximal tubular $Na^+/H^+$ exchange, there is clear evidence that kinase A (cAMP) activation leads to inhibition of $Na^+/H^+$ exchange ([2, 15, 38]; for review see [13]). However, for activation of kinase C and regulation of renal proximal tubular $Na^+/H^+$ exchange, present available reports are contradictory. In in vitro microperfused rabbit proximal tubules, addition of phorbol esters led to an inhibition of volume reabsorption, $Na^+$-bicarbonate reabsorption and NaCl reabsorption [3]; these phenomena are best explained by inhibition of $Na^+/H^+$ exchange. In canine proximal tubular segments, cytoplasmic pH rose after incubation with phorbol esters, indicating activation of $Na^+/H^+$ exchange [23]. Finally, in transiently opened renal brush border membranes or reconstituted proteoliposomes, activation of kinase C by micromolar concentrations of phorbol esters led to an activation of $Na^+/H^+$ exchange [36, 37]. In addition, it should be mentioned that in non-polarized cells a stimulation of kinase C was always found to be associated with an activation of $Na^+/H^+$ exchange (for review see [13]).

In the present experiments we show that in OK cells PTH inhibits $Na^+/H^+$ exchange and that maximal doses of cAMP or phorbol ester, respectively, lead to an inhibition of $Na^+/H^+$ exchange by 40–50%, irrespective of the use of agonist. Together with data reported earlier [30], we conclude that the intracellular regulatory cascades in PTH action on $Na^+/H^+$ exchange and $Na^+$/phosphate co-transport are very similar, i.e. at low (physiological) PTH concentrations, the kinase C pathway might be especially important in PTH-dependent control of proximal tubular $Na^+/H^+$ exchange.

## Materials and methods

### *Cells and cell culture*

An uncloned OK cell line [17] was obtained from Dr. D. G. Warnock, San Francisco, Calif, at serial passage 79. A cloned, single cell isolate was selected from the wild-type based on its ability to express PTH-sensitive $Na^+$/phosphate co-transport and to adapt to low phosphate media (J. Forgo, J. Biber and M. Murer, unpublished observation). Cells were maintained in culture in Dulbecco's modified Eagle's medium (DMEM/Hams F12) supplemented with 10% fetal calf serum (FCS), 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethane-sulphonic acid), 2 mM glutamine, 50 IU/ml penicillin and 50 μg/ml streptomycin in a humidified atmosphere of 5% $CO_2$, 95% air at 37°C. Subculturing was performed as previously described [22]. All studies were performed using cells between passage 3 and 12 (single cell clone).

### *Cell suspension*

*$Na^+/H^+$ exchange studies.* For experimental purposes $3 \times 10^6$ cells were plated in 75 $cm^2$ culture flasks (Corning, New York) and grown to confluency for 4–5 days. The monolayers were washed twice with phosphate-buffered saline and were submitted to mild trypsinization. Trypsinization was carried out by incubating the cells with 5 ml phosphate-buffered saline (PBS) containing 0.1% trypsin plus 0.5 mM EDTA (ethylenediaminetetraacetic acid) for 1 min. Trypsin medium was then removed and replaced by 1 ml of the same medium for 5 min at 37°C. At the end of the incubation period, cells were diluted into 20 ml of Na-medium (see "Solutions") containing 5 mg ovomucoid trypsin inhibitor. Cells were allowed to recover for 3 h at 25°C while being gently shaken. Subsequent determination of $Na^+/H^+$ exchange activity was performed at 25°C.

Analogous to the subculturing of cells used for $Na^+$/phosphate co-transport studies (see below), sensitivity of $Na^+/H^+$ exchange to PTH was determined in cellular suspensions created from cells maintained at normal culture medium or at DMEM/Hams F12 supplemented with 20 mM HEPES and 0.1% bovine serum albumin (BSA) (deprived of bicarbonate and FCS) 12 h prior to use. PTH dose-response data accumulated under both conditions indicated no specific requirement for BSA (data not shown). Therefore the incubation step in BSA medium over night was not included in the protocol for $Na^+/H^+$ exchange activity studies.

*$Na^+$/phosphate co-transport studies.* Cells plated at $1.8 \times 10^6$ cells/10-cm-diameter plastic petri dish were maintained in culture until confluency; prior to use they were incubated for 12 h with DMEM/Hams F12 (minus $HCO_3^-$, minus FCS), 20 mM HEPES, 0.1% BSA at 37°C. Depending on the experiments, cell suspensions were created either by mild trypsinization or by using the $Ca^{2+}$ chelator EDTA [22]. If cells were suspended by the EDTA method, monolayers were washed twice with PBS and exposed to 2.5 ml of 5 mM EDTA in PBS for 12 min at 37°C. At the end of this incubation period 5 ml DMEM/Hams F12 supplemented with 0.1% BSA was added and the cells were suspended using a syringe with a large-bore needle. EDTA was then removed by centrifugation, and the cells were washed twice with DMEM/Hams F12 plus 0.1% BSA. The final pellet was resuspended in an appropriate volume of DMEM/Hams F12 plus 0.1% BSA to give a final concentration of 2.5 mg protein/ml. If monolayers were to be exposed to trypsin, cells were treated exactly as indicated above ($Na^+/H^+$ exchange studies) except that trypsin was removed by centrifugation and cell concentration was adjusted to 2.5 mg protein/ml DMEM/Hams F12 plus 0.1% BSA. Suspended cells were always allowed to re-equilibrate for 3 h at 37°C prior to the transport measurement.

### *Measurement of transport*

*Measurement of $Na^+$/phosphate co-transport.* For studies of $Na^+$/phosphate co-transport activity as a function of PTH concentration, re-equilibrated cells were exposed to PTH for 2 h at 37°C prior to phosphate uptake measurements. The rational for a 2-h incubation with PTH is based on time-course studies of the activity of $Na^+$/phosphate co-transport in the presence and absence of PTH [10, 22]. In these experiments, it was shown that PTH decreased $Na^+$/phosphate co-transport activity maximally after 120 min of exposure with no further decrease after an additional 60 or 120 min. The respective values of phosphate uptake in control samples remained unchanged during the experimental time of 4 h (data not

shown; see [22]). Phosphate uptake was measured by adding 0.1 ml of $^{32}$[P]-$PO_4$ uptake medium (1.5 μCi/10.1 ml) to 0.1 ml aliquots of suspended cells for 5 min at 37°C. Uptake of $^{32}PO_4$ was stopped by addition of 1 ml ice-cold solution (137 mM NaCl, 14 mM Tris/HCl pH 7.4, 5 mM $K_2HPO_4$). $^{32}$[P]-$PO_4$ external to the cells was removed by centrifugation. Cells were washed once with stop solution and lysed for 1 min with 0.5 ml perchloric acid. Membrane fragments were sedimented and 150 μl of the resultant supernatant was assayed for radioactivity by liquid scintillation spectrometry. At a 5-min uptake period sodium-independent uptake of phosphate was less than 10% of the uptake in the presence of sodium, and was not affected by any of the experimental manoeuvres performed (data not shown; see [22]).

