cAMP-dependent and -independent downregulation of type II Na-Pi cotransporters by PTH

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PFister, Markus F., JUTKA FORGO, Urs ZIEGLER, Jürg BIBER, and Heini MURER. cAMP-dependent and -independent downregulation of type II Na-Pi cotransporters by PTH. Am. J. Physiol. 276 (Renal Physiol. 45): F720–F725, 1999.—Parathyroid hormone (PTH) leads to the inhibition of Na-Pi cotransport activity and to the downregulation of the number of type II Na-Pi cotransporters in proximal tubules, as well as in opossum kidney (OK) cells. PTH is known also to lead to an activation of adenylyl cyclase and phospholipase C in proximal tubular preparations, as well as in OK cells. In the present study, we investigated the involvement of these two regulatory pathways in OK cells in the PTH-dependent downregulation of the number of type II Na-Pi cotransporters. We have addressed this issue by using pharmacological activators of protein kinase A (PKA) and protein kinase C (PKC), i.e., 8-bromo-cAMP (8-BrCAMP) and β-12-O-tetradeanol/phorbol 13-acetate (β-TPA), respectively, as well as by the use of synthetic peptide fragments of PTH that activate adenylyl cyclase and/or phospholipase C, i.e., PTH-(1–34) and PTH-(3–34), respectively. Our results show that PTH signal transduction via cAMP-dependent, as well as cAMP-independent, pathways leads to a membrane retrieval and degradation of type II Na-Pi cotransporters and, thereby, to the inhibition of Na-Pi cotransport activity. Thereby, the cAMP-independent regulatory pathway leads only to partial effects (∼50%).

opossum kidney cells; protein kinase A; protein kinase C; phorbol ester; parathyroid hormone (3–34)

PARATHYROID HORMONE (PTH) is an important regulator of renal proximal tubular P i reabsorption. By interacting with the PTH/PTH-related peptide receptor, PTH leads to the acute inhibition of the sodium-dependent P i transport across the proximal tubular brush-border membrane (11, 12). Inhibition of the proximal tubular Na-P i cotransport by PTH is characterized by a decrease in the maximal transport rate (V max), as well as by a decreased expression of the type II Na-P i cotransporter in the brush borders of renal proximal tubules (6). Recent experiments on opossum kidney (OK) cells, a cell line exhibiting many of the characteristics of renal proximal tubule cells (2, 14–16, 19–24), provided insight into mechanisms involved in PTH-dependent regulation of Na-P i cotransport activity. It was found that PTH leads to the endocytosis and lysosomal degradation of the type II Na-P i cotransporter and, thereby, to the inhibition of the apical Na-P i cotransport activity (8, 15, 16). Recent experiments provided evidence that PTH also leads to the endocytosis and lysosomal degradation of the type II Na-P i cotransporter in vivo in rat renal proximal tubules (7).

Intracellular signaling pathways leading to the inhibition of the proximal tubular Na-P i cotransport and to the downregulation of the number of type II Na-P i cotransporters by PTH are incompletely understood. Experiments on renal proximal tubular preparations and OK cells provided firm evidence for PTH-mediated stimulation of adenylyl cyclase (1, 13) and phospholipase C/protein kinase C (PKC) (1, 5, 13). Moreover, it was found that, in OK cells, the inhibitory effect of PTH on apical Na-P i cotransport activity can be mimicked by 8-bromo-cAMP (8-BrCAMP) and phorbol esters, pharmacological activators of protein kinase A (PKA) and PKC, respectively (3, 4, 8, 9, 17). It has also been shown that the PTH fragment PTH-(3–34) is able to inhibit OK cell Na-P i cotransport activity through cAMP-independent pathways; however, maximal inhibition induced by PTH-(3–34) is significantly less (∼50%) than that obtained by exposure to PTH-(1–34) (3, 4, 8). From these data, it has been inferred that inhibition of the apical Na-P i cotransport by PTH is likely to involve activation of adenylyl cyclase/PKA and/or phospholipase C/PKC. In the present study, we examined the involvement of these two regulatory pathways in the PTH-dependent downregulation of the number of type II Na-P i cotransporters in OK cells.

