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Research Article

Sodium-phosphate cotransport in the PTH-responsive opossum kidney (OK) cell line is inhibited by PTH, cAMP, and activators of protein kinase C. In order to probe the role of cAMP, we stably transfected OK cells with an expression vector for a cAMP-binding mutation of the murine protein kinase A regulatory subunit. Two-dimensional electrophoresis of cAMP-binding proteins from transfected cells indicated a 20-fold overexpression of the mutant regulatory unit. Protein kinase A from these cells had a 20-fold increase in the concentration of cAMP required for half-maximal activation, 2.8 microM vs. 0.15 microM for wild type cells. In the transfected cells, Na-phosphate cotransport was insensitive to up to 1 mM 8-Br-cAMP and 1 microM PTH, while these same agonists caused a significant inhibition of transport in the wild type cells. The effects on Na-phosphate cotransport of the protein kinase C activators oleoyl-acetyl glycerol and tetradecanoyl-phorbol acetate, which were marked in the wild type cells, were still present, although attenuated, in the transfected mutants. With prolonged passage, the cAMP-insensitive phenotype reverted to wild type cAMP sensitivity despite continued selection for the cotransfected neo marker. The revertant cells had a normal cAMP requirement for half-maximal activation of protein kinase A, 0.13 microM, and the PTH and cAMP-sensitive inhibition of Na-phosphate cotransport was restored. We suggest that an intact and normally cAMP-sensitive protein kinase A […]

Transfection-mediated Expression of a Dominant cAMP-resistant Phenotype in the Opossum Kidney (OK) Cell Line Prevents Parathyroid Hormone-induced Inhibition of Na-phosphate Cotransport

A Protein Kinase-A-mediated Event

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Abstract

Sodium-phosphate cotransport in the PTH-responsive opossum kidney (OK) cell line is inhibited by PTH, cAMP, and activators of protein kinase C. In order to probe the role of cAMP, we stably transfected OK cells with an expression vector for a cAMP-binding mutation of the murine protein kinase A regulatory subunit. Two-dimensional electrophoresis of cAMP-binding proteins from transfected cells indicated a 20-fold overexpression of the mutant regulatory unit. Protein kinase A from these cells had a 20-fold increase in the concentration of cAMP required for half-maximal activation, 2.8 μM vs. 0.15 μM for wild type cells. In the transfected cells, Na-phosphate cotransport was insensitive to up to 1 mM 8-Br-cAMP and 1 μM PTH, while these same agonists caused a significant inhibition of transport in the wild type cells. The effects on Na-phosphate cotransport of the protein kinase C activators oleoyl-acetyl glycerol and tetradecanoyl-phorbol acetate, which were marked in the wild type cells, were still present, although attenuated, in the transfected mutants. With prolonged passage, the cAMP-insensitive phenotype reverted to wild type cAMP sensitivity despite continued selection for the cotransfected neo marker. The revertant cells had a normal cAMP requirement for half-maximal activation of protein kinase A, 0.13 μM, and the PTH and cAMP-sensitive inhibition of Na-phosphate cotransport was restored. We suggest that an intact and normally cAMP-sensitive protein kinase A pathway is an absolute requirement for PTH inhibition of Na-phosphate cotransport in the OK cell. (*J. Clin. Invest.* 1990. 86:1442–1450.) Key words: cAMP • protein kinase A • OK cell • transfection • parathyroid hormone

Introduction

The renal effects of parathyroid hormone on Na coupled-phosphate transport and Na/H exchange have been extensively studied in the proximal-tubule-like opossum kidney cell line (OK[1] cells) (1, 2, 3). In this model, PTH and cAMP decrease both Na/H antiporter activity and Na coupled-phosphate transport. cAMP-mediated pathways are clearly important as effectors of PTH action, but several lines of evidence have suggested the possibility of other intermediaries. First, PTH increases intracellular calcium and causes phosphoinositide turnover in the OK cell (4). Although recent evidence suggests that intracellular calcium is not a mediator of these effects, protein kinase C (PKC) activation may be required for the inhibition of transport (5–7). Second, there is a several log order disparity between the concentrations of PTH required for half-maximal transport inhibition versus those needed for half-maximal cAMP generation (5, 6, 8). This dissociation may, however, merely indicate that only a small increment in cAMP concentration is adequate to activate protein kinase A (PKA) for PTH-induced effect on transport to be manifest (9, 10). Most studies of these relationships have used pharmacological inhibitors of cAMP generation (e.g., dideoxyadenosine) or of protein kinase C activation (e.g., prolonged phorbol ester incubation or staurosporine) to probe these mechanisms.

The effects of cAMP are known to be mediated through the PKA system. PKA exists as a heterotetramer of two regulatory subunits (R^I or R^{II}), which on binding two cAMP molecules per R unit dissociate from two catalytic units (C) (11). The C units, only active when dissociated from R, are then free to phosphorylate their target substrates. R and C subunits form a very high affinity tetramer and under normal circumstances the bulk of the catalytic units are complexed to R in the form R_2C_2, a state in which the catalytic C units are inactive (11). Many somatic mutations which disrupt cell PKA activity have been described and have been found to affect R units (12–15). Recently, McKnight et al. have cloned both the native mouse R^I unit and several mutant R isoforms which had been isolated by Steinberg et al. (14, 16). These mutations are characterized by decreased cAMP binding affinity to one or both of the two cAMP binding sites of R and consequently, a marked increase in the cAMP concentration needed to dissociate R from C and activate PKA (17). Recent studies by McKnight et al. have demonstrated that when cAMP binding-defective R units are overexpressed by DNA-mediated transfection, phenotypic cAMP resistance occurs in the overexpressing cells. Therefore expression of these mutant R units confers a dominant cAMP-resistant phenotype on a variety of transfected cells (16, 18).

Herein we report an approach to dissect the importance of the PKA pathway in PTH action by DNA-mediated gene transfer of dominant cAMP resistance in OK cells. By overexpressing a mutant type I regulatory subunit of PKA (R^{I*}), which is deficient in cAMP binding, phenotypic cAMP resistance was achieved in OK cells. The resulting 20-fold reduction in the sensitivity of the PKA system to cAMP abolished the PTH and cAMP effects on Na-phosphate transport. These findings suggest that an intact and normally cAMP-sensitive

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1. *Abbreviations used in this paper:* C, catalytic unit; DAG, diacylglycerol; DDA, dideoxyadenosine; IP_3, inositol Tris phosphate; OAG, oleoyl-acetyl glycerol; OK, opossum kidney; OK-HL, OK cells cotransfected with pHLREV and pSV2NEO; OK-wt, OK wild type cells; pHLREV, an expression vector for the A or site I mutant of the mouse R^I unit under the control of the Harvey sarcoma virus LTR; PKA and PKC, protein kinase A and C; PMA, phorbol myristate acetate; R, regulatory subunit; R^{I*}, mutant type I regulatory subunit of PKA.

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PKA system is required for PTH to inhibit Na-phosphate cotransport in the OK cell.

Methods

Cell culture. A single cell clone of OK cells which is responsive to PTH was the starting material for all studies. They were maintained in DME, 5% fetal bovine serum; 5% NuSerum (Collaborative Research, Inc., Bedford, MA); glucose, 5 g/liter; $NaHCO_3$, 24 mM; Hepes, 12.5 mM; glutamine, 2 mM; penicillin, 100 U/ml; streptomycin, 100 μg/ml; in 5% CO_2:95% air. Cells were passed weekly.

