Biochem. J. (1988) 251, 207–213 (Printed in Great Britain)

Intracellular cascades in the parathyroid-hormone-dependent regulation of Na^+/phosphate cotransport in OK cells

Kerstin MALMSTRÖM,* Gerti STANGE and Heini MURER†
Department of Physiology, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

Parathyroid hormone (PTH) increased intracellular cyclic AMP and reduces Na^+/phosphate cotransport activity in OK cells [Malmström & Murer (1986) Am. J. Physiol. **251**, C23–C31; Caverzasio, Rizzoli & Bonjour (1986) J. Biol. Chem. **261**, 3233–3237]. It was also shown that PTH activates phosphoinositide metabolism in OK cells [Hruska, Moskowitz, Esprit, Civitelli, Westbrook & Huskey (1987) J. Clin. Invest. **79**, 230–239]. In the present paper we show that tumour-promoting phorbol esters are effective in reducing Na^+/phosphate cotransport. The Ca^{2+} ionophores A23187 and ionomycin had only a small effect on Na^+/phosphate cotransport; added together, A23187 and phorbol esters showed a synergistic action. Phorbol esters and phorbol esters plus ionomycin stimulated prostaglandin synthesis as well as cyclic AMP production; acetylsalicylic acid prevented phorbol-ester-induced prostaglandin synthesis and cyclic AMP production, but had no effect on inhibition of Na^+/phosphate cotransport. In suspensions of OK cells, PTH and thrombin produced a rise in intracellular Ca^{2+}. In contrast with PTH, thrombin did not elevate cellular cyclic AMP in suspended OK cells. PTH and thrombin reduced Na^+/phosphate cotransport in suspended OK cells. It is suggested that two regulatory cascades are involved in PTH action on Na^+/phosphate cotransport: cyclic AMP/kinase A and Ca^{2+}/diacylglycerol/kinase C.

INTRODUCTION

Recently, we and others have shown that parathyroid hormone (PTH) induces a concentration- and time-dependent inhibition of Na^+/phosphate cotransport in an established kidney cell line derived from an American opossum (OK). PTH was found to act specifically on Na^+/phosphate cotransport without affecting other Na^+/cotransport systems, such as Na^+-coupled hexose or neutral amino acid transport. Evidence was presented for an involvement of cyclic AMP/kinase A in the regulation of Na^+/phosphate cotransport in OK cells (Malmström & Murer, 1986*b*; Caverzasio *et al.*, 1986). Recently, Hruska *et al.* (1987) showed that PTH activates phosphoinositide metabolism in OK cells, in primary cultures of proximal tubular cells and in basolateral membranes from canine proximal tubular segments. Therefore, PTH activates two intracellular messenger systems. Accordingly, it was of interest to analyse the importance of the Ca^{2+}/diacylglycerol (DAG) protein kinase C pathway in the regulation of the Na^+/phosphate cotransport. In a preliminary report, we have described that various tumour-promoting phorbol esters also reduce Na^+/phosphate cotransport (Malmström & Murer, 1986*a*), and after the present manuscript was submitted two reports on TPA-induced inhibition of Na^+/phosphate cotransport in OK cells appeared (Nakai *et al.*, 1987; Cole *et al.*, 1987). In the present study we have extended these observations and have established in more detail the role of the protein kinase C regulatory pathway in the regulation of Na^+/phosphate cotransport.

EXPERIMENTAL

Materials

All cell culture supplies were purchased from Amimed, Basle, Switzerland. Synthetic bovine parathyroid hormone [bPTH-(1–34)] was obtained from Bachem, Basle, Switzerland; radiolabelled material was from New England Nuclear; phorbol esters and A23187 were from Sigma; thrombin and ionomycin were from Calbiochem; H7 was from Seikaguka Kogyo Co. and 1-oleoyl-2-acetylglycerol (OAG) was from Molecular Probes, Junction City, OR, U.S.A.

Cell culture

OK cells were maintained in serial culture using Dulbecco's modification of Eagle's Minimum Essential Medium (DMEM) and Hams F12 (1:1) supplemented with 2 mM-glutamine, 50 i.u. of penicillin/ml, 50 μg of streptomycin/ml and 10% (v/v) foetal calf serum. The buffer system included 22 mM-$NaHCO_3$ and 20 mM-Hepes and the cultures were kept at 37 °C in a humidified atmosphere of CO_2/air (1:19). Cells between passage numbers 80 and 100 were used and they were subcultured using 0.1% trypsin and 0.5 mM-EDTA in $Ca^{2+}+Mg^{2+}$-free phosphate-buffered saline. For experimental purposes cells were plated at a density of $(1–2)\times10^5$ cells/2 ml in 35 mm diameter Petri dishes or $(1–2)\times10^6$ cells/16 ml in 100 mm diameter Petri dishes (NUNC, Roskilde, Denmark). Confluency was reached after 4 days and the monolayers were used routinely after 5 days. At 15–20 h

Abbreviations used: PTH, parathyroid hormone; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; H7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride: IBMX, 3-isobutyl-1-methylxanthine; DAG, diacylglycerol; OAG, 1-oleoyl-2-acetylglycerol; BSA, bovine serum albumin; IP_3, inositol trisphosphate.