MATERIALS AND METHODS

Cells. All cell culture supplies were obtained from Life Technologies (Basel, Switzerland). OK cells (clone 3B/2) were maintained in DMEM/Ham's F-12 medium (1:1), supplemented with 10% FCS, 22 mM NaHCO 3, 20 mM HEPES, and 2 mM L-glutamine in a humidified atmosphere of 95% air-5% CO 2 at 37°C.

SDS-PAGE and immunoblotting. SDS-PAGE and immunoblotting were performed as previously described (16). For preparation of total cellular homogenates, cells were grown to confluency on 6-cm petri dishes (Corning), washed twice with PBS, and scraped into 2 ml of PBS. The scraped cells were centrifuged for 10 min at 3,000 rpm in an Eppendorf centrifuge at 4°C. The supernatant was sucked off and 250 µl of 50 mM mannitol, 10 mM HEPES-Tris, pH 7.2, was added to each sample. The samples were homogenized 10 times with a 1-ml syringe connected to a 25-gauge needle. Twenty-five micrograms of total protein of cell homogenates were used for SDS-PAGE (9%) and subsequent transfer to nitrocellulose (0.45 µm; Schleicher and Schuell). We blocked nonspecific binding by incubating the nitrocellulose at room temperature for 2 h in TBS (0.9% NaCl, 10 mM Tris-HCl, pH 7.4)
containing 5% nonfat dry milk and 1% Triton X-100 (Blotto-TX-100, pH 7.4). We detected the type II Na-Pi cotransporter (NaPi-4) using a polyclonal antiserum raised against the COOH-terminal 12 amino acids of the published NaPi-4 sequence (antiserum dilution 1:4,000) (15, 16, 24). Incubation with the primary antibody took place overnight at 4°C. The nitrocellulose was washed four times with TBS/10% Blotto-TX-100 (pH 7.4) and incubated for 1 h with Blotto-TX-100 (pH 7.4) at room temperature. Thereafter, the nitrocellulose was incubated with a 1:10,000 dilution of an anti-rabbit IgG labeled with horseradish peroxidase (Amersham) in Blotto-TX-100 (pH 7.4) for 2 h at room temperature. The nitrocellulose was washed four times with TBS and the signals were detected by enhanced chemiluminescence (Amersham), according to the manufacturer’s protocol, with the use of Kodak X-OMAT AR films.

Immunofluorescence. Immunofluorescence was performed as previously described (8, 15, 16). We grew 3B/2 OK cells to confluency on coverslips. After cells were washed three times with PBS containing 0.5 mM MgCl₂ and 1 mM CaCl₂, cells were fixed for 10 min at room temperature with PBS supplemented with 3% paraformaldehyde, washed three times with PBS, incubated 10 min with 20 mM l-glycine in PBS, and washed again three times with PBS. Permeabilization was performed by an incubation for 30 min with PBS containing 0.1% saponin (PBS-saponin). After one wash with PBS-saponin, cells were incubated with anti-NaPi-4 antiserum (8, 15, 16) at a dilution of 1:100 in PBS-saponin for 1 h at room temperature and washed three times with PBS-saponin. Thereafter, the cells were incubated with a fluorescein isothiocyanate-conjugated anti-rabbit IgG (dilution 1:50; Dakopatts, Denmark) and phalloidin rhodamine (dilution 1:50; Calbiochem) in PBS-saponin. After incubation for 30 min in the dark, cells were washed three times with PBS-saponin and once with PBS. We mounted coverslips using Dako-Glycergel (Dakopatts, Denmark) plus 2.5% 1,4-diazabicyclo[2.2.2]octane (Sigma) as a fading retardant. Immunofluorescence was revealed by confocal microscopy (Zeiss laser-scanning microscope 310; Zeiss, Oberkochen, Germany).