Transfection and selection. OK cells were transfected with pHLREV, an expression vector for the A or site I mutant of the mouse R^I unit under the control of the Harvey sarcoma virus long terminal repeat (LTR), and pSV2NEO (100 and 5 μg, respectively). Transfection was done by electroporation as described by Chu (19), and cells were selected in 800 μg/ml of G418 (20). G418-resistant colonies were expanded, pooled, and frozen. Thereafter, cells used for experiments described were maintained in G418-free medium for at least three passages before experiments. This plasmid was provided by Dr. G. S. McKnight, Univ. of Washington, Seattle, WA, and was propagated, prepared, and purified by standard techniques (21).

Protein kinase A measurement. Cells were grown to confluence in 150-cm^2 tissue culture dishes, washed three times with iced PBS, scraped from the plate, and centrifuged at 200 *g* for 3 min. The pellet was disrupted by freeze-thawing in MES (pH 7.4), 10 mM; EDTA, 1 mM; DTT, 1 mM; 1,10 phenanthroline, 1 mM; benzamidine, 1 mg/ml; PMSF, 0.2 mM; acid-washed charcoal, 2 mg/ml. The homogenate was centrifuged at 100,000 *g* for 1 h at 4°C. PKA activity in the cytosolic supernatant was measured by the phosphorylation of the synthetic substrate Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly; Peninsula Laboratories, Inc., Belmont, CA) (22, 23). The assay solution contained Tris (pH 7.4), 25 mM; Mg-acetate, 12.5 mM; DTT, 12.5 mM; Kemptide, 37.5 μM; NaF, 0.5 mM; isobutyl methyl xanthine (IBMX), 1 mM; ATP, 250 μM; [γ^{32}P]ATP (10 Ci/mmol), 20 μCi/ml. cAMP was added to achieve concentrations ranging from 0.1 nM to 0.1 mM. 40 μl of homogenate was mixed with 160 μl of assay solution in microcentrifuge tubes and incubated at room temperature for 5 min. 50-μl samples were then transferred to strips of phosphocellulose paper (P81; Whatman Laboratory Products, Inc., Clifton, NJ). The strips were washed four times in iced 75 mM phosphoric acid, dried, and counted in Safety-Solve scintillation fluid (Research Products International, Mount Prospect, IL). Proteins were measured by the Lowry method or with BCA reagent (Pierce Chemical Co., Rockford, IL). PKA activity was expressed as nanomoles ^{32}P-incorporated/mg protein per 5 min. Incorporation of ^{32}P in samples without homogenate was subtracted from all other values.

Column chromatography. Cells were grown to confluence on 550-cm^2 plates, washed 3 times with PBS, scraped, and centrifuged at 200 *g* for 3 min at 4°C. After resuspension in disruption buffer (MES [pH 7.4], 20 mM; EDTA, 1 mM; 1,10 phenanthroline, 1 mM; PMSF, 1 mM), cells were sonicated for 15 s on ice. Homogenate was cleared by centrifugation at 5,000 *g* for 10 min and the supernatant loaded on a DEAE-cellulose column (5 ml bed vol). The column was washed with Na-free buffer and eluted with MES (pH 6.4), 20 mM; DTT, 1 mM; EDTA, 1 mM; PMSF, 1 mM, using a linear NaCl gradient (0–0.4 M). An aliquot of each fraction collected was assayed for PKA activity.

Photoaffinity labeling and two-dimensional gel analysis of cAMP-binding protein. Homogenate was obtained as described above and concentrated with a centrifugal ultrafilter (Ultrafree-MC; Millipore/Continental Water Systems, Bedford, MA, 30,000-D molecular mass cutoff). Homogenate was incubated in the dark at 4°C with 10 μM 8-azido-^{32}P-cAMP (≈ 50 Ci/mmol; ICN Radiochemicals, Costa Mesa, CA) for 1 h followed by 10 min exposure to 254 nM UV light (UVS 54 hand-held lamp at 10-cm illumination distance; UV Products). The reaction mixture was concentrated and subjected to two-dimensional gel electrophoresis followed by autoradiography. To specifically quantitate radioactivity over regions of interest, gels were directly quantitated by counting with an AMBIS system 2-dimensional scintillation scanner (AMBIS, Inc., San Diego, CA).

Na-dependent phosphate cotransport. Cells were grown to confluence in multiwell clusters (Costar Data Packaging Corp., Cambridge, MA, or Nunc, Roskilde, Denmark). They were incubated at 37°C for 4 h following addition of medium containing 8-Br-cAMP, PTH, the phorbol ester PMA (10^{-7}M), or the diacylglycerol analogue OAG (250 μg/ml). Previous studies had determined that this time frame afforded maximal modulation of transport by all of these agents. Uptake medium "A" contained Na, 144 mM; Hepes (pH 7.4), 24 mM; Cl, 128 mM; Ca, 1 mM; Mg, 1 mM; K, 4.2 mM; PO_4, 0.12 mM; [^{32}P]orthophosphate, 1 μCi/ml. Uptake medium "B" was identical, except for *N*-methyl-D-glucamine replacing Na. Cells were washed and then incubated briefly at 37°C on a heating pad with preuptake medium "C" (equivalent to "B" without PO_4). After this, medium was replaced with either solution A (+Na), or solution B (−Na). After 10 min, uptake was terminated by washing five times with 4°C $MgCl_2$, 0.1 M; and Hepes (pH 7.4), 1 mM. The wells were aspirated and the remaining contents were dissolved in 0.1% SDS overnight. Aliquots were counted for ^{32}P as well as protein measurement. The Na-independent uptake was subtracted from all other values. Uptake was calculated as nanomoles phosphate/mg protein per unit time. Preliminary experiments indicated that Na-phosphate cotransport was linear for > 15 min using these protocols.

cAMP generation. Cells were grown to confluence in multiwell clusters as previously described. They were incubated with DME buffered with 25 mM Hepes (pH 7.4) and containing 1 mg/ml bovine serum albumin at 37°C in CO_2-free air for 1 h. The medium was then replaced with PTH-containing medium (0.1–1,000 nM) also containing 1 mM IBMX. After 5 min incubation, cells were washed with iced PBS, and 1 ml absolute ethanol was added. After 1 h at 4°C the ethanol was transferred to microcentrifuge tubes and centrifuged at 43,000 *g*. The supernatant was evaporated to dryness in a Speedvac (Savant Instruments, Inc., Hicksville, NY). Dried samples were reconstituted and cAMP was assayed by competitive protein binding using a kit (Amersham Corp., Arlington Heights, IL).

Measurement of diacylglycerol content. Cells were grown in 150-cm^2 dishes to confluence and gently scraped from the plates, rinsed three times in Hepes-buffered DME containing 1 mg/ml fatty acid-free bovine serum albumin, and resuspended in the same medium at the approximate ratio of 150-cm^2 growing area/0.5 ml. 200 μl of cell suspension was added to 100 μl medium with or without 300 nM PTH in a microcentrifuge tube at 37°C. After 10 s 200 μl of this suspension was removed and pipetted into 800 μl of chloroform:methanol:1 M HCl (2:1:.1). A parallel sample was centrifuged, the cell pellet dissolved in 0.1% SDS, and protein determined. Quantitative lipid extraction and determination of diacylglycerol with *Escherichia coli* diacylglycerol kinase was performed as described by Preis et al. (24). Results were expressed as pmol/mg protein.