* Present address: Department of Biology, MIT, Cambridge, MA 02139, U.S.A.
† To whom correspondence and reprint request should be addressed.

before the experiment, the growth medium was changed to serum-free bicarbonate-free medium containing 0.1% bovine serum albumin (BSA). BSA was omitted in the experiments with Ca^{2+} ionophores. All additions were made as portions directly to the medium. The incubations were carried out at 37 °C.

Cell suspension

The cell monolayers were disrupted into single cells by using the Ca^{2+}-chelator EDTA. Briefly, monolayers grown in 100 mm diameter Petri dishes were rinsed twice with phosphate-buffered saline, and 2.5 ml of 5 mM-EDTA/phosphate-buffered saline was added to the monolayers kept at 37 °C for 12 min. Subsequently, 10 ml of either a salt solution (A) containing, in mM: NaCl, 130; KCl, 4; $CaCl_2$, 1; $MgSO_4$, 1; glucose, 18; Hepes, 20; NaH_2PO_4, 1; pH 7.4 or DMEM/Hams F12 (1:1)/20 mM-Hepes/0.1% BSA was added and the rounded cells were suspended into single cells with agitation using a syringe with a large-bore needle. After washing the cells twice with the suspension solution, they were suspended to (1–2) $\times 10^6$ cells/ml in the appropriate experimental solution.

Transport studies

Monolayers. Transport of solutes was determined as described earlier (Malmström & Murer, 1986*b*). Briefly, the medium was aspirated off and the monolayer was rinsed once with a substrate-free 'uptake' medium containing, in mM: NaCl (or *N*-methyl-D-glucamine hydrochloride), 137; KCl, 5.4; $CaCl_2$, 2.8; $MgSO_4$, 1.2; Hepes/Tris, 10; pH 7.4. The complete 'uptake' medium contained also a substrate, either 0.1 mM-$KH_2{}^{32}PO_4$ (0.5 μCi/ml) or 0.1 mM-[^{14}C]α-methyl D-glucopyranoside (0.4 μCi/ml). After a predetermined time the uptake was stopped by aspirating off the 'uptake' medium, washing the monolayers four times with an ice-cold stop solution containing 137 mM-NaCl and 14 mM-Tris/HCl, pH 7.4. Subsequently, the monolayer was solubilized in 1 ml of 0.5% Triton X-100 and aliquots of 150 or 300 μl were used for determining the accumulated substrate by liquid-scintillation counting. The Na^+-independent phosphate uptake in OK cells is less than 1% of the uptake in presence of Na^+ and can thus be neglected (Malmström & Murer, 1986*b*). Accordingly, the reported Na^+/phosphate cotransport is the uptake measured in a Na^+-containing medium.

Suspension. The suspended cells were left to recover in DMEM/Hams F12 (1:1)/20 mM-Hepes/0.1% BSA for 3 h before adding either PTH or thrombin. The phosphate uptake was determined in the 'uptake' medium (see above for monolayers) supplemented with 0.1 mM radioactively labelled phosphate. The reaction (50 μl of cell suspension and 50 μl of medium) was stopped by adding 1 ml of ice-cold stop solution (see above) and trapping the cells on Sartorious filters (0.65 μM). The filters were washed with 4 ml of ice-cold stop solution and the retained radioactivity was determined by liquid-scintillation counting.

Intracellular Ca^{2+} determination

Cells (2×10^6) were suspended in 2 ml of salt solution A (see above) containing 1.25 μM of the acetoxymethyl ester of Fura-2 (Fura-2/AM). After a 20–30 min loading time at 37 °C, the cells were collected by centrifugation (12000 rev/min for 5 s in an Eppendorf centrifuge), resuspended in 50 μl of salt solution A and added to 1.95 ml of prewarmed salt solution A. The cells were kept in suspension by continuous stirring. Changes in fluorescence were measured in two consecutive runs (excitation 340 nm, emission 505 nm, and excitation 380 nm, emission 505 nm) in an Schimadzu RF 510 spectrofluorimeter (slit 3 nm for excitation and 10 nm for emission). The free Ca^{2+} concentration was calculated by using the ratio method as described by Grynkiewicz *et al.* (1985) and using dye released from digitonin-treated cells to determine the minimum ($R_{min.}$) and maximum ($R_{max.}$) fluorescence ratios. In time-course experiments on hormonal responses (Fig. 1), free Ca^{2+} concentrations could be calculated from the fluorescence ratios (F_{340}/F_{380}) as obtained in two consecutive runs (340 nm/380 nm excitation).

Cyclic AMP determination

Cell monolayers that had been treated with different agents were rinsed twice with an ice-cold buffer containing, in mM: Tris/HCl, 50; β-mercaptoethanol, 16; theophylline, 8; pH 7.4. The reaction was stopped by dropping the Petri dishes directly into liquid N_2. The monolayers were kept at −80 °C until cyclic AMP was determined. The cells were scraped into 0.55 ml of the above buffer containing 0.1 mM-HCl, sonicated, neutralized with 50 μmol of NaOH and the disrupted cells were removed by a short centrifugation step (12000 rev./min for 5 s in an Eppendorf centrifuge). Cyclic AMP was measured in aliquots of the supernatant using a competitive protein binding assay as described by Brown *et al.* (1972). For cell suspensions a similar method was used. Cells were left to recover in DMEM/Hams F12 (1:1)/20 mM-Hepes/0.1% BSA for 3 h and the cells suspended in buffer A were incubated with the different agents. Subsequently, the cells were sedimented (12000 rev./min for 5s in an Eppendorf centrifuge) and the cyclic AMP was determined as described above.