Scanning electron microscopy. OK cell monolayers grown on coverslips were prefixed with 0.25% glutaraldehyde in 0.16 M cacodylate buffer (pH 7.2) for 30 min at room temperature, postfixed with 2% glutaraldehyde in 0.16 M cacodylate buffer for 30 min at room temperature, and washed three times with 0.16 M cacodylate buffer (pH 7.2) for 5–10 min. Thereafter, monolayers were osmicated with 1% OsO₄ in 0.16 M cacodylate buffer (pH 7.2) for 1 h at 37°C, washed three times with 0.16 M cacodylate buffer, dehydrated in an acetone series, and dried by the critical point method. The specimens were then examined in a scanning electron microscope (Philips, Eindhoven).

Phosphate uptake measurements. Na⁺-dependent transport of phosphate was measured as previously described (15).

Agonist treatment of OK cells. Incubation of OK cells with PTH, 8-BrcAMP, and β-12-O-tetradecanoylphorbol 13-acetate (β-TPA) was performed as previously described (9, 20). Briefly, 10⁻⁶ M PTH (dissolved in 10 mM acetic acid), 200 nM 8-BrcAMP (dissolved in water), or the corresponding amount of vehicle (controls) was directly added to the culture medium, and cells were incubated with these agonists for the appropriate time.

cAMP determination. Cell monolayers were exposed to hormone for 5 min in the presence of 1 mM 3-isobutyl-1-methylxanthine (IBMX). Cells were washed and disrupted as previously described (9). We determined total intracellular cAMP concentration by using the test kit from NEN Life Science Products (NEK033).

RESULTS AND DISCUSSION

Pharmacological activation of PKA or PKC mimics the inhibition of the apical Na-Pi cotransport activity by PTH in OK cells (3, 4, 8, 9, 17, 18). The PTH analog PTH-(1–34) is able to partially mimic the action of PTH-(1–34) (3, 4). However, PTH-(3–34)-induced inhibition of Na-Pi cotransport occurs in the complete absence of cellular accumulation of cAMP and is only ~50% (at a concentration of 10⁻⁶ M) compared with transport inhibition obtained at maximal concentrations of PTH (10⁻⁸ M) (3, 4). In addition to the aforementioned effects on Na-Pi cotransport, it was observed that PTH-(3–34) leads to a dose-dependent stimulation of PKC activity in OK cells in the absence of any stimulation of adenylyl cyclase activity (1). PTH-(1–34)-induced inhibition of Na-Pi cotransport in OK cells was dependent on “intact” PKA (10, 23) and PKC (18) regulatory pathways. More recently, it was shown that PTH-(1–34)-induced inhibition of OK cell apical Na-Pi cotransport is related to membrane retrieval of the specific type II Na-Pi cotransporter protein (15), followed by its lysosomal degradation (8, 16).

In the present study, we examined whether pharmacological activation of PKA or PKC mimics the down-
regulation of the number of type II Na-Pi cotransporters by PTH. Confluent OK cell monolayers were treated for 4 h with either 10^{-8} M PTH-(1–34) or 10^{-6} M PTH-(3–34), 4 h with 10^{-4} M 8-BrcAMP, 2 h with 200 nM β-TPA, or with the corresponding vehicle for the appropriate time. After these treatments, we compared Na-Pi cotransport activity with the expression of type II Na-Pi cotransporter protein.

In Fig. 1, it can be seen that treating cells with 8-BrcAMP, as well as with β-TPA, mimics not only the PTH-dependent inhibition of Na-Pi cotransport activity but also the PTH-dependent reduction in the amount of the type II Na-Pi cotransporter protein. Parallel immunofluorescence experiments (Fig. 2) revealed that, under control conditions, the type II Na-Pi cotransporter is localized at the apical membrane, within distinct clusters, and that the immunofluorescence staining for the transporter coincides with the F-actin staining at the apical surface (15). Treating cells with PTH-(1–34) (10^{-8} M, 4 h) led to an almost complete reduction in the expression of the Na-Pi cotransporter at the apical membrane of OK cells (Fig. 2). These PTH effects were mimicked in cells treated with 8-BrcAMP (10^{-4} M, 4 h) or with β-TPA (200 nM, 4 h; Fig. 2).

A comparison of the transport activity with the transporter protein content (Fig. 1) shows that PTH-(1–34) and 8-BrcAMP led to an almost complete disappearance of the transporter protein, with