Phosphoinositide turnover. Cells were incubated for 72 h in inositol-free medium containing 10 μCi/ml of ^{3}H-*myo*-inositol. The medium was supplemented with 10% fetal bovine serum which had been dialyzed against PBS. Cells were then exposed to 100 nM PTH for 45 s or 5 min. The exposure was terminated by aspiration of medium and addition of 10% TCA. After centrifugation for 5 min at 10,000 *g*, the supernatant was extracted six times with diethyl ether. The resultant solution was neutralized to pH 6–7 with 0.15 M NH_4OH, and applied to Dowex resin in the formate form in disposable columns. IP_2 and IP_3 were separated by eluting with increasing concentrations of ammonium formate in 100 mM formic acid. Scintillation counting was performed on all fractions. Proteins were measured from cell pellets dissolved in 0.1% SDS using the BCA protein assay reagent (Pierce).

Results

Characterization of transfected mutant R^I unit of PKA in OK cells. OK cells cotransfected with pHLREV and pSV2NEO

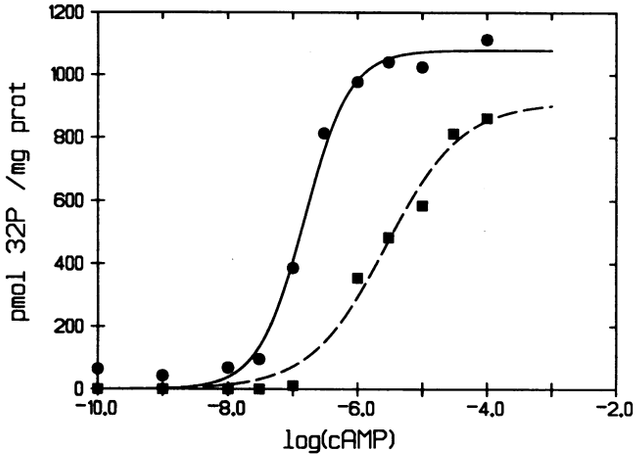

Figure 1. Sensitivity of protein kinase A from OK-wt and OK-HL cells to activation by cAMP. High-speed supernatants from OK-wt and OK-HL cell homogenates were prepared as described in Methods. Aliquots of homogenate were assayed for their ability to phosphorylate the synthetic PKA substrate Kemptide as described. The solid line and broken lines represent the calculated activation curve of protein kinase A from OK-wt and OK-HL cells, respectively, and was calculated using least-squares minimization of the logarithmic transformation of the Hill equation. Points were derived from two experiments per cell type.

(OK-HL) were selected in G418 and resistant colonies pooled and frozen at early passage. Parallel cultures transfected with only 100 μg of pSV2NEO acquired stable G418 resistance at a frequency of $\sim 5 \times 10^{-4}$. Transfected cells displayed some morphological differences under phase microscopy and grew at a slower rate than wild type OK cells (OK-wt).

The cAMP sensitivity of PKA in OK-wt and OK-HL cells was determined in order to demonstrate that phenotypic cAMP resistance was achieved. The sensitivity of PKA in the cytoplasmic fraction of wild type OK cells (OK-wt) and OK-HL cell homogenates is shown in Fig. 1. PKA in OK-wt cells displayed a half-maximal cAMP activation of kinase activity ($EC_{.5}$) of 0.15 μM cAMP which is characteristic of type I PKA in most cells. In contrast, PKA in OK-HL cells had a 20-fold higher $EC_{.5}$, 2.8 μM cAMP. The V_{max} was only slightly reduced in OK-HL cells as compared to OK-wt cells. It is important that the Hill slopes[2] calculated from the activation curves were 1.29 and 0.68 in OK-wt and OK-HL cells, respectively. The native R^I subunit has two, nonequivalent, cAMP-binding sites, and $R_2{}^IC_2$ is known to exhibit a > 1 Hill coefficient for cAMP activation of PKA activity (17). In contrast, the OK-HL cells whose phenotype should assume that of the mutant R^I (R^{I*}) should reflect the fact that each R^{I*} unit has only one functional cAMP-binding site. The Hill slope of ≈ 0.7 is concordant; Hill slopes obtained using PKA reconstituted in vitro from purified catalytic and either R^I or R^{I*} units have been determined to be 1.58 and 0.58, respectively (17).

To further characterize the difference in the bulk PKA population, cell extracts from OK-wt and OK-HL cells were chromatographed on DEAE cellulose and eluted with a NaCl gradient. Under these conditions only the intact R_2C_2 tetramer is recovered by salt elution (13, 25). It can resolve type I from type II PKA, known mutants of R^I, and has been used to resolve the partial cAMP-bound from cAMP-free PKA. Fig. 2 shows the elution profile of PKA activity. OK-wt cell PKA activity eluted as a single broad peak between 0.13–0.2 M NaCl. In contrast, OK-HL cell PKA eluted at a higher concentration (0.2–0.26 M NaCl). These findings are consistent with a change in the ion exchange properties of the PKA holoenzyme: virtually all of the intact PKA holoenzyme-containing R subunits differed in ion exchange properties from the wild type.

A direct demonstration of coexpression of normal and mutant R^I units was done by photoaffinity labeling R^I subunits with 8-azido-[^{32}P]cAMP and resolving the labeled products by two-dimensional gel electrophoresis (26, 27). R^I, the dominant species in the OK cell, represents the bulk of the cAMP binding activity in the OK cell. It can be visualized in two forms of slightly differing isoelectric point in the upper panel of Fig. 3, migrating at $\sim$ 48 kD. These two forms represent the non and autophosphorylated forms of R^I (14, 15). In addition, two lower molecular weight forms, $\sim$ 31 kD, are present and probably represent a well defined proteolytic product of R (28). This proteolytic fragment is characterized as retaining the two cAMP binding sites and is therefore labeled by the photoaffinity probe. Photoaffinity labeling of an equal amount of OK-HL extract is shown in the lower panel of Fig. 3. This gel demonstrates the presence of R^I and its phosphorylation product, in addition to another set of labeled proteins, migrating $\approx$ 0.5-pH units more alkaline but at the same molecular weight. This represents the mutant R^{I*}. The alkaline pH shift is comparable to that described by Steinberg et al., who developed the cAMP-resistant S49 lymphoma cells from which this R^{I*} was cloned (14). Direct two-dimensional surface scintillation counting over the dried gel with an AMBIS counter was

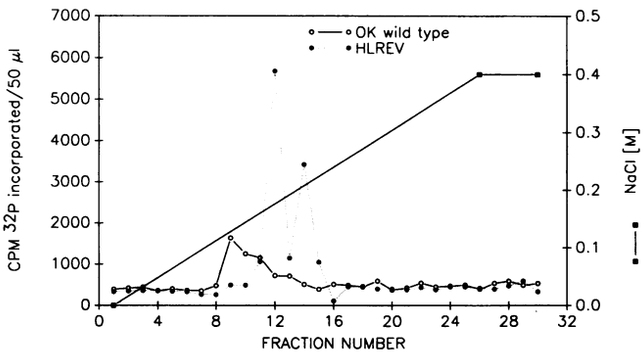

Figure 2. Elution profile of OK-wt and OK-HL cell protein kinase A holoenzyme from DEAE cellulose. Homogenates from OK-wt and OK-HL cells were prepared as described, applied to a DEAE cellulose column, and eluted with a 0–0.4 M NaCl gradient. Equal amounts of each fraction were assayed directly for protein kinase A activity as described. The solid and dotted lines represent elution profiles for OK-wt and OK-HL cells, respectively.

2. The PKA activity was expressed as a function of the $\log_{10}$ of the cAMP concentration. It was fitted to the logarithmic transformation of the Hill equation: activity = $\text{activity}_{max}/(10^{\wedge(-N_H \cdot \log [cAMP]-pK)}+1)$, where N_H is the Hill coefficient and pK is the log of an arbitrary coefficient. The log of the cAMP concentration for half-maximal activity is given as $-pK/N_H$. The data were fitted to this logarithmic transformation of the Hill equation using the program MINSQ.