Prostaglandin E_2 determination

Prostaglandin E_2 production was determined in the medium of treated cell monolayers by using a radioimmunoassay (New England Nuclear).

Protein determination

Protein content was determined by a modified Lowry method (Dulley & Grieve, 1975).

RESULTS

Effect of PTH and thrombin on intracellular Ca^{2+} concentration

The effect of PTH on the intracellular Ca^{2+} concentration was measured in suspended cells loaded with Fura-2. Fig. 1 shows a typical trace at 340 nm excitation and 505 nm emission. Immediately after exposing the suspended cells to PTH the Ca^{2+} concentration increased 3–4-fold. The change was transient and within 45 s the original Ca^{2+} level or a somewhat lower level was resumed. When PTH was added to cells suspended in a Ca^{2+}-free medium, a similar change of intracellular Ca^{2+} was found (results not shown), suggesting that PTH released Ca^{2+} from an intracellular store. Hruska *et al.* (1987) reported similar changes in the intracellular Ca^{2+}

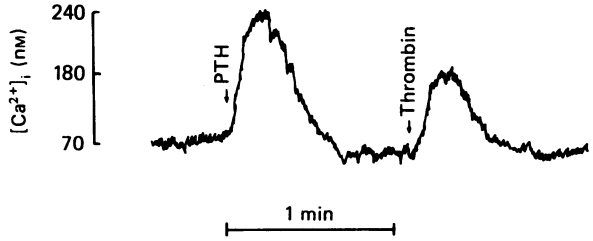

Fig. 1. Intracellular Ca^{2+} level in OK cells

The intracellular Ca^{2+} concentration was measured in suspended cells, which had been loaded with 1.25 μM of the acetoxymethylester of Fura-2 for 20–30 min at 37 °C. Treatments: 10^{-7} M-PTH; 2.5 units of thrombin/ml. The trace shown is the fluorescence changes at 340 nm excitation and 505 nm emission.

concentration and they also measured changes in the diacylglycerol (DAG) and inositol trisphosphate (IP_3) levels after exposure to PTH. PTH thus activates phosphoinositide metabolism in OK cells.

Fig. 1 shows also that addition of thrombin caused a transient increase in cytosolic Ca^{2+} in suspended OK cells. This increase in intracellular Ca^{2+} was also observed in the absence of extracellular Ca^{2+}(results not shown). Thrombin is known to stimulate phosphoinositide metabolism in many cell types (Berridge & Irvine, 1984; Nishizuka, 1984) and can, therefore, be used to evaluate further the role of Ca^{2+} and protein kinase C in regulation of Na^+/phosphate cotransport in OK cells.

Effect of thrombin and OAG on Na^+/phosphate cotransport

Since thrombin affected the intracellular Ca^{2+} levels in a similar way to PTH (Fig. 1) we pursued the experiments by looking at its effect on Na^+/phosphate cotransport. When analysed in monolayers, thrombin was unable to reduce Na^+/phosphate cotransport (Table 1). The synthetic diacylglycerol OAG, which directly activates protein kinase C (Nishizuka *et al.*, 1984; Castagna *et al.*, 1982), reduced the transport rate in monolayers; at the highest concentrations used a significant reduction was observed (100 μM) (Table 1).

The increases of intracellular Ca^{2+} concentration by PTH and thrombin (Fig. 1) were measured in suspended OK cells. Therefore, the lack of an effect of thrombin on Na^+/phosphate cotransport in monolayers could be due to an inaccessibility of thrombin to its site of action. We have therefore performed transport studies using suspended cells (Fig. 2). After suspending the cells by the Ca^{2+}-chelation method we left the cells to recover for 3 h in serum-free culture medium. As with uptake in OK cell monolayers (Malmström & Murer, 1986*b*), the phosphate influx into suspended cells was entirely Na^+-dependent (results not shown), and the initial influx rate (5 min) was constant when analysed after 3, 4 or 5 h of recovery and about twice the influx rate as observed without recovery (Fig. 2). The Na^+-dependent influx rate in suspended OK cells was about 1 nmol/5 min per mg, protein, i.e. much smaller than in the monolayer experiments. This apparent low transport activity in suspended cells can either be related to the presence of a significant amount of cell debris in suspension, and/or to incomplete separation of cells by the filtration procedure,

Table 1. Effect of thrombin and OAG on Na^+/phosphate cotransport

Cell monolayers were incubated with different concentrations of thrombin or OAG for 2 h prior to determining the Na^+/phosphate cotransport. The data are means ± S.D. for the numbers of experiments shown in parentheses: $*P > 0.5$, $**P > 0.2$, $***P > 0.001$.