*Measurement of $pH_i$ and $Na^+/H^+$ exchange activity.* $Na^+/H^+$ exchange activity and intracellular pH ($pH_i$) measurements in suspended cells were performed as described previously [25] using the fluorescent probe 2′,7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein (BCECF). In short 2 ml aliquots of re-equilibrated cells ($1.8 \times 10^6$ cells/2 ml) were exposed to 0.41 μM BCECF acetoxymethyl ester (Molecular Probes, Eugene, Ore) for 45–60 min at 25°C. Extracellular BCECF was removed by centrifugation for 2 s at 10000 rpm (Eppendorf microfuge). Cells were resuspended in 60 μl "Na-medium" for calibration purposes and resting $pH_i$ measurements. Of these samples, 50 μl was transferred into 2 ml medium in a standard fluorometer cuvette and was analysed for changes in fluorescence at 25°C. If $Na^+/H^+$ exchange activity in response to an acid load was to be analysed, $NH_4Cl$ (concentration given in figure legends) was added to the cells during the last 15 min of the dye-loading period. Similarly, if PTH effects (or effects of other compounds) were being studied, OK cells were exposed to the hormone 15 min prior the onset of data collection (i.e. during the BCECF loading period). At the end of this interval, BCECF was removed and cells were treated as above, except that they were resuspended in 60 μl Na-medium containing an appropriate amount of $NH_4Cl$. $Na^+/H^+$ exchange activity was measured using a Schimadzu RF 510 apparatus. Samples were excited at 500 nm (with a 3.0 nm slit width), and emission was recorded at 530 nm (10.0 nm sit width). Calibration of the absorbance of BCECF in terms of $pH_i$ was performed as described previously [25, 27]. Briefly, absorbance of BCECF was assessed by releasing trapped dye with digitonin (0.025%) and sequential titration of dye with $HNO_3$ to a given pH value (measured by an electrode implanted into the cuvette). Changes in absorbance caused by variations of the total dye amount between individual samples were normalized by measuring the fluorescence at the isosbestic point of the dye [25].

*Measurement of $Ca_i$.* Cell grown to confluency in a 75 $cm^2$ culture flask were suspended from the culture flask using the $Ca^{2+}$ chelator EDTA. Cells were washed twice with PBS and exposed to 5 mM EDTA in PBS (2.5 ml) for 12 min. Isolated cells were diluted in 10 ml Na-medium and centrifuged at 500 rpm for 3–4 min. Immediately after, cells were resuspended in 10 ml Na-medium and incubated at 37°C for 3 h with continuous shaking.

Loading of cells (2-ml aliquots) with the fluorescent $Ca^{2+}$ indicator fura-2 (dissolved 1:1:30 in dimethyl sulfoxide (DMSO), pluronic acid, FCS) was started by adding a small volume (usually 1/25) of the acetoxy-methyl ester (fura-2/AM; Molecular Probes, Eugene, Ore, USA) to the cellular suspension. The final concentration of fura-2 in the incubation media was 1.25 μM. The temperature of incubation was 37°C and the length of incubation ranged between 10 and 20 min. Prior to fluorescent measurement fura-loaded cells were washed twice in Na-medium. Between each washing step a centrifugation step of 2 s at 10000 rpm was included in order to remove extracellular dye.

Fluorescence was measured with a spectrofluorometer (Schimadzu, Model RF 510) on a 2-ml sample at 37°C. Samples were excited alternatively at 340 and 380 nm (band width 3 nm) and emission was monitored at 505 nm (band width 10 nm). Fluorescence signals were corrected for autofluorescence and were calibrated in terms of $Ca_i$ as described previously [31].

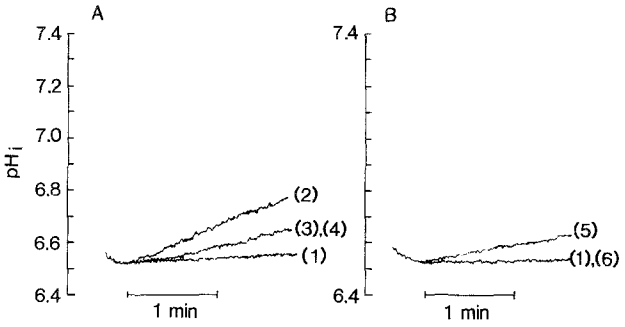


**Fig. 1 A, B.** Fluorescent intensity tracing of BCECF loaded cells. OK cells loaded with 0.41 μM BCECF were exposed for 15 min to 20 mM $NH_4Cl$ (*traces 1–6*), $1.2 \times 10^{-7}$ M PTH (*trace 3*); $1.2 \times 10^{-6}$ M PTH (*trace 4*); 10 μM EIPA (*trace 6*). After removal of BCECF, cells were resuspended in TMACl-medium plus 20 mM $NH_4Cl$ (*traces 1, 5, 6*), or Na-medium plus 20 mM $NH_4Cl$ (*traces 2–4*) and were transferred to the fluorometer cuvette to initiate acidification. Fluorescence was recorded as a function of time. The traces given are from a representative experiment. Values given below are mean ± SEM. **A** Recovery of $pH_i$ after intracellular acidification – effect of PTH. In the absence of $Na^+$ (TMACl-medium) there was no recovery of $pH_i$ from the intracellular acid load (*trace 1*). In the presence of $Na^+$ (Na-medium) the decrease in $pH_i$ was followed by a recovery of $pH_i$. At 140 mM $Na^+$ (Na-medium) $pH_i$ recovered at a rate of 0.124 ± 0.009 pH/min ($n$ = 3) (*trace 2*). In the presence of $1.2 \times 10^{-7}$ M PTH (*trace 3*) or $1.2 \times 10^{-6}$ M PTH (*trace 4*) the $pH_i$ recovery rate was decreased to 0.071 ± 0.004 pH/min ($n$ = 3) and to 0.068 ± 0.004 pH/min ($n$ = 3), respectively, and was not significantly different for the two PTH concentrations. **B** Recovery of $pH_i$ after intracellular acidification – effect of EIPA. At 20 mM $Na^+$, $pH_i$ recovery rate was 0.090 pH/min (*trace 5*) and resembled 2/3 the rate at saturating $Na^+$ concentrations (140 mM $Na^+$). At 20 mM $Na^+$ plus 10 μM EIPA no recovery of $pH_i$ from intracellular acid load was observed (*trace 6*)

If $Ca_i$ was to be buffered with the $Ca^{2+}$ chelator BAPTA (1,2-bis(2-aminophenoxy)ethane-N,N,N′,N-tetraacetic acid), which is reported to have a high selectivity for $Ca^{2+}$ over $Mg^{2+}$ and $H^+$ and does not release protons on binding $Ca^{2+}$ [34], cells were exposed simultaneously to the AM ester derivatives of fura-2 and BAPTA (Molecular Probes, Eugene, USA) (10 μM loading concentration).