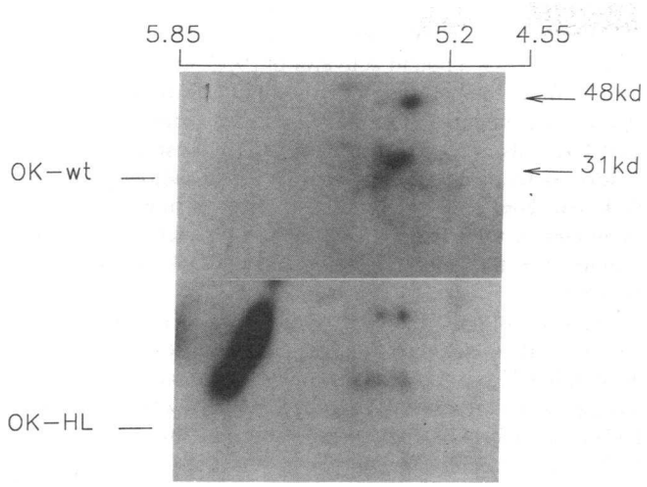

Figure 3. Demonstration of protein kinase A regulatory subunits in OK-wt and OK-HL cells by 8-azido-^{32}P-cAMP photoaffinity labeling and two-dimensional electrophoresis. Extracts from OK-wt and OK-HL cells were incubated with $\approx$ 10 μM 8-azido-[^{32}P]cAMP at 4°C followed by exposure to 254 nM ultraviolet light, and subjected to two-dimensional electrophoresis. (*Top*) 2-d autoradiographic exposure of OK-wt extract. (*Bottom*) A 1-d exposure of the same amount of OK-HL extract. Molecular weights were determined from known standards included in a parallel lane of the second dimension. The nominal isoelectric point scale at the top of the figure was calculated from protein standards run in parallel lanes of the isoelectric focusing dimension.

performed. The R^{I*} area contained 9.8-fold the ^{32}P counts as the native R^I area. Since the R^{I*} has only one functional cAMP binding site (12, 17), the other having been destroyed by mutation, analysis of this gel indicates a 19.6-fold overexpression of R^{I*} compared to the native R^I.

Finally, we measured the increase in PKA activity ratio (9) in both OK-wt and OK-HL cells with and without 100 nM PTH. The PKA activity ratio is the ratio of kinase activity in the absence of added cAMP divided by that in the presence of maximal cAMP. As such, when in the absence and presence of an agonist, it gives an estimate of how much dissociation of PKA has occurred as a result of addition of an agonist. The PKA ratio in OK-wt cells increased 67% after 1 h of 100 nM PTH exposure. In OK-HL cells, the activity ratio increased only 11% after the same PTH treatment. Therefore, similar PTH concentrations induced a much larger dissociation of PKA in the OK-wt cells than the OK-HL line, supporting the contention of relative cAMP resistance.

In summary, these data demonstrate that: (*a*) R^{I*} was $\approx$ 20-fold overexpressed relative to the native R^I unit; (*b*) the overexpressing cells demonstrated intact PKA in which the bulk ion-exchange properties were altered, indicating that almost all of the PKA contained a mutant R^{I*} unit; (*c*) the cAMP-dependence of activation of PKA was shifted to the "right," $\approx$ 20-fold less sensitive to cAMP and less cooperative with respect to cAMP binding; and (*d*) the total PKA activity at maximal cAMP concentration was relatively preserved. Therefore, the phenotype of the transfected OK-HL population is that expected from dominant expression of the R^{I*} unit. In this sense dominant indicates that although a normally cAMP-sensitive R^I unit was present, the observed cAMP sensitivity was that characteristic of the less cAMP-sensitive mutant R^{I*} unit. Therefore, the overexpression is phenotypically dominant (15, 16). Having induced relative cAMP resistance, we evaluated the effect of agonists on Na-phosphate cotransport.

Effects of cAMP-insensitive PKA on PTH- and cAMP-induced inhibition of Na-phosphate cotransport. Preliminary experiments indicated that the PTH effect to decrease Na-phosphate cotransport was maximal between 3 and 4 h, and therefore all experiments were done at 4 h. OK-wt and OK-HL cells were exposed to PTH, 1 mM cAMP, 250 μg/ml oleoyl-acetyl glycerol (OAG), or 10^{-7} M phorbol myristate acetate (PMA) after which Na-phosphate cotransport was measured. Fig. 4 (*top*) shows the effect of these agonists on Na-phosphate cotransport in OK-wt cells. PTH produced a dose-dependent inhibition of Na-phosphate cotransport with maximal inhibition of 28% of transport occurring at 1,000 nM PTH and half-maximal inhibition between 1 and 10 nM. The maximal concentrations of cyclic AMP, OAG, and PMA used in these studies each decreased Na-phosphate cotransport by over 50%. In contrast, the responses of OK-HL cells to maximal concentrations of these agonists is shown in Fig. 4 (*bottom*). In these cells, expressing a dominant cAMP-resistance mutation, there was no effect of 1 mM cAMP or of 1,000 nM PTH. Lower

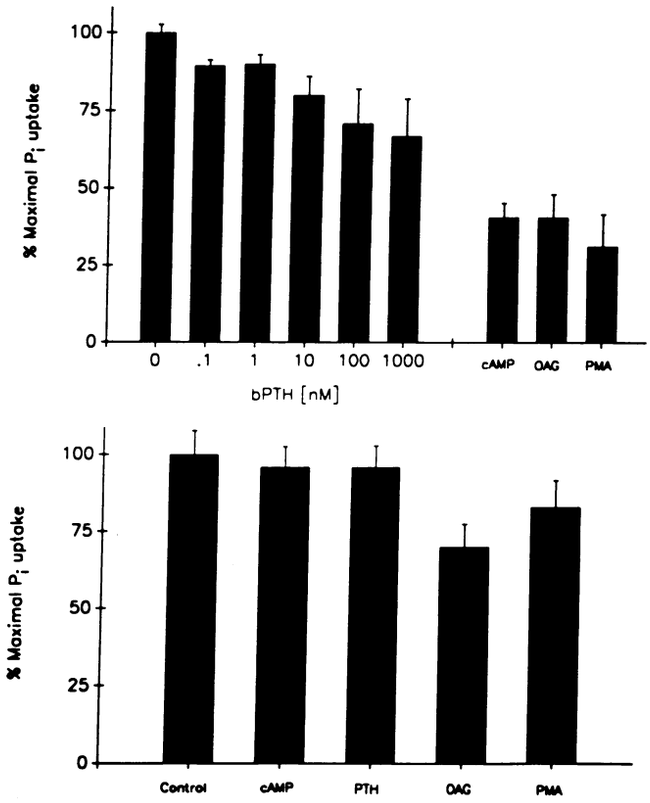

Figure 4. (*Top*) Effect of agonists on Na-phosphate cotransport in OK-wt cells. OK cells were incubated with 1-34 bPTH in the concentrations shown as well as 8-Br-cAMP, 1 mM; tetradecanoyl phorbol acetate (PMA), 10^{-7} M; and oleoyl acetyl glycerol (OAG), 250 μg/ml, for 4 h. The Na-dependent component of phosphate uptake was determined as described in 24-well clusters. Data points represent three sets of 4-well uptakes. Error bars represent standard error. (*Bottom*) Effect of agonists on Na-phosphate cotransport in OK-HL cells. The experiments were performed as in the upper panel except that OK-HL cells were used.

concentrations of PTH (data not shown) also had no effect on Na-phosphate cotransport. In other experiments OK-HL cells were exposed to maximal concentrations of cAMP or PTH for 6 h without an inhibition of Na-phosphate cotransport. OAG and PMA did decrease Na-phosphate cotransport significantly, but with a smaller effect than in the OK-wt cells.