Condition	Phosphate uptake (nmol/3 min per mg of protein)
No addition	6.3±0.8
Thrombin	
0.8 unit/ml (3)	6.9±0.5
2.5 units/ml (3)	7.5±0.1
No addition (6)	9.7±1.5
OAG	
5 μM (6)	9.4±1.8*
10 μM (6)	9.4±1.8*
50 μM (6)	8.7±1.5**
100 μM (6)	8.3±0.9***

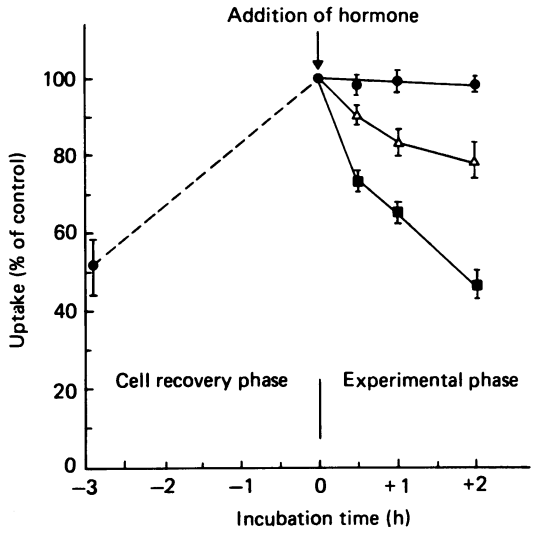

Fig. 2. Effect of PTH and thrombin on Na^+/phosphate cotransport in OK cells kept in suspension

Prior to the addition of the hormone the cells were allowed to recover for 3 h from the 'stress' exerted by bringing cells in suspension (cell recovery phase). At time zero an aliquot of buffer (control, ●) or PTH (■, final concn. 10^{-7} M) or thrombin (△, final concn. 2.5 units/ml) was added and Na^+/phosphate cotransport activity was measured at the times indicated (experimental phase). For further details see the text. The data are means ± S.E.M. for four determinations.

and/or to cell damage during the filtration step. PTH or thrombin was added after 3 h of recovery and, 30 min later, Na^+-dependent phosphate transport was reduced by 24±7% ($n = 4$) by PTH and 10±3% ($n = 4$) by thrombin. After 1 h treatment, the reduction was 32±6% for PTH and 20±7% for thrombin; after 2 h, inhibition was 55±7% and 22±9% respectively (Fig. 2). These observations suggest that thrombin can inhibit phosphate transport.

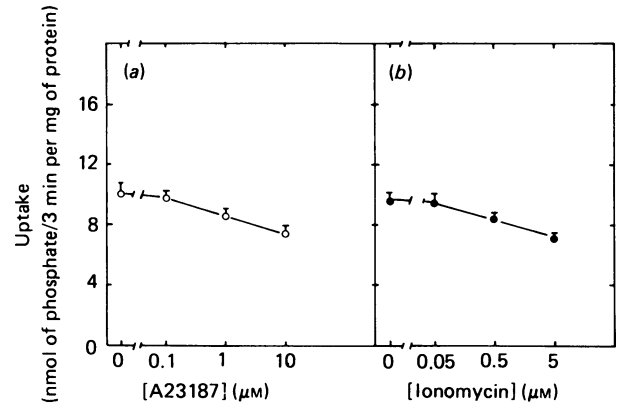

Fig. 3. Effect of Ca^{2+} ionophores on Na^+/phosphate cotransport

Cell monolayers were incubated with various concentrations of (*a*) A23187 and (*b*) ionomycin for 4 h prior to determining the phosphate uptake. The data are means ± S.D. for six determinations.

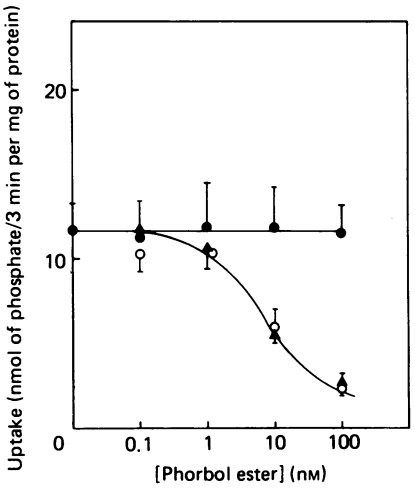

Fig. 4. Effect of phorbol esters on Na^+/phosphate cotransport

Cell monolayers were incubated with various concentrations of phorbol esters for 2 h prior to determining the phosphate uptake: ●, 4 α-phorbol 12,13-didecanoate; ○, 4 β-phorbol 12,13-didecanoate; ▲, TPA. The data are means ± S.D. for six determinations.

Effect of Ca^{2+} ionophores and phorbol esters on Na^+/phosphate cotransport

The intracellular Ca^{2+} concentration can be transiently increased by exposing the cells to a low concentration of Ca^{2+} ionophores such as A23187 or ionomycin (e.g. at ionomycin concentrations between 0.5 and 1 μM the intracellular Ca^{2+} concentration in suspended OK cells rose to values between 500 and 600 nM and returned to values slightly above basal values within 2–3 min; results not shown). Na^+/phosphate cotransport was determined in cell monolayers which had been incubated with different concentrations of ionomycin (5×10^{-9}–5×10^{-6} M) and of A23187 (10^{-7}–10^{-5} M) for 2 h. Both drugs inhibited Na^+/phosphate cotransport in a concentration-dependent manner (Fig. 3). However, the degree of inhibition provoked by the Ca^{2+} ionophores was small (11% after 2 h using the largest concentrations, 5×10^{-6}M-ionomycin or 10^{-5} M-A23187) as compared with the inhibition caused by PTH (30–40% after 2 h using 10^{-10} M-PTH; Malmström & Murer, 1986*b*). We have also analysed for an effect of the ionophores at various concentrations after shorter exposure times (2, 5, 15, 30 and 60 min) and did not find inhibitions larger than that observed in Fig. 3 (results not shown).