*Solutions.* Na-medium contained: 140 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM $NaH_2PO_4$, 18 mM glucose, buffered with 20 mM HEPES to pH 7.4. In the solution designed to study proton leakage, tetramethylammonium ($TMA^+$) replaced $Na^+$ (TMACl-medium). The $^{32}$[P]-$PO_4$ uptake medium contained: 140 mM NaCl (or 140 mM TMACl), 4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.1 mM $NaH_2{}^{32}PO_4$ (or 0.1 mM $KH_2{}^{32}PO_4$), 18 mM glucose, buffered with 20 mM HEPES to pH 7.4. For $Ca_i$ measurements the following solutions were prepared: 10 mM digitonin in DMSO; 5 mM ionomycin in DMSO; 250 mM EGTA in 2.5 M Tris, pH > 8.0.

## Results

### *Basic observations*

Figure 1 illustrates the basic experimental protocol and the basic experimental observations. Cells were suspended by trypsinization and allowed to recover for 3 h at 25°C in Na-medium (see Materials and methods for details). The resting $pH_i$ value under these conditions was $pH_i$ = 7.21 ± 0.02; $n$ = 13. After acid loading (20 mM

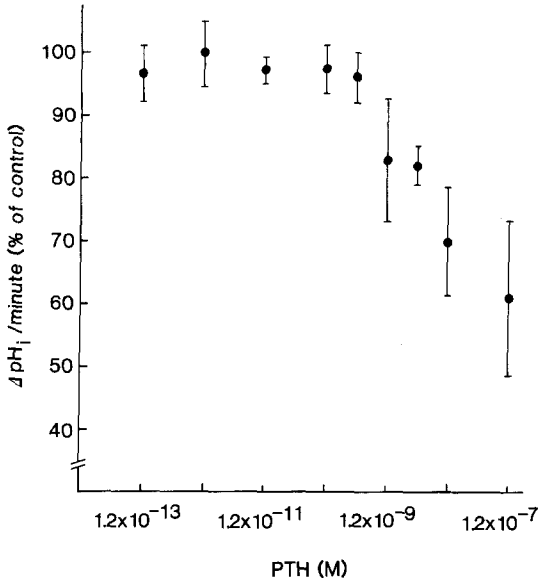


**Fig. 2.** Dose dependency of PTH on the $pH_i$ recovery from an acid load. Cells were loaded with BCECF and exposed to PTH and 20 mM $NH_4Cl$ as described. To sustain the ammonia repulse prior recording cells were resuspended in 60 ml Na-medium plus 20 mM $NH_4Cl$. Cellular acidification and subsequent recovery of $pH_i$ from the acid load was introduced by injection of the sample (50 µl) in 2 ml Na-medium in the fluorometer cuvette. The initial rate of $pH_i$ recovery was measured and is presented as change in fluorescence per minute relative to the rate without PTH (control). Values are the mean ± SEM ($n$ = 4)

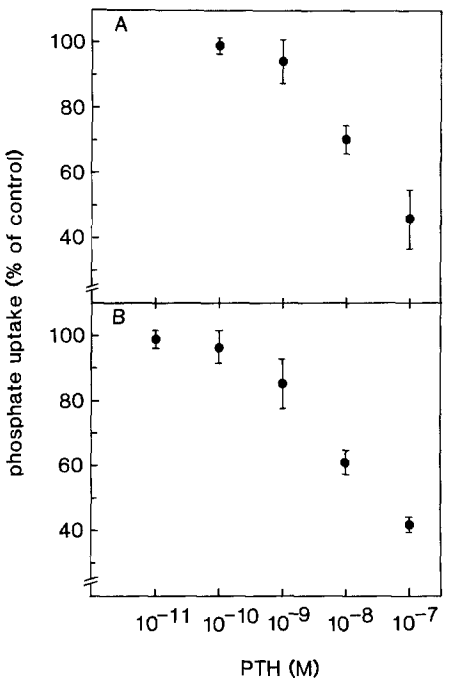


**Fig. 3 A, B.** Concentration dependency of PTH on $Na^+$-dependent phosphate uptake. **A** Dose-response relationship between phosphate uptake and PTH as measured in suspensions created by trypsinization. **B** Dose-response relationship between phosphate uptake and PTH by OK cells placed in suspension using the $Ca^{2+}$ chelator EDTA. Cells were preincubated for 2 h with the indicated concentrations of PTH. The $^{32}$[P]-$PO_4$ uptake time was 5 min. $^{32}$[P]-$PO_4$ uptake was measured with 140 mM $Na^+$ in th extracellular medium. The rate of uptake (100%) by control cells was 0.602 + 0.128 (**A**) and 0.585 + 0.053 nmol/mg protein (**B**). Each experiment was carried out in quadruplicate. The data represent the mean ± SEM

$NH_4Cl$ for 15 min followed by $NH_4Cl$ removal; see Materials and methods), the cells resumed a low $pH_i$ ($pH_i$ = 6.62 ± 0.08; $n$ = 13). In the absence of sodium in the extracellular solution (TMACl-medium), there was no recovery from the acid load, i.e. there is apparently no significant proton leak under these conditions; addition of sodium resulted in an immediate extrusion of protons, which was faster at 140 mM sodium as compared to 20 mM sodium; sodium-dependent recovery from the acid load was fully sensitive to 10 µM ethylisopropyl-amiloride (EIPA), a competitive inhibitor of $Na^+/H^+$ exchange [11, 35]. Thus, the observed sodium-dependent recovery is mainly due to the activity of the $Na^+/H^+$ exchanger.

Preincubation of the cells for 15 min with PTH results in an inhibition of the sodium-dependent recovery from the acid load; $1.2 \times 10^{-7}$ M and $1.2 \times 10^{-6}$ M PTH provoked an identical reduction in proton efflux rate, i.e. these hormone concentrations apparently show the maximal effect. In other words, PTH given at maximal dose only leads to a partial inhibition (see below) of $Na^+/H^+$ exchange activity in OK cells. The resting $pH_i$ value was not influenced by PTH ($pH_i$ = 7.18 ± 0.02; $n$ = 3, data not shown).