In postulating that we had interrupted PTH effect by expressing this mutation, it was necessary to demonstrate that the OK-HL population had PTH receptor coupled to adenylate cyclase. Fig. 5 shows the cell cAMP content of OK-wt and OK-HL cells 5 min after addition of increasing PTH concentrations. The cAMP-generation curves of both populations are comparable with a half-maximal cAMP production between 1 and 10 nM. The kinetics of Na-phosphate cotransport were also determined. At 100 mM external Na, the K_m for phosphate was 99 and 131 μM for phosphate in OK-wt and OK-HL cells, respectively; the V_{max} was also comparable. Therefore, the Na-phosphate cotransporter appeared to be similar in both cell types with respect to external phosphate kinetics and maximal transport activity.

Role of activators of PKC and Ca_i in the PTH-induced alterations in Na-phosphate cotransport. Both OAG and protein kinase C-activating phorbol esters have been shown to decrease Na-phosphate cotransport in the OK cell suggesting that activation of PKC could play a role in this PTH action. PTH exposure has been shown in some studies to increase the release of labeled inositol Tris phosphate (IP_3) and diacylglycerol over a short (30 s–5 min) time course (4–6). In order to determine whether these cells possessed a PTH-associated pathway capable of activating protein kinase C, we measured the total diacylglycerol content and ^{3}H-IP_3 release from OK-wt and OK-HL cells after PTH exposure.

Total diacylglycerol (DAG) content of both OK-wt and OK-HL cells was measured 10 s after addition to either a control medium or 100 nM PTH. In five paired experiments on OK-wt cells, the basal DAG content was 2,582±191 (SEM) pmol/mg protein. PTH treatment increased measured DAG by 27±6% (SEM) (P, 0.01; paired t test). Similarly, in OK-HL cells (basal DAG 1,972±207 pmol/mg protein, n, 3) 10 s of PTH exposure increased DAG by 20±2% (P, .002; paired t test). Thus DAG, a potent activator of PKC, was released over a rapid time course after PTH addition. Two groups have reported increments in DAG release in the range of 40–100% from OK cells in response to PTH (4, 5). These studies were performed by evaluating the appearance of radiolabeled arachidonate from prelabeled cells. It is not clear how the smaller increment in total DAG mass we found relates to the larger increment in the arachidonate-labeled pool which was found by others.

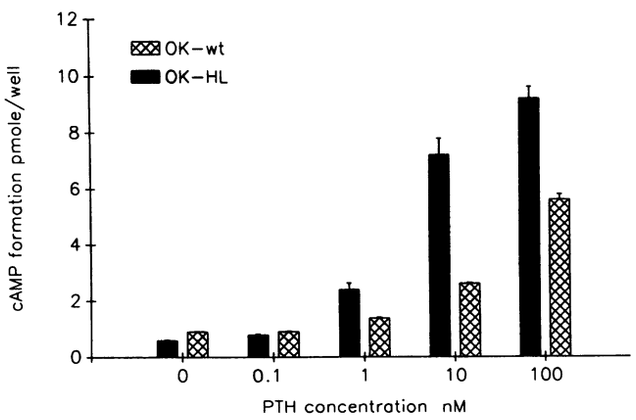

Figure 5. PTH-sensitive cAMP production from OK-wt and OK-HL cells. OK-wt and OK-HL cells were grown in multiwell clusters and exposed to the indicated concentrations of PTH for 5 min. The cells were rinsed and intracellular cAMP was recovered and determined as described in Methods. The data points represent at least 8 wells per point.

Inositol phosphates were determined in OK-wt and OK-HL cells metabolically labeled with 10 μCi/ml ^{3}H-*myo*-inositol for 72 h in inositol-free medium containing 10% dialyzed fetal bovine serum. After labeling, cells were exposed to PTH (1 μM) for 45 s and 5 min followed by inactivation with trichloroacetic acid. The content of ^{3}H activity coeluting with IP_2 and IP_3 was determined by ion exchange chromatography. In both the OK-wt and OK-HL cells, we were unable to determine a change in the ^{3}H elution profile with PTH treatment of either 45 s or 5 min. In several experiments, we found no PTH-induced increment in IP_2 or IP_3 release in either the clonally derived OK-wt cells or in the OK-HL transfectants.

PTH has also been reported to be accompanied by a small increase in intracellular calcium in the OK cell. This presumably arises via an IP_3-mediated mechanism. However, recent evidence suggests that changes in Na-phosphate cotransport occur independently of these fluxes. Even though we were unable to measure an IP_3 increment, we determined whether a calcium flux was retained in these cells. OK-wt cells and OK-HL cells were grown on glass coverslips and intracellular calcium (Ca_i) was directly measured by fura II fluorescence (29). In OK-wt cells addition of 100 nM PTH produced an increment in Ca_i of 75–130 nM at 45 s, and slowly returned towards baseline. In the OK-HL cells, a smaller increment in Ca_i occurred after a similar time course. However, this smaller transient did not return to baseline during the observation period. Thus in both OK-wt and OK-HL cells, 100 nM PTH produced a small increment in intracellular calcium over a brief time course; however, over a 2-min period, Ca_i remained elevated in the OK-HL cells. It is unclear whether the very small Ca_i transient was the result of an IP_3 transient we were unable to measure, or to other mechanisms.

The transfection-mediated overexpression of the mutant R^{I*} subunit proved to be unstable over extended passages. We initially elected not to isolate single cell subclones of transfected cells because previous experiments (data not included) involving expression of other exogenous genes in OK cells had demonstrated that loss of expression occurred over a similar number of passages when no selective pressure for expression existed. This was found to be true both in whole populations and single-cell subclones. In addition to loss of expression related to the instability of transfected genes, the cAMP-resistant phenotype would seem to be disadvantaged because of a slower growth rate than the wild type. The later passages of revertant OK-HL cells remained resistant to G418, the selection for the cotransfected NEO marker, yet expression of the mutant phenotype was lost. This emphasizes that the overexpressed R^{I*} phenotype was selectively disadvantaged over the wild type, probably because of its slower growth rate. After $\approx$ 35 passages, the population of OK-HL cells reverted to the parental phenotype; its growth rate accelerated and became comparable to OK-wt cells, and the cAMP sensitivity of PKA

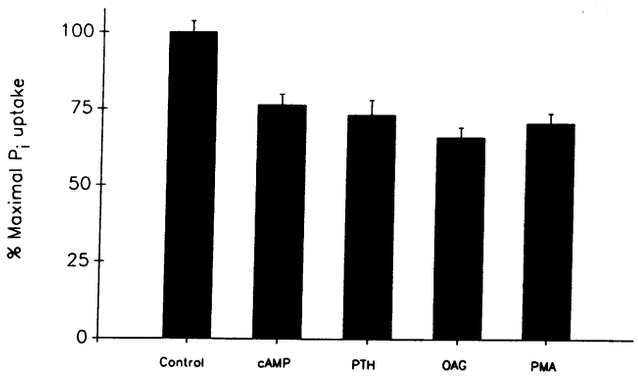

Figure 6. Response of Na-phosphate cotransport in OK-HL cells which had reverted to parental phenotype. Na-phosphate cotransport was determined as described in the previous figures in OK-HL cells whose PKA phenotype had reverted to parental cAMP sensitivity after prolonged passage. The agonist concentrations and exposure times are as described in Fig. 4.

reverted to the parental sensitivity, displaying half-maximal activation at 0.13 μM cAMP. The sensitivity of Na-phosphate cotransport to PTH and cAMP in these revertant cells is depicted in Fig. 6. Thus, expression of cAMP resistance of PKA was associated with a loss of both cAMP and PTH sensitivity; reversion of this mutation restoring cAMP sensitivity of PKA restored the cAMP and PTH sensitivity of Na-phosphate cotransport. This reversion persisted despite continued selection in G418. This finding strongly supports the proposition that the essential differences between the OK-HL population and the parental OK-wt cells were expression of the R^{I*} and cAMP desensitization, and not other features which might have been unknowingly selected for. The reversion we evaluated was fortuitous. We initially attempted to use the heavy metal inducible metallothionein promoter construct reported by McKnight et al. (16) and recently exploited by Bringhurst et al. (30) in PTH-responsive osteosarcoma cells, to bring about a rapid induction of cAMP resistance by adding the inducer zinc. However, we found that in OK cells, the metallothionein constructs expressed poorly. Therefore, this strategy could not be used to define, over a short time course, the isolated role of PKA.