Table 2. Synergistic action of ionomycin and phorbol ester on Na^+/phosphate and Na^+/α-methyl D-glucopyranoside cotransport

Cell monolayers were incubated with 5×10^{-7} M-ionomycin, 5×10^{-8} M-4β-phorbol 12,13-dibutyrate (PBu), or the two drugs combined, for 2 h prior to determining the phosphate uptake. The data are means ± S.D. for the numbers of experiments shown in parentheses: $*P > 0.5$.

Condition	Uptake (nmol/3 min per mg of protein)	
	Phosphate	α-Methyl glucoside
No addition (6)	8.4±0.7	1.05±0.21
Ionomycin (6)	7.9±0.7	1.02±0.35*
PBu (6)	7.1±0.7	1.03±0.34*
Ionomycin+PBu (6)	4.8±0.4	0.93±0.32*

Incubation of OK cell monolayers with TPA for 2 h decreased phosphate uptake in a concentration-dependent way. The inhibition was evident at concentrations larger than 10^{-9} M and the maximal response was seen at 10^{-7} M-TPA (Fig. 4). 4β-Phorbol 12,13-didecanoate, a phorbol ester which expresses similar tumour-promoting activity (Castagna *et al.*, 1982; Nishizuka *et al.*, 1984) inhibited Na^+/phosphate cotransport indistinguishably from TPA. However, 4α-phorbol 12,13-didecanoate, which has no tumour-promoting activity (Castagna *et al.*, 1982; Nishizuka *et al.*, 1984), did not affect phosphate uptake either at low (10^{-10} M) or at high (10^{-7} M) concentrations. We have also analysed the time course of Na^+/phosphate cotransport inhibition by phorbol esters; for TPA (10 nM), half-maximal inhibition was obtained at about 20 min of incubation, whereas for 4 β-phorbol 12,13-didecanoate (10 nM), half-maximal inhibition was at about 45 min of incubation ($n = 2$; results not shown).

We further analysed whether Ca^{2+} and phorbol esters act synergistically, as has been described for other hormonal regulatory systems (Rasmussen & Barrett, 1984). We used the phorbol ester 4β-phorbol 12,13-dibutyrate, which has a slightly lower tumour-promoting activity than TPA (Nishizuka *et al.*, 1984). The results presented in Table 2 show that Na^+/phosphate cotransport was minimally inhibited at suboptimal concentrations of ionomycin and by 15% in the presence of 4β-phorbol dibutyrate alone, whereas the simultaneous addition of the two drugs caused a 42% inhibition. Ca^{2+} can thus intensify the action of phorbol esters on Na^+/phosphate cotransport. In analogy with PTH (Malmström & Murer, 1986*b*; Caverzasio *et al.*, 1986), ionomycin and/or phorbol esters inhibited Na^+/phosphate cotransport specifically without reducing other Na^+/cotransport systems, e.g. Na^+/α-methyl glucoside (Table 2).

Table 3. Prostaglandin E_2 production in OK cells

The cells were preincubated for 1 h with 1 mM-acetylsalicylic acid, prior to the 2 h incubation with 10^{-8} M-TPA + 5×10^{-7} M-ionomycin. The data are means ± S.D. of three determinations.

	Prostaglandin E_2 (pg/mg of protein)		Phosphate uptake (nmol/3 min per mg of protein)	
Condition	Control	TPA + ionomycin	Control	TPA + ionomycin
No addition	187 ± 11	1443 ± 34	6.21 ± 0.94	3.47 ± 0.13
1 mM-Acetylsalicylic acid	205 ± 22	220 ± 20	6.00 ± 0.91	3.58 ± 0.54

Table 4. Effect of acetylsalicylic acid on cyclic AMP production in OK cells after treatment with PTH and phorbol esters

The monolayers were incubated with DMEM/Hams F12 (1:1)/20 mM-Hepes/0.1% BSA for 12 h. At 5 min prior to the addition of the agents (TPA or PTH), 1 mM-IBMX (control) or 1 mM-IBMX plus 1 mM-acetylsalicylic acid was added and cyclic AMP was determined after 5 min of incubation in the presence of PTH (10^{-7} M) or TPA (10^{-7} M). Values are means from quadruplicate determinations. S.D. values were lower than 10% for all values reported.

	Cyclic AMP (pmol/5 min per mg of protein)	
Condition	Control	Acetylsalicylic acid (1 mM)
No addition	3.85	4.61
PTH(10^{-7} M)	220.67	244.35
TPA (10^{-7} M)	12.0	4.77