Figure 2 shows a dose-response curve for PTH-dependent inhibiton of sodium-dependent $pH_i$ recovery from an acid load. The rate of $pH_i$ recovery ($\Delta pH_i$/min) in the presence of 140 mM sodium and in the absence of PTH was for each individual experiment assumed to correspond to maximal activity (100%) for the particular cell suspension. As can be seen, PTH concentrations of $10^{-9}$ M and higher lead to progressive inhibition of $Na^+/H^+$ exchange. At $1.2 \times 10^{-7}$ M PTH $Na^+/H^+$ exchange activity decreased to 50–70% of the value of cells without PTH. Higher concentrations of PTH ($1.2 \times 10^{-6}$ M) did not cause any further reduction in $pH_i$ recovery rate (Fig. 1).

Interestingly, the dose-response curve given in Fig. 2 for PTH inhibition of $Na^+/H^+$ exchange indicates a rather low sensitivity to PTH. Previous work on monolayer cultures measuring amiloride-sensitive $^{22}Na^+$ influx, however, provided evidence for a different PTH concentration-response relationship (25% inhibition of $Na^+$ uptake at $10^{-10}$ M PTH) [29]. Moreover, our previous work on PTH inhibition of $Na^+$/phosphate cotransport in monolayer cultures demonstrated half maximally inhibitory effects at $10^{-12}$ M to $10^{-11}$ M PTH [8, 9, 19, 30]. Compared to the findings on PTH sensitivity of the $Na^+/H^+$ exchange the observations on $Na^+$/phosphate co-transport are concordant with a high-affinity hormone interacting site. The alteration in activity of $Na^+/H^+$ exchange, however, may be related either to a different hormone interaction or may result from the different experimental system (OK cells in suspension) used for the study of $Na^+/H^+$ exchange activity. To investigate for a role of the experimental system in the discrep-

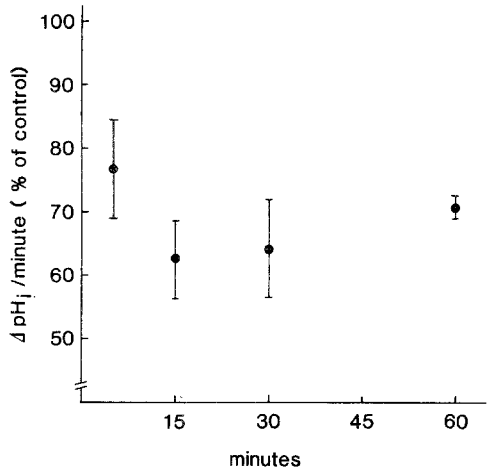


**Fig. 4.** Time course of the PTH inhibition of $Na^+/H^+$ exchange. Cellular $pH_i$ recovery from an acid laod was analysed exactly as described in Fig. 2. Cells were exposed during the indicated incubation periods and prior to the start of data collection to $1.2 \times 10^{-7}$ M PTH. Results are presented as a percentage of the respective control determination (from the same preparation and analysed in the same way). Each point is the mean ± SEM of five separate experiments

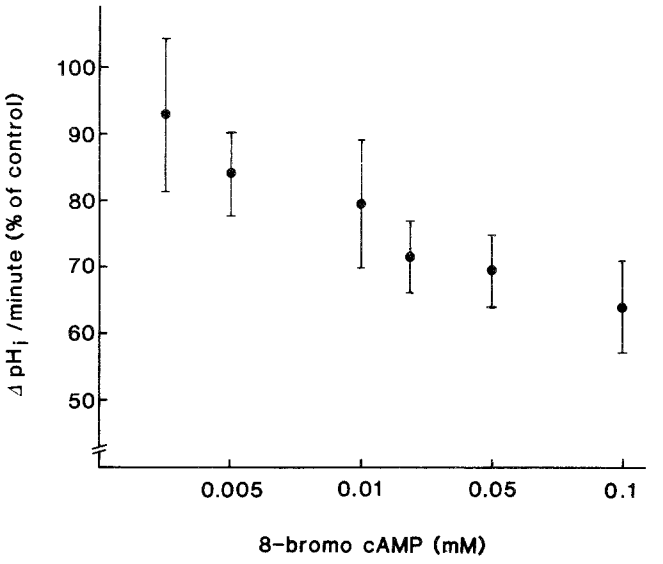


**Fig. 5.** Dose dependency of 8-bromo-cAMP on the $pH_i$ recovery from an acid load. Cellular $pH_i$ recovery from an acid load was analysed exactly as described in Fig. 2. Cells were exposed to the indicated concentrations of 8-bromo-cAMP for the last 15 min interval of dye loading. Values are mean ± SEM ($n = 4$)

ancy between hormone-induced changes in $Na^+/H^+$ exchange activity and $Na^+$/phosphate co-transport activity we have created dose-response curves for PTH inhibition of $Na^+$/phosphate co-transport in cell suspensions. To allow comparison of the PTH response we have attempted to measure $Na^+$/phosphate co-transport activity under closely similar experimental conditions as reported for $Na^+/H^+$ exchange activity. To exclude the possibility of receptor damage by trypsinization, we have compared the PTH response in cell suspensions obtained either by trypsinization (Fig. 3A) or by the EDTA/$Ca^{2+}$ chelation method (Fig. 3B). The data indicate that PTH affects $Na^+$/phosphate co-transport with an analogous potency as it reduces $Na^+/H^+$ exchange activity. The effect of PTH is independent of the method used to make the cellular suspension (trypsin versus EDTA/$Ca^{2+}$ chelation method). Thus, the low sensitivity to PTH inhibition of $Na^+/H^+$ exchange seems to be related to some unknown factors within our experimental protocol (cellular suspensions).

We have also analysed the time course of PTH inhibition of $Na^+/H^+$ exchange (Fig. 4). Maximal inhibition was achieved within 15 min. This "fast" inhibition is in marked contrast to inhibition of $Na^+$/phosphate co-transport, which requires 2–3 h for maximal effects to develop in both monolayers and cell suspensions [19, 20, 22].

*Intracellular mediation*

In view of PTH-dependent production of intracellular messenger (see above; [6, 8, 9, 14, 30, 31]), inhibition of $Na^+/H^+$ exchange can be related either to cAMP-dependent activation of kinase A, DAG-dependent activation of kinase C or to $Ca^{2+}$-calmodulin-dependent reactions.