Discussion

The essential observation of this study is that induction of relative biological insensitivity to cAMP abolishes both the PTH and cAMP inhibition of Na-phosphate cotransport in the OK cell line. Our conclusion is that an intact and normally cAMP-responsive PKA pathway is mandatory for the PTH inhibition of Na-phosphate cotransport. A corollary is that if non-cAMP-associated pathways, such as PKC or Ca_i, are involved in this process, their inhibition of Na-phosphate cotransport requires PKA activity normally responsive to cAMP.

The OK cell has been extensively investigated as a model for hormone modulation of membrane transport in which PTH and cAMP inhibit both Na/H exchange and Na-phosphate cotransport. Two general lines of evidence have been cited to suggest that non-cAMP-mediated pathways may be involved in the PTH effect. First, although cAMP mimics the PTH inhibition of transport, a persistent observation is the disparity between the dose response curves for PTH-associated cAMP generation and PTH effects on transport; concentrations of PTH which do not produce a measurable rise in cellular cAMP content may affect OK cell Na/H exchange and Na-phosphate cotransport. In this vein, Cole et al. observed that hormones such as vasoactive intestinal polypeptide raise OK-cell cAMP but do not, or only minimally, inhibit transport (8). In another study, these same authors also identified clonal lines of the OK cell with an intact PTH-coupled adenylate cyclase yet no alterations of Na-phosphate cotransport in response to PTH or cAMP, leading them to conclude that other signaling mechanisms must be involved in the PTH effect (31).

Martin et al. offer a rebuttal to arguments which center on the disparity between the PTH dose response on cAMP generation and the inhibition of Na-phosphate cotransport (9). These authors determined the relationship between inhibition of Na-phosphate cotransport and the PKA activity ratio in response to PTH and antagonists of PTH action. The PKA activity ratio (the ratio between free catalytic C activity and inactive but recruitable R_2C_2) is a measure of the degree of "activation" of PKA by cAMP. They used 2',5'-dideoxyadenosine (DDA), an inhibitor of cAMP formation, and 3-34-[Nle^8, Nle^{18}, Tyr^{34}]bPTH amide (3-34-bPTH), a PTH analogue that results in no measurable cAMP formation. These authors determined that inhibition of Na-phosphate cotransport was unrelated to the amount of intracellular cAMP generated in response to agonists but was proportional to the alteration in PKA activity ratio, the degree of activation of PKA. Using PTH plus DDA or 3-34-bPTH alone, PKA was clearly activated in the presence of reduced or unmeasurable increments of cAMP generation. The finding of this study was that there was cAMP effect in the absence of large or detectable changes in intracellular cAMP. In this setting, PKA activation is likely to be more reflective of the effect of intracellular cAMP than is the measurement of the cAMP level itself. Accordingly, these authors suggest that a PKA-independent mechanism need not be invoked to explain the disparity between dose-response curves for cAMP generation and for inhibition of Na-phosphate cotransport.

Protein kinase C or intracellular calcium mediation of PTH effects has been suggested for several reasons. PTH produces a small, rapid turnover of IP_3 and diacylglycerol release in OK cells associated with a small intracellular calcium transient (4–7). Further, a brief activation of PKC, measured as a partition from soluble to membrane-bound form, has been reported to occur seconds after addition of PTH to OK cells (32). In some studies, an increase in IP_3 turnover and release of diacylglycerol are evident at sub-nanomolar PTH concentrations, below those at which an elevation of cAMP content is present. (Although we found a significant increase in total DAG after PTH, it should be noted that we found no increment in IP_3 release and that other laboratories have also had difficulty in demonstrating this effect [Dr. R. A. Nissenson, UCSF, personal communication]). Since partial inhibition of Na-phosphate cotransport is evident at these low PTH concentrations, it has been suggested that PKC is the primary mediator of PTH inhibition of Na-phosphate cotransport and the role of PKA has been questioned (5).

While there is no question that activators of protein kinase C (phorbol esters and permeable diacyl glycerol) inhibit Na-phosphate cotransport in the OK cell, the evidence that PKC

activation is necessary for the PTH effect is based on two less convincing observations. Quamme et al. (6) noted that staurosporine, a fungal protein kinase inhibitor, abolished the PTH effect on Na-phosphate cotransport in a dose-related manner and attributed this to inhibition of PKC, based on staurosporine's greater specificity for PKC. However, the primary data in the literature suggest that staurosporine is a relatively nonselective inhibitor of both PKA and PKC; the K_i of staurosporine for PKA and PKC differs by less than an order of magnitude (33, 34). At the concentrations reported, both PKA and PKC would be inhibited. Therefore, staurosporine does not seem a useful probe to differentiate the action of these two kinases.

In the same report, it was found that PKC downregulation by prolonged phorbol ester incubation rendered the OK cell Na-phosphate cotransporter insensitive to all agonists including PTH, cAMP, and phorbol ester rechallenge. This finding indicates that an intact PKC pathway is needed for modulation of Na-phosphate cotransport by PTH, cAMP, and, trivially, activators of PKC. However, rather than acting as a pharmacological or kinetic inhibitor, phorbol ester downregulation of PKC is associated with marked absolute loss of most PKC activity and protein (35, 36). As noted by these authors, this maneuver does not separate the necessity for PTH-induced PKC activation from the need for a basal level of PKC activity.

The intervention described in this study is the induction of a decrease in the cAMP sensitivity of PKA by expression of a cAMP-insensitive PKA regulatory unit. We pursued this approach to avoid some of the ambiguities inherent with the use of pharmacological kinase inhibitors. The nature of the overexpressed R^{I*} subunit and the phenotype of the resultant PKA reported herein was fortuitous; even though the mutant R^{I*} unit was overexpressed and the cAMP-sensitivity of PKA reduced, the total PKA activity (at maximal cAMP) was relatively preserved. The mechanisms of cAMP insensitivity presumably reflect several features of PKA biology. First, since the mutant R^{I*} was $\approx$ 20-fold overexpressed relative to wild type R^I, it was likely that most of the R_2C_2 tetramers would contain the mutant. This suggestion is borne out by the ion exchange elution profile of intact PKA reported. This degree of overexpression may not have been necessary to attain this effect. S49 lymphoma mutants with allelic expression of equal amounts of normal and defective R units also express decreased kinase activity (15). Second, this complex was more stable, less likely to dissociate by virtue of a decreased cAMP sensitivity; a 20-fold increase in the cAMP concentration needed for half-maximal activation of PKA. This feature probably explains the greater recovery of intact PKA tetramers by ion exchange chromatography in the OK-HL cells; PKA from the mutants was less likely to dissociate during preparation and more likely to be recovered from the ion exchange column. Finally, as evidenced by a decreased Hill coefficient for cAMP activation, lack of intrasubunit cooperativity results in a smaller increment in PKA activity for a given increment in cAMP concentration; a decrease in biological amplification of cAMP effect.