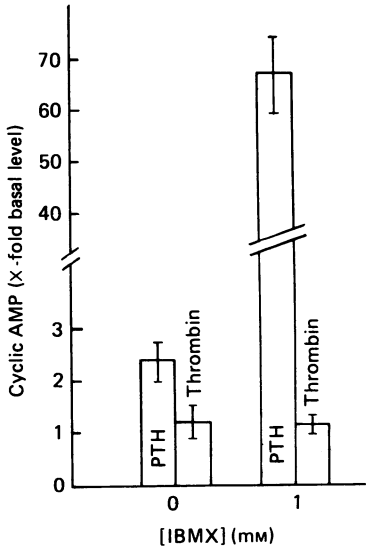

Fig. 5. Effect of PTH and thrombin on cyclic AMP levels in suspended OK cells

Prior to the addition of the hormone the cells were allowed to recover for 3 h from the 'stress' exerted by bringing cells in suspension. Cells were then incubated in the presence or absence of 1 mM-IBMX and in the absence or presence of either 10^{-7} M-PTH or 2.5 units of thrombin/ml. Cyclic AMP was determined after 5 min of incubation. Values are means ± S.D. for quadruplicate determinations. The basal level in the absence of IBMX was 3.0 pmol/5 min per mg of protein and in the presence of IBMX 7.3 pmol/5 min per mg of protein.

Effect of phorbol esters on prostaglandin production

The actions of phorbol esters are multivalent. For example, they stimulate the production of prostaglandin E_2 in bone cells (Tashjian *et al.*, 1978) and in mesangial cells (Pfeilschifter *et al.*, 1986). We measured prostaglandin E_2 production in cell monolayers that had been incubated with TPA alone (results not shown) or TPA + ionomycin. These conditions were found to inhibit Na^+/phosphate cotransport markedly (see Table 2 and Fig. 4). Incubation with TPA + ionomycin resulted in an elevated prostaglandin E_2 synthesis (Table 3) which could be blocked by preincubating the cells with 1 mM-acetylsalicylic acid (Table 3). The Na^+/phosphate cotransport activity was measured in parallel experiments, and the reduction of the transport rate induced by TPA + ionomycin was as large in the absence as in the presence of acetylsalicylic acid (Table 3). It could, therefore, be ruled out that phorbol esters act via increased prostaglandin E_2 synthesis with a subsequent stimulation of adenylate cyclase; it was found previously that prostaglandin E_2 leads to increased cellular cyclic AMP levels in OK cells (Malmström & Murer, 1986*b*).

Effect of PTH and thrombin on cellular cyclic AMP production

Prostaglandin E_2 was shown to increase cyclic AMP levels in OK cells (Malmström & Murer, 1986*b*). Thus, it was important to analyse cellular cyclic AMP levels in the presence and absence of acetylsalicylic acid in order to decide whether an effect of an agent on cellular cyclic AMP levels was mediated by increased levels of prostaglandin E_2, i.e. indirectly. Incubation of OK cell monolayers for 5 min in the presence of the phosphodiesterase inhibitor IBMX with 10^{-7} M-PTH produced a more than 50-fold increase in cellular cyclic AMP content; this increase was not significantly altered by the presence of 1 mM-acetylsalicylic acid (Table 4). The PTH effect on Na^+/phosphate cotransport was not prevented by acetylsalicylic acid (results not shown). Also, the TPA produced an about 3-fold elevation of cellular cyclic AMP. This elevation was to a large extent prevented by 1 mM-acetylsalicylic acid, suggesting that it was mostly related to the increased cellular prostaglandin E_2 level (Table 3). [It should be noted that in many

different cell types kinase C activation had an influence on adenylate cyclase activity and thus on cellular cyclic AMP (e.g. Bell *et al.*, 1985; Bell & Brunton, 1986; Yoshimasa *et al.*, 1987; Rozengurt *et al.*, 1987; Jakobs *et al.*, 1985). In OK cells we measured the cyclic AMP level after pretreating the cell monolayers with TPA for 3 h and there was a small but significant increase of cyclic AMP production (3–4-fold); the cyclic AMP signal produced by PTH was also potentiated after pretreating the cell monolayers with TPA (1.5-fold; results not shown).] Finally, we have also measured the effect of thrombin and PTH on cellular cyclic AMP levels in OK cells kept in suspension (Fig. 5). Similar to the finding in monolayers, PTH provoked a marked increase in cyclic AMP, especially in the presence of IBMX; thrombin was unable to produce an increase in cellular cyclic AMP, both in the presence and absence of IBMX.

DISCUSSION

The data presented suggest that PTH activates two messenger systems in OK cells, the cyclic AMP system and the Ca^{2+}/DAG system. This is in agreement with the recent observation by Hruska *et al.* (1987). PTH led to a rise in intracellular Ca^{2+} (released from an intracellular store) (Fig. 1), with a simultaneous rise of DAG and of IP_3 (Hruska *et al.*, 1987). The intracellular Ca^{2+} returned to resting levels within 45 s. The previously described activation of adenylate cyclase (maximal activiation at 2–5 min; Teitelbaum & Strewler, 1984; Malmström & Murer, 1986*b*), appears to be preceded by the activation of phosphoinositide metabolism. The receptor site coupled to adenylate cyclase (Teitelbaum & Strewler, 1984) apparently coexists with a second receptor site, coupled to phosphoinositide metabolism. Whether the two receptors can be distinguished by their cellular location (apical versus basolateral) and/or by their affinity remains to be determined.

Application of low concentrations of A23187 or ionomycin to OK cells induced a transient increase of intracellular Ca^{2+} (results not shown), but had only minimal effects on the transport rate at prolonged incubation periods (Fig. 2) as well as after short exposure to the ionophores (results not shown). Thus, a transient increase in intracellular Ca^{2+} concentration is apparently not a sufficient signal to induce a regulatory cascade resulting in reduction of Na^+/phosphate cotransport. From this it might be speculated that calmodulin-coupled reactions (Rasmussen & Barrett, 1984) are not important in the PTH-dependent regulation of Na^+/phosphate cotransport in OK cells.