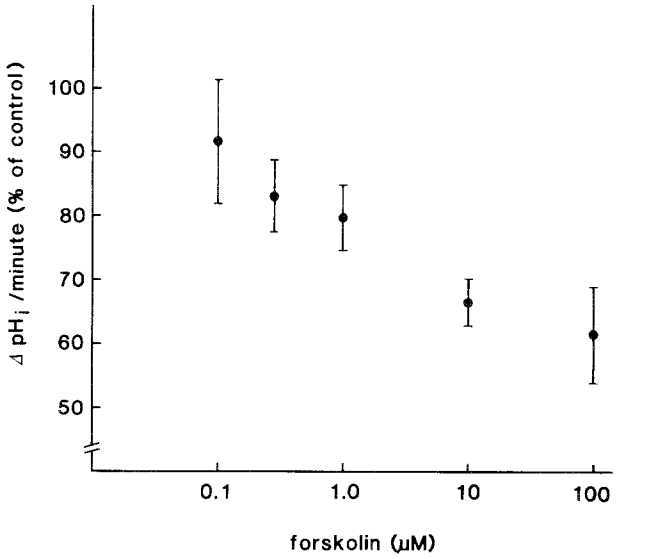


**Fig. 6.** Dose-response relationship between forskolin and $Na^+/H^+$ exchange activity. Suspended cells were preincubated for 15 min with varying concentrations of forskolin. $Na^+/H^+$ exchange activity was determined as indicated in Fig. 2. Values are mean ± SEM ($n = 4$)

The role of kinase A (cAMP) was evaluated by either 8-bromo-cAMP or forskolin, an activator of the catalytic subunit of the adenylate cyclase [18]. The dose-response relationship between 8-bromo-cAMP (after 15 min of exposure to the agonist) and $Na^+/H^+$ exchange activity is given in Fig. 5. A similar set of experiments is shown in Fig. 6 for forskolin. Maximal inhibition of $Na^+/H^+$ exchange occurred at agonist concentrations between 10 and 100 μM forskolin (Fig. 6) or 0.01–0.1 mM 8-bromo-

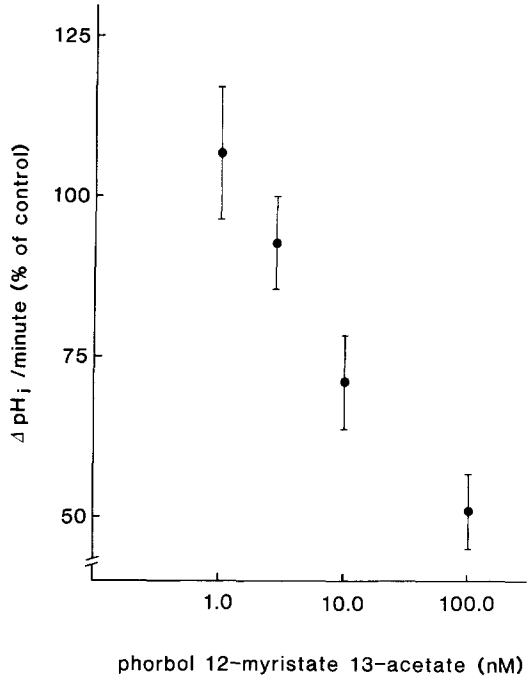


**Fig. 7.** Dose dependency of phorbol 12-myristate 13-acetate (TPA) on $pH_i$ recovery. Pretreatment of cells with increasing concentrations of TPA was carried out for 15 min prior to the determinations of $pH_i$ recovery. For further experimental details see Fig. 2. Values are mean ± SEM ($n = 4$)

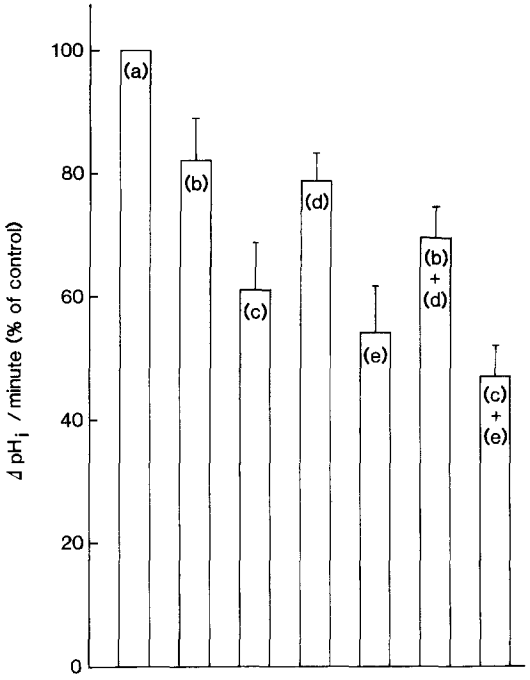


**Fig. 8.** Combined effects of forskolin and 4β-phorbol 12,13-dibutyrate (PDB) in both submaximal and maximal doses. Recovery from an acid load was examined as described in previous figures. Effectors were added to the incubation medium 15 min prior to the data collection. Results are expressed as a percentage of control values. The symbols ($a-e$) refer to the recovery rate of OK cells ($a$) in the absence of effectors, ($b$) in the presence of 0.5 μM forskolin, ($c$) in the presence of 50 μM forskolin; ($d$) in the presence of 10 nM PDB, ($e$) in the presence of 100 nM PDB, ($b + d$) in the presence of submaximal doses of forskolin and PDB, and ($c + e$) in the presence of maximal doses of forskolin and PDB

cAMP, respectively, where approximately 40% of the $Na^+/H^+$ exchange activity was inhibited (Fig. 5). Thus, a cAMP-dependent process (kinase A) has the capacity to reduce $Na^+/H^+$ exchange to a similar extent as observed with maximal PTH concentrations (Figs. 1, 2).

The role of kinase C was evaluated by applying different phorbol esters at different concentrations for 15 min prior to the analysis of $Na^+/H^+$ exchange activity. Phorbol esters are known activators of the kinase C regulatory pathway [5, 28]. As an example for these studies, the dose-response dependency of phorbol 12-myristate 13-acetate (TPA) on $pH_i$ recovery rate is shown in Fig. 7. After application of 100 nM TPA ~ 50% inhibition of $Na^+/H^+$ exchange occurred. Separate experiments (data not shown) documented that 100 nM TPA is a maximal concentration, i.e. the effect cannot be increased by increasing the phorbol ester concentration. Results similar to those reported for phorbol 12-myristate 13-acetate were obtained for 4β-phorbol 12,13-dibutyrate (data not shown; see Fig. 8). Furthermore, 4α-phorbol 12,13-didecanoate, which is reported to be inactive with respect to kinase C activation [5] had no effect on $Na^+/H^+$ exchange activity (data not shown).