A potential question that arises is why no inhibition of Na-phosphate cotransport was evident in OK-HL cells at very high 8-Br-cAMP concentrations (1 mM) or 1,000 μM PTH even though the in vitro sensitivity of PKA was decreased only 20-fold. Although this question is not directly answerable by the experiments reported herein, several points should be considered. First, although PTH induces a dose-responsive increment in measured cell cAMP, these determinations are usually made in the presence of phosphodiesterase inhibitors. In the original description of the OK cell PTH-responsive adenylate cyclase, Teitelbaum et al. (37) noted that in the absence of phosphodiesterase inhibitors, only a very small, very transient increment in cellular cAMP content could be measured even with maximal PTH concentrations. Given the increase in $EC_{.5}$ for cAMP we measured in vitro and the decrease in cooperativity of activation, it is possible that even high concentrations of PTH did not induce a sufficient rise in cell cAMP to activate the mutant PKA. Second, the degree of overexpression of R^{I*} relative to the native R^I is estimated to be $\approx$ 20-fold. In normal cells R and C are expressed in roughly equal proportions. In the OK-HL cell population reported here, the overexpression of R^{I*} was unaccompanied by an overexpression of C as evidenced by the roughly normal total PKA activity measured at maximal cAMP concentrations. Therefore there was probably a large pool of free R^{I*} units capable of reforming stable complexes with C, making the release of free C (the cAMP effector) less likely.[3] Thus the mutant cells described herein retained potential PKA activity while demonstrating relative cAMP resistance. This is in contrast to the maneuver of PKC-inhibition phorbol ester downregulation; a setting in which almost all potential PKC activity is lost from the pool of recruitable kinase.

There is a large literature dealing with interactions of the PKA and PKC-signaling pathways and precedent is available for virtually every permutation of interaction. Many of these events involve PKA inhibition of PKC effect and vice versa (38–40). More germane to the present studies are several examples in which activation of either PKA or PKC may independently produce the same or similar biological effect without an absolute requirement for activation of the complementary pathway (41–46). PTH inhibition of Na-phosphate cotransport in the OK cell may represent a situation in which two distinctly controlled kinase activities produce the same biological effect. In summary, exogenous agonists that directly and specifically activate either PKA or PKC result in an inhibition of OK cell Na-phosphate cotransport supporting the contention that two potential mechanisms for this effect exist in the OK cell. The finding that in transfected, cAMP-resistant cells Na-phosphate cotransport is unresponsive to maximal levels of cAMP and a range of PTH concentrations suggests that an intact and normally cAMP-sensitive PKA pathway is an absolute requirement for the PTH effect on Na-phosphate cotransport. Loss of the cAMP-resistant phenotype with repeated passage was associated with a return of cAMP and PTH sensitivity, strongly suggesting that the perturbation of the

3. This large pool of free R^{I*} units would also have been present in the in vitro assay mixture in which PKA activation was measured. Operationally, given the previously determined intracellular vol/mg protein of OK cells (2) and volumes used for cell disruption and the dilution factor in the actual PKA assay, the R^I, R^{I*}, and C units contained in a cell monolayer would have been diluted 80–100-fold from their distribution in total cell water, by the time of PKA assay. Although we do not address the kinetic mechanism of association, the formation of an R_2C_2 complex, a quaternary-molecular reaction, presumably shows a greater than first order dependence on R and C concentration. Therefore, the degree of intracellular cAMP resistance, in which R and C concentrations were higher, may have been greater than the $\approx$ 20-fold decrease in PKA sensitivity measured in the diluted in vitro system.

PKA system was responsible for these effects. Definition of the independent role, if any, of PKC in the PTH action on OK cell Na-phosphate cotransport was not unequivocally determined by these studies and must await the availability of more specific tools.

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References

1. Caverzasio, J., and J. P. Bonjour. 1985. Inhibition of phosphate transport by parathyroid hormone in the opossum kidney cell line. *Kidney Int.* 27:111a. (Abstr.)

2. Pollock, A. S., D. G. Warnock, and G. J. Strewler. 1985. Parathyroid hormone inhibition of Na+/H+ antiporter activity in a cultured renal cell line. *Am. J. Physiol.* 250:F217–F235.

3. Miller, R. T., and A. S. Pollock. 1987. Modification of the internal pH sensitivity of the Na/H antiporter by parathyroid hormone in a cultured renal cell line. *J. Biol. Chem.* 262:9115–9120.

4. Hruska, K. A., D. Moskowitz, P. Esbrit, R. Civitelli, S. Westbrook, and M. Huskey. 1987. Stimulation of inositol trisphosphate and diacylglycerol production in renal tubular cells by parathyroid hormone. *J. Clin. Invest.* 79:230–239.

5. Quamme, G., J. Pfeilschifter, and H. Murer. 1989. Parathyroid hormone inhibition of Na/phosphate cotransport in OK cells: generation of second messengers in the regulatory cascade. *Biochem. Biophys. Res. Commun.* 158:951–957.

6. Quamme, G., J. Pfeilschifter, and H. Murer. 1989. Parathyroid hormone inhibition of Na/phosphate cotransport in OK cells: requirement of protein kinase-C dependent pathway. *Biochim. Biophys. Acta.* 1013:159–165.

7. Quamme, G., J. Pfeilschifter, and H. Murer. 1989. Parathyroid hormone inhibition of Na/phosphate cotransport in OK cells: intracellular [Ca2+] as a second messenger. *Biochim. Biophys. Acta.* 1013:166–172.

8. Cole, J. A., L. R. Forte, S. Eber, P. K. Thorne, and R. E. Poelling. 1988. Regulation of sodium-dependent phosphate transport by parathyroid hormone in opossum kidney cells: adenosine 3',5'-monophosphate-dependent and independent mechanisms. *Endocrinology.* 122:2981–2989.

9. Martin, K. J., C. L. McKonkey, J. C. Garcia, D. Montani, and C. R. Betts. 1989. Protein kinase-A and the effects of parathyroid hormone on phosphate uptake in opossum kidney cells. *Endocrinology.* 125:295–301.

10. Middleton, J. P., C. B. Dunham, J. J. Onorato, D. A. Sens, and V. W. Dennis. 1989. Protein kinase A, cytosolic calcium, and phosphate uptake in human proximal renal cells. *Am. J. Physiol.* 257:F631–F638.

11. Taylor, S. S., and P. H. Stafford. 1978. Characterization of adenosine 3',5'-monophosphate dependent protein kinase and its dissociated subunits from porcine skeletal muscle. *J. Biol. Chem.* 253:2284–2287.

12. Ogreid, D., S. O. Doskeland, K. B. Groman, and R. A. Steinberg. 1988. Mutations that prevent cyclic nucleotide binding to binding sites A or B of type I cyclic AMP-dependent protein kinase. *J. Biol. Chem.* 263:17397–17404.

13. Singh, T. J., J. Hochman, R. Verna, M. Chapman, I. Abraham, I. H. Pastan, and M. M. Gottesman. 1985. Characterization of a cyclic AMP-resistant Chinese hamster ovary cell mutant containing both wild-type and mutant species of type I regulatory subunit of cyclic AMP-dependent protein kinase. *J. Biol. Chem.* 260:13927–13933.