Na^+/phosphate cotransport in OK cells was reduced after incubating the cells for an extended period of time with phorbol esters expressing tumour-promoting activity. The reduction induced by phorbol esters was potentiated by the simultaneous addition of Ca^{2+} ionophores such as A23187 and ionomycin (Table 2), indicating that activation of kinase C and Ca^{2+} mobilization have a synergistic effect, as reported for several other hormonal systems (Rasmussen & Barrett, 1984). The phorbol-ester-induced inhibition of Na^+/phosphate cotransport was also observed in the presence of 1 mM-acetylsalicylic acid, which prevented both phorbol-ester-induced generation of cyclic AMP and phorbol-ester-induced production of prostaglandin E_2. Thus activation of kinase C apparently leads in a cyclic AMP-independent manner to inhibition of Na^+/phosphate cotransport.

The role of protein kinase C in regulation of Na^+/phosphate cotransport was further evaluated by activating the phosphoinositide metabolism by thrombin. In suspended OK cells, addition of thrombin caused a transient rise in Ca^{2+} (Fig. 1) and a time-dependent decrease in Na^+/phosphate cotransport (Fig. 2). We were unable to detect a thrombin-dependent increase in cellular cyclic AMP (Fig. 5). This observation again supports the view that activation of kinase C is capable, independent of cyclic AMP production, of reducing Na^+/phosphate cotransport. The lack of an effect of thrombin in the monolayer experiments suggests that the thrombin effect is apparently related to an event (receptor interaction) occurring not at the apical but rather at the basolateral cell surface. For PTH, which acts in cell monolayers as well as in suspended cells, it must be assumed that receptors are present either on the apical membrane or that the hormone is able to reach the basolateral compartment.

In previous reports it was shown that increased cyclic AMP levels in OK cells are associated with a specific reduction in Na^+/phosphate cotransport (Malmström & Murer, 1986*b*; Caverzasio *et al.*, 1986). In the present report we show that manoeuvres which are known to activate kinase C in many different cell types are also associated with inhibition of Na^+/phosphate cotransport. Hruska *et al.* (1987) have shown previously that addition of cyclic AMP to OK cells did not produce an increase in IP_3. Thus, the cyclic AMP (kinase A) as well as the Ca^{2+}/DAG (kinase C) regulatory cascades apparently both have the capacity to inhibit Na^+/phosphate cotransport in OK cells. In a study on different renal cell lines, it was previously shown (Malmström & Murer, 1986*b*) that the capacity of increased cyclic AMP levels to reduce Na^+/phosphate cotransport is specific for OK cells and is not observed in, e.g., LLC-PK_1 cells. The same is true for the kinase C regulatory branch; in LLC-PK_1 cells and cultured mouse kidney cells, TPA produced stimulation of Na^+/phosphate cotransport (Mohrmann *et al.*, 1986; Kinoshita *et al.*, 1986), whereas in OK cells inhibition was observed (see above). This suggests that OK cells, but not LLC-PK_1 cells, contain a Na^+/phosphate cotransport system with regulatory properties similar to that of the mammalian proximal tubule (for review see Gmaj & Murer, 1986).

The capacity of two regulatory cascades in regulation of Na^+/phosphate cotransport does not necessarily imply that they are of equal importance in the PTH regulation of Na^+/phosphate cotransport. In an attempt to establish whether one of the two regulatory cascades is more important, we have used several inhibitors of protein kinase C, such as calmodulin antagonists (Hidaka *et al.*, 1984; Mori *et al.*, 1980; Schatzman *et al.*, 1981), polypeptides with hydrophobic regions (Kuo *et al.*, 1983), isoquinolino sulphonamide derivatives (Hidaka *et al.*, 1984) and sphingosine (Hannun *et al.*, 1986). Although all have been reported to inhibit kinase C activity, their action is not very specific and other regulatory systems (e.g. kinase A; Hidaka *et al.*, 1984) are affected as well. Furthermore, in the situation of two regulatory cascades with the same final effect, it cannot be ruled out that by inhibiting one pathway the second one is amplified. Our results (not shown) indicated no effect of H7 (Hidaka *et al.*, 1984) on the PTH response in either cell monolayers

or suspended cells. Even if we assume that the inhibitor could enter the cell, our negative observations do not imply that PTH regulation of Na^+/phosphate cotransport occurs in the absence of kinase C activity (for the reasons mentioned above). A separation of the two regulatory cascades certainly needs further work, e.g. with PTH fragments (Habener *et al.*, 1984) or with cloned cell lines eventually showing separation of the two receptors into individual clones. A two-receptor model for the action of PTH has been proposed for the action of the hormone on osteoblasts with respect to elevation of both intracellular cyclic AMP and free Ca^{2+} (Löwik *et al.*, 1985).