The role of an increase in $Ca_i$ on inhibition of $Na^+/H^+$ exchange activity was evaluated in the following way (Table 1). First, we studied the effect of clamping $Ca_i$ by ionomycin in the presence of high extracellular $Ca^{2+}$. Ionomycin did not induce an inhibition of $Na^+/H^+$ exchange, nor did it prevent the effect of PTH on $Na^+/H^+$ exchange (Table 1A). $Ca_i$ rose by a factor of 10.5 and PTH did not alter $Ca_i$ under these conditions (Table 1A). Second, in cells loaded with 10 μM BAPTA, the PTH effect on $Na^+/H^+$ exchange was still observed (Table 1B). In BAPTA-loaded cells $Ca_i$ was lowered to 72% of the control value and addition of PTH failed to increase $Ca_i$ significantly (Table 1B). These studies indicate that transient elevations in $Ca_i$ are not necessary for PTH inhibition of $Na^+/H^+$ exchange.

To analyse for an interaction (synergism and/or additivity) between the two regulatory cascades (kinase A and kinase C) showing inhibitory potency, we have studied the combined effects of forskolin and 4β-phorbol 12,13-dibutyrate (Fig. 8) at intermediate as well as at maximal concentrations. Maximal concentrations of phorbol ester (100 nM) and forskolin (50 μM) lead to the previously observed maximal inhibition of $Na^+/H^+$ exchange of about 40–50%. Maximal concentrations of both agents applied together do not increase the inhibition of $Na^+/H^+$ exchange. Furthermore, inhibitory effects of both agonists at intermediate concentrations were not significantly different from the effects of either of the agonists examined alone. These data suggest that the two regulatory cascades are most likely operating independent of each other to inhibit an identical "population" of $Na^+/H^+$ exchangers, which is responsible for about 40–50% of the total $Na^+/H^+$ exchange capacity of the OK cell plasma membrane. The lack of a synergistic effect of forskolin and 4β-phorbol 12,13-dibutyrate on $Na^+/H^+$ exchange, when combined at submaximal concen-

**Table 1.** Correlation between $Ca_i$ and $Na^+/H^+$ exchange activity
**A** Effect of ionomycin and/or PTH on $Ca_i$ and on rate of $pH_i$ recovery from an acid load

| | $\Delta pH_i/dt$ | $Ca_i$ |
|---|---|---|
| | (% of control) | |
| Control | 100 | 100 |
| Ionomycin (5 μM) | 128.7 ± 8.5 | 1048.8 |
| PTH ($1.2\times10^{-9}$ M) | 81.0 ± 7.8 | ND |
| PTH ($1.2\times10^{-7}$ M) | 66.3 ± 8.1 | 155.5 |
| Ionomycin (5 μM) plus PTH ($1.2\times10^{-9}$ M) | 87.0 ± 6.5 | ND |
| Ionomycin (5 μM) plus PTH ($1.2\times10^{-7}$ M) | 68.3 ± 8.4 | 1048.8 |

**B** Effect of BAPTA on $Ca_i$ and $pH_i$ recovery in the presence and absence of PTH

| | | |
|---|---|---|
| Control | 100 | 100 |
| BAPTA (10 μM) | 96.6 | 72.7 |
| PTH ($1.2\times10^{-7}$ M) | 50.0 | 162.9 |
| BAPTA (10 μM) plus PTH ($1.2\times10^{-7}$ M) | 63.2 | 72.7 |

ND = Not determined
OK cells were loaded with 0.41 μM BCECF, 1.25 μM fura, and 10 μM BAPTA as described under Materials and methods. Analysis of the cellular $pH_i$ recovery from an acid load was performed as described in Fig. 2. The acid load was imposed by incubation with 20 mM $NH_4Cl$ during the last 15-min interval of dye-loading. Various effectors were added to the incubation media 15 min prior to the start of data collection. The initial rate of $pH_i$ recovery was measured. Results are expressed as a percentage of control values. $pH_i$ recovery rates under control conditions were 0.101 ± 0.006 (Table 1 A; $n = 5$) and 0.118 pH/min (Table 1 B, mean of triplicate assays in one experiment). Analysis of $Ca_i$ was performed as described under Materials and methods. Cells were washed and resuspended in Na-medium containing 1.0 mM $Ca^{2+}$. Changes in $Ca_i$ in response to $1.2\times10^{-7}$ M PTH or 5 μM ionomycin were determined at the peak with the 340/380 nm ratio technique. Values given in Table 1 A and B are means of triplicate assays in one experiment, and are expressed as a percentage of the respective control values. The basal $Ca_i$ of OK cells used for the experiments given in Table 1 A and B was 200.7 nM and 186.1 nM, respectively

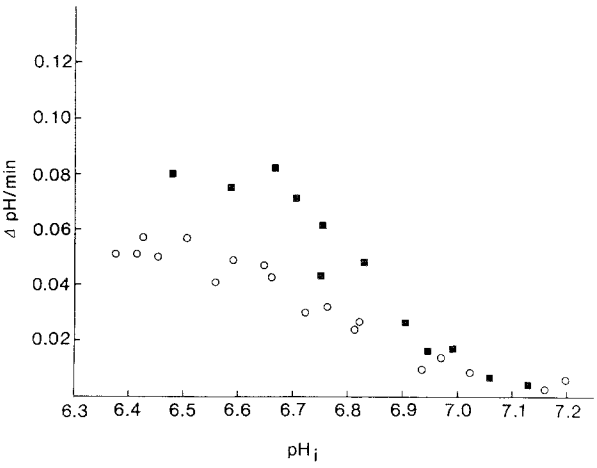


**Fig. 9.** Effect of PTH on the $pH_i$ sensitivity of $Na^+/H^+$ exchange. Cells were exposed to $1.2\times10^{-7}$ M PTH as described. Variations in the magnitude of the initial acid load were imposed by incubation with 5–20 mM $NH_4Cl$ during the last 15-min interval of dye-loading. Acid-loaded cells were examined for the rate of $pH_i$ recovery, as specified in Fig. 2. Data points represent values of four independent experiments ■ = control, ○ = PTH, $1.2\times10^{-7}$ M