14. Murphy, C. A., and R. A. Steinberg. 1985. Hotspots for spontaneous and mutagen-induced lesions in regulatory subunit of cyclic AMP-dependent protein kinase in S49 mouse lymphoma cells. *Somatic Cell Mol. Genet.* 11:605–615.

15. Steinberg, R. A., P. H. O'Farrell, U. Freidrich, and P. Coffino. 1977. Mutations causing charge alterations in regulatory subunits of the cAMP-dependent protein kinase of cultured S49 lymphoma cells. *Cell.* 10:381–391.

16. Clegg, C. H., L. A. Correll, G. G. Cadd, and G. S. McKnight. 1987. Inhibition of intracellular cAMP-dependent protein kinase using mutant genes of the regulatory type I subunit. *J. Biol. Chem.* 262:13111–13119.

17. Woodford, T. A., L. A. Correll, G. S. McKnight, and J. D. Corbin. 1989. Expression and characterization of mutant forms of the type I regulatory subunit of cAMP-dependent protein kinase. *J. Biol. Chem.* 264:13321–13328.

18. Mellon, P. A., C. A. Clegg, L. A. Correll, and G. S. McKnight. 1989. Regulation of transcription by cyclic AMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA.* 86:4887–4891.

19. Chu, G., H. Hayakawa, and P. Berg. 1987. Electroporation for the efficient transfection of mammalian cells with DNA. *Nucleic Acids Res.* 15:1311–1326.

20. Southern, P. J., and P. Berg. 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early promoter region. *J. Mol. App. Genet.* 1:327–341.

21. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.

22. Kemp, B. E., D. J. Graves, E. Benjamini, and E. G. Krebs. 1977. Role of multiple basic residues in determining the substrate specificity of cyclic AMP-dependent protein kinase. *J. Biol. Chem.* 252:4888–4894.

23. Maller, J. L., B. E. Kemp, and E. G. Krebs. 1978. In vivo phosphorylation of a synthetic peptide substrate of cyclic AMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA.* 75:248–251.

24. Preis, J. E., C. R. Loomis, R. M. Bell, and J. E. Neidel. 1987. Quantitative measurement of sn-1,2-diacylglycerol. *Methods Enzymol.* 141:294–300.

25. Rannels, S. R., C. E. Cobb, L. R. Landiss, and J. D. Corbin. 1985. The regulatory subunit monomer of cAMP-dependent protein kinase retains salient kinetic properties of the native dimeric subunit. *J. Biol. Chem.* 260:2432–3430.

26. Walter, U., P. Kanof, H. Schulman, and P. Greengard. 1978. Adenosine 3':5'-monophosphate receptor proteins in mammalian brain. *J. Biol. Chem.* 253:6275–6280.

27. Walter, U., I. Uno, A. Y. C. Liu, and P. Greengard. 1977. Study of autophosphorylation of isoenzymes of cyclic AMP-dependent protein kinases. *J. Biol. Chem.* 252:6588–6590.

28. Saraswat, L. D., M. Filutowicz, and S. B. Taylor. 1986. Expression of the type I regulatory subunit of cAMP-dependent protein kinase in Escherichia coli. *J. Biol. Chem.* 261:11091–11096.

29. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca2+ indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440–3450.

30. Bringhurst, F. R., J. D. Zajac, A. S. Daggett, R. N. Skurat, and H. M. Kronenberg. 1989. Inhibition of parathyroid hormone responsiveness in clonal osteoblast cells expressing a mutant form of a 3',5'-cyclic adenosine monophosphate-dependent protein kinase. *Mol. Endocrinol.* 3:60–67.

31. Cole, J. A., L. R. Forte, W. J. Krause, and P. K. Thorne. 1989.

Clonal sublines that are morphologically and functionally distinct from parental OK cells. *Am. J. Physiol.* 256:F672–F679.

32. Tamura, T., H. Sakamoto, and C. R. Filburn. 1989. Parathyroid hormone 1-34, but not 3-34 or 7-34, transiently translocates protein kinase C in cultured renal OK cells. *Biochem. Biophys. Res. Commun.* 159:1352–1358.

33. Meyer, T., U. Regenass, D. Fabro, E. Alteri, J. Rosel, M. Muller, G. Caravatti, and A. Matter. 1989. A derivative of staurosporine (CGP 41 251) shows selectivity for protein kinase C inhibition and in vitro anti-proliferative as well as in vivo anti-tumor activity. *Int. J. Cancer.* 43:851–856.

34. Ruegg, U. T., and G. M. Burgess. 1989. Staurosporine, K-252 and UCN-01: potent but non-specific inhibitors of protein kinases. *Trends Pharmacol. Sci.* 10:218–220.

35. Ballester, R., and O. M. Rosen. 1985. Fate of immunoprecipitable protein kinase C in GH3 cells treated with phorbol 12 myristate 13-acetate. *J. Biol. Chem.* 260:15194–15199.

36. Rodriguez-Pena, A., and E. Rozengurt. 1984. Disappearance of Ca-sensitive, phospholipid-dependent protein kinase activity in phrobol ester-treated 3T3 cells. *Biochem. Biophys. Res. Commun.* 120:1053–1059.

37. Teitelbaum, A. P., and G. J. Strewler. 1984. Parathyroid hormone receptors coupled to cyclic adenosine monophosphate formation in an established renal cell line. *Endocrinology.* 114:980–985.

38. Otte, A. P., P. Van-Run, M. Heideveld, R. Van-Driel, and A. J. Durston. 1989. Neural induction is mediated by cross-talk between protein kinase C and cyclic AMP pathways. *Cell.* 58:641–648.

39. Supattapone, S., S. K. Sanoff, A. Theibert, S. K. Joseph, J. Steiner, and S. H. Snyder. 1988. Cyclic AMP-dependent phosphorylation of a brain inositol triphosphate receptor decreases its release of calcium. *Proc. Natl. Acad. Sci. USA.* 85:8747–8750.

40. Dawson, G., and P. McAtee. 1989. Differential regulation of basic protein phosphorylation by calcium phospholipid and cyclic AMP-dependent protein kinases. *J. Cell. Biochem.* 40:261–269.

41. Kitamura, K., K. Knagawa, H. Matsuo, and K. Uyeda. 1988. Phosphorylation of myocardial fructose-6-phosphate,2-kinase:fructose-2,6-bisphosphatase by cAMP-dependent protein kinase and protein kinase C. *J. Biol. Chem.* 263:16796–16801.

42. Shirakawa, F., and S. B. Mizel. 1989. In vitro activation and nuclear translocation of NF-κB catalyzed by cyclic AMP-dependent protein kinase and protein kinase C. *Mol. Cell Biol.* 9:2424–2430.

43. Issaad, C., M. A. Ventura, and P. Thomopoulos. 1989. Biphasic regulation of macrophage attachment by activators of cyclic adenosine monophosphate-dependent kinase and protein kinase C. *J. Cell Physiol.* 140:317–322.

44. Jahn, H., W. Nastainczyk, A. Rohrkasten, T. Schneider, and F. Hofmann. 1988. Site-specific phosphorylation of the purified receptor for calcium-channel blockers by cAMP- and cGMP-dependent protein kinases, protein kinase C, calmodulin-dependent protein kinase II and casein kinase II. *Eur. J. Biochem.* 178:535–542.

45. Waseem, A., and H. C. Plafrey. 1988. Erythrocyte adducin. *Eur. J. Biochem.* 178:563–573.

46. Ling, E., Y. N. Danilov, and C. M. Cohen. 1988. Modulation of red cell band 4.1 function by cAMP-dependent kinase and protein kinase C phosphorylation. *J. Biol. Chem.* 263:2209–2216.