In conclusion, it is shown that two independent regulatory cascades can mediate the PTH action on Na^+/phosphate cotransport. The phorbol-ester-induced inhibition of Na^+/phosphate cotransport, most likely mediated by kinase C activation, as well as inhibition related to increased cyclic AMP levels are independent of each other. Both effects, similar to the effect of PTH, are reversible and inhibition is protein-synthesis-independent (Caverzasio *et al.*, 1986; Malmström & Murer, 1986*a*,*b*, 1987). Recovery from PTH inhibition is protein-synthesis-dependent, and it has been suggested that the carrier protein itself or a component necessary for its activity is finally irreversibly inactivated (Malmström & Murer, 1987).

The OK cells were a kind gift from Dr. D. Warnock, San Fransisco, CA, U.S.A. We thank Mr. Hans Niederberger and Ms. Ingrid Weissbrodt for determining the prostaglandins. The financial support of SOLCO, Basle and the Swiss National Foundation (Grant nos. 3.881.085 and 3.881.185) is gratefully acknowledged.

REFERENCES

Bell, J. D. & Brunton, L. L. (1986) J. Biol. Chem. **261**, 12036–12041

Bell, J. D., Buxton, I. L. O. & Brunton, L. L. (1985) J. Biol. Chem. **260**, 2625–2628

Berridge, M. J. & Irvine, R. F. (1984) Nature (London) **312**, 315–321

Brown, B. L., Elkins, R. P. & Albano, J. D. H. (1972) Adv. Cyclic Nucleotide Res. **2**, 25–40

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. & Nishizuka, Y. (1982) J. Biol. Chem. **257**, 7847–7851

Caverzasio, J., Rizzoli, R. & Bonjour, J.-P. (1986) J. Biol. Chem. **261**, 3233–3237

Cole, J. A., Ebber, S. L., Toelling, R. E., Thorne, P. K. & Forte, L. R. (1987) Am. J. Physiol. **253**, E221–E227

Dulley, J. T. & Grieve, P. A. (1975) Anal. Biochem. **64**, 135–141

Gmaj, P. & Murer, H. (1986) Physiol. Rev. **66**, 36–70

Grynkiewicz, G., Poenie, M. & Tsien, R. Y. (1985) J. Biol. Chem. **260**, 3440–3450

Habener, J. F., Rosenblatt, M. & Potts, J. T., Jr. (1984) Physiol. Rev. **64**, 985–1053

Hannun, J. A., Loomis, C., Merrill, A. H., Jr. & Bell, R. M. (1986) J. Biol. Chem. **261**, 12604–12609

Hidaka, H., Inagaki, M., Kawamoto, S. & Sasaki, Y. (1984) Biochemistry **23**, 5036–5041

Hruska, K. A., Moskowitz, D., Esprit, P., Civitelli, R., Westbrook, S. & Huskey, M. (1987) J. Clin. Invest. **79**, 230–239

Jakobs, K. H., Bauer, S. & Watanabe, Y. (1985) Eur. J. Biochem **151**, 425–430

Kinoshita, I., Fukase, M., Hishikawa, R., Jamatani, J. & Fujita T. (1986) Biochim. Biophys. Res. Commun. **136**, 177–182

Kuo, J. F., Raynor, R. L., Mazzei, G. J., Schatzman, R. C., Turner, R. S. & Kem, W. R. (1983) FEBS Lett. **153**, 183–186

Löwik, C. W. G. M., van Leeuven, J. P. T. H., van der Meer, J. M., van Zweelen, J. K., Scheven, B. A. A. & Herrmann-Erlee, J. M. (1985) Cell Calcium **6**, 311–326

Malmström, K. & Murer, H. (1986*a*) Experientia **42**, 639 (abstr.)

Malmström, K. & Murer, H. (1986*b*) Am. J. Physiol. **251**, C23–C31

Malmström, K. & Murer, H. (1987) FEBS Lett. **216**, 257–260

Mohrmann, I., Mohrmann, M., Biber, J. & Murer H. (1986) Biochim. Biophys. Acta **860**, 35–43

Mori, T., Takai, Y., Minakuchi, R., Yu, B. & Nishizuka, Y. (1980) J. Biol. Chem. **255**, 8378–8380

Nakai, M., Kinoshita, J., Fukase, M. & Fujita, J. (1987) Biochim. Biophys. Res. Commun. **145**, 303–308

Nishizuka, Y. (1984) Science **225**, 1365–1370

Nishizuka, Y., Takai, Y., Kishimoto, A., Kikkawa, U. & Kaibucchi, K. (1984) Recent Progr. Horm. Res. **40**, 301–345

Pfeilschifter, J., Kurtz, A. & Bauer, Ch. (1986) Biochem. J. **234**, 125–130

Rasmussen, H. & Barrett, P. Q. (1984) Physiol. Rev. **64**, 938–984

Rozengurt, E., Murray, M., Zachary, J. & Collins, M. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 2282–2286

Schatzman, R. C., Wise, B. C. & Kuo, J. F. (1981) Biochem. Biophys. Res. Commun. **98**, 669–676

Tashjian, A. H., Jr., Ivey, J. L., Delclos, B. & Levine, L. (1978) Prostaglandins **16**, 221–232

Teiltelbaum, A. P. & Strewler, G. J. (1984) Endocrinology (Baltimore) **114**, 980–985

Yoshimasa, T., Sibley, D. R., Bouvier, M., Lefkowitz, R. J. & Caron, M. G. (1987) Nature (London) **327**, 67–70

Received 15 June 1987/12 October 1987; accepted 27 November 1987