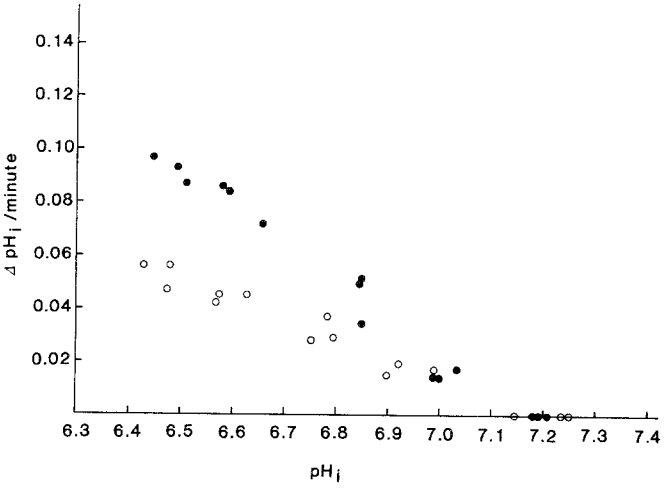


**Fig. 10.** Effect of forskolin on the $pH_i$ sensitivity of $Na^+/H^+$ exchange. 50 μM forskolin was present in the incubation media for 15 min before performing a study. The rate $pH_i$ recovery was measured exactly as reported in the legend to Fig. 9. Data were collected from three individual experiments. ● = control; ○ = forskolin, 50 μM

trations, suggests that the regulatory cascades of kinase A and kinase C operate independently (with no detectable interaction) to inhibit $Na^+/H^+$ exchange. Theoretically, submaximal concentrations should at least result in an additive effect if the two cascades are separate. It should be mentioned, however, that such additive effects are difficult to detect because we are working on the slopes of two dose-response relationships. This experimental difficulty is even increased by the experimental scatter between individual experimental runs; certainly the quality of the data seems to permit the conclusion that there is no "cross-talk" between the two regulatory cascades, i.e. the partial activation of one is unable to potentiate the inhibitory action of the second regulatory cascade.

### *Effects on $pH_i$ sensitivity of $Na^+/H^+$ exchange*

In non-polarized cells, a variety of agents are known to activate $Na^+/H^+$ exchange by shifting the set point towards more alkaline values. Since these agents alter intracellular proton sensitivity of the $Na^+/H^+$ exchanger, an effect of the respective agent on resting $pH_i$ (especially in $HCO_3^-$-free media) would be expected (for review see [13]). Our results from fluorometric measurements of $pH_i$, however, demonstrate that neither of the agonists tested (PTH, phorbol esters, forskolin, cAMP; see above and data not shown) had an influence on resting $pH_i$ in OK cells, suggesting that mechanisms other than a change in set point contribute to the observed inhibition of $Na^+/H^+$ exchange by PTH. This conclusion was directly supported by the analysis of the $pH_i$ sensitivity of $Na^+/H^+$ exchange under three conditions: (a) in the presence and absence of PTH, (b) in the presence and absence of

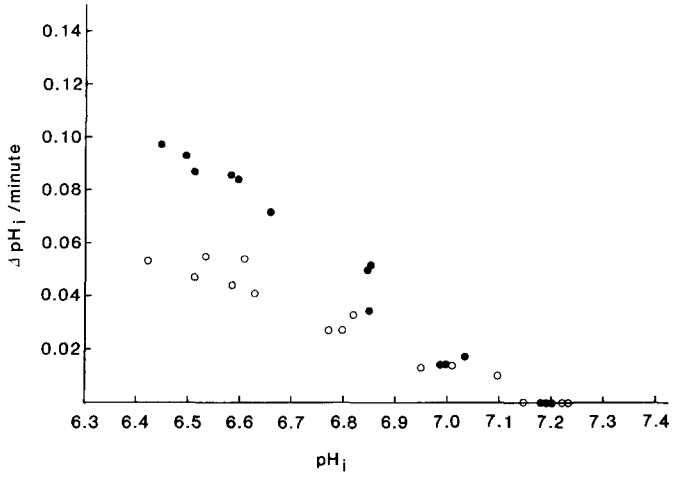


**Fig. 11.** Effect of 8-bromo-cyclic AMP on the $pH_i$ sensitivity of $Na^+/H^+$ exchange. Details of the experimental procedure are specified in Fig. 9. OK cells were preincubated with 0.1 mM 8-bromo-cAMP for 15 min. Data points represent values of three independent experiments. ● = control; ○ = 8-bromo-cyclic AMP, 0.1 mM

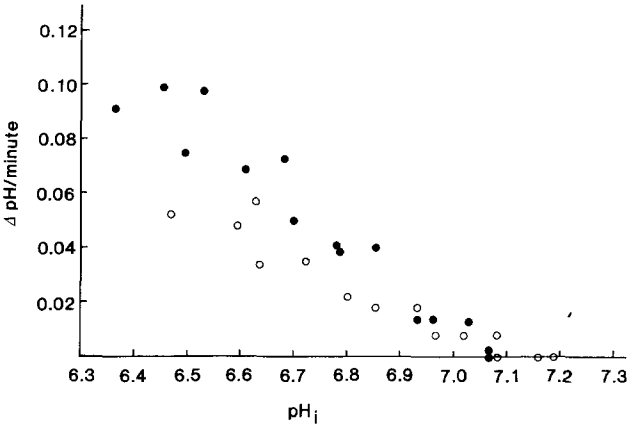


**Fig. 12.** Effect of phorbol 12-myristate 13-acetate (TPA) on the $pH_i$ sensitivity of $Na^+/H^+$ exchange. Initial rates of cellular $pH_i$ recovery from different $pH_i$ values were measured as detailed in Fig. 9. TPA (100 nM) was present in the incubation media for 15 min before performing a study. Data points represent values of three independent experiments. ● = control; ○ = phorbol 12-myristate 13-acetate, 100 nM

forskolin or 8-bromo-cyclic AMP, and (c) in the presence and absence of phorbol 12-myristate 13-acetate. Figures 9–12 summarize these results; the data strongly suggest that the change in activity of $Na^+/H^+$ exchange with different agonists is not due to a shift in the set point. As the intracellular buffer capacity is increased by lowering intracellular pH from 7.2 to 6.5 (more than 2-fold; D. Krayer and H. Murer, unpublished observation), the slopes of the $pH_i$-dependence (Figs. 9–12) represent significant underestimates of the actual alterations in transport rates.

## Discussion

The data presented demonstrate that PTH inhibits $Na^+/H^+$ exchange in OK cells, a proximal tubular like cell line. In this cell line PTH is known to increase cAMP, diacylglycerol, $IP_3$ and $Ca_i$. In the present study we have demonstrated that an increase in cAMP as well as in DAG is of functional importance in controlling $Na^+/H^+$ exchange. We found that pharmacological activation of kinase A (forskolin, 8-bromo-cAMP) as well as pharmacological activation of kinase C (phorbol esters) led to an inhibition of $Na^+/H^+$ exchange with maximal effects similar to that observed for PTH, which decreases total $Na^+/H^+$ exchange capacity of the OK cell plasma membrane up to 50%. Since we observed no synergistic or additive effects when the combined effect of agonists was studied, we conclude that the regulatory cascades of kinase A and C are operating independently of each other. Potentially, these cascades could act at the same final target site. According to our findings, it seems likely that an increase of $Ca_i$ is not directly involved in PTH-dependent control of $Na^+/H^+$ exchange activity. Finally, we have shown that inhibition of $Na^+/H^+$ exchange is not related to a shift in set point.

Since in the present study, $Na^+/H^+$ exchange activity with or without agonists was generally analysed at 140 mM NaCl, i.e. $V_{max}$ concentration, ($K_t$ $Na^+$ = 35 mM; Montrose and Murer, to be published), and at a pH of 7.4 in the external medium (cuvette solution), as well as at identical initial $pH_i$ values ($pH_i = 6.62 \pm 0.08$, for the experiments presented in Figs. 2–8; $n = 13$), effects due to different transmembrane proton concentration differences can be excluded. Thus, the reduced $Na^+/H^+$ exchange activity observed in all the experimental conditions above can be related either to a decrease in the $V_{max}$ of the $Na^+/H^+$ exchanger or to a decrease in the affinity for protons at the internal membrane surface [24]. Our data do not permit to distinguish clearly between these possibilities. The fact, however, that the agonist-induced changes, when expressed in relative terms (rate of inhibition of $pH_i$ recovery relative to the rate without any agonist at any given $pH_i$), are similar at the lowest $pH_i$ values and at intermediate $pH_i$ (Figs. 9–12) supports the hypothesis that the action of agonists on $Na^+/H^+$ exchange may be via an alteration in $V_{max}$ of the transporter rather than a change in affinity for protons at the cytoplasmic membrane surface.

The response of OK cells to PTH reported here is in agreement with the PTH-dependent inhibition of $Na^+/H^+$ exchange in brush border membranes of proximal tubule [15] and has been described previously in OK cell monolayers and suspensions [7, 24, 26, 29]. The effect of PTH is mimicked by cAMP and cAMP analogues and explains the regulation of proximal tubular bicarbonate reabsorption by these agonists (for review see [2]). With respect to activation of kinase C by phorbol esters and inhibition of $Na^+/H^+$ exchange, our results are in contrast with those reported for $Na^+/H^+$ exchange in nonpolarized cells, where kinase C activation results in a stimulation of $Na^+/H^+$ exchange (for review see [13]). It should be noted that in the cell system we used, down-regulation of protein kinase C by phorbol ester does not

provide an explanation for the observed reduction of $Na^+/H^+$ exchange seen with TPA, since down-regulation of protein kinase C in the OK cell line occurs after incubation times much longer than those used in the present studies [32]. Furthermore, the phorbol-ester-dependent inhibition of $Na^+/H^+$ exchange was observed after incubation with the $\beta$-analogue but not with the $\alpha$-analogue, which is in full agreement with known properties of kinase C action. With respect to effects of kinase C activation on $Na^+/H^+$ exchange located in the apical membrane of epithelial cells, our results are in agreement with those obtained for $Na^+/H^+$ exchange in proximal colon [1] and in descending colon [10]. In the isolated perfused tubule (rabbit, convoluted tubule) phorbol esters also inhibit volume reabsorption, sodium bicarbonate and sodium chloride reabsorption, which are all functionally related to the apical $Na^+/H^+$ exchange [3]. However, our results are in contrast with recent studies by Weinman and coworkers, who showed kinase-C-dependent activation of the renal proximal tubular $Na^+/H^+$ exchange in transiently opened brush border membranes (less than 15% stimulation at 1000 nM TPA) [36] or in reconstituted renal proximal tubular $Na^+/H^+$ exchange activity (less than 40% stimulation at 1000 nM TPA [37, 38]. Mellas and Hammerman [23] reported alkalinization of cytosolic pH after incubation of isolated canine proximal tubule segments with phorbol esters, consistent with an activation of $Na^+/H^+$ exchange. We have no explanations for the discrepancies between these latter results [23, 36–38] and those obtained in the intact perfused tubules [3] or in the OK cells as presented in this study.

In some tissues protein kinase C activation has been reported to enhance cAMP production in response to agonists [16]. In the OK cell line such an effect of phorbol ester is unlikely. In our previous studies on TPA effects on cAMP levels in OK cells we have shown that 100 nM TPA (equivalent to the highest concentration of phorbol ester used in the present study) increases cellular cAMP content in the presence of a phosphodiesterase inhibitor by a factor of 4 compared to a 50-fold increase seen with maximal PTH concentrations [22]. Furthermore, the effect of TPA to augment cAMP content was entirely dependent on the presence of the phosphodiesterase inhibitor (unpublished observations), whereas PTH or forskolin induced an increase of the cAMP level even in the absence of the phosphodiesterase inhibitor [19]. According to these results it is rather unlikely that phorbol esters exert their effects on $Na^+/H^+$ exchange through an enhancement of cAMP production.

One could speculate that in our cell system activation of a regulatory cascade might have effects on some alternate $pH_i$ recovery mechanisms. A possible contribution of a $pH_i$ recovery mechanism other than the $Na^+/H^+$ exchange to the observed inhibition of $pH_i$ recovery rate in response to TPA, however, seems to be unlikely, since the experiments reported in our study were performed in the nominal absence of $HCO_3^-$. Evidence for $Na^+/H^+$ exchange to be the important $pH_i$-regulating mechanism in our system is further brought about by the fact that $pH_i$ recovery is completely dependent on $Na^+$ (no recovery in TMACl-medium, Fig. 1 A). In addition, the recovery rate with 20 mM $Na^+$ ceases in the presence of 10 μM EIPA (Fig. 1 B). Furthermore, microfluorometric studies (single cell analysis in monolayer configuration) on effects of TPA or forskolin on $pH_i$ recovery in the absence of sodium revealed that neither of the compounds alters the degree of cellular acidification or the "proton leak" measured under sodium-free conditions (Helmle-Kolb, Montrose and Murer, to be published). Taken together these data confirm that the reported inhibition of $pH_i$ recovery by phorbol ester and forskolin or 8-bromo-cAMP is mainly due to an inhibition of $Na^+/H^+$ exchange by either of the effectors.

In summary, $Na^+/H^+$ exchange in OK cells is inhibited by PTH. This inhibition can be mimicked by pharmacological activation of kinase A or kinase C, respectively. The preferential use of either the kinase A or kinase C regulatory cascade in mediating PTH control of $Na^+/H^+$ exchange activity is given by the PTH-dependent liberation of intracellular mediators, such as cAMP and DAG. Previously, we and others have suggested a preferential liberation of the phospholipase C related mediators at low physiological PTH concentrations [30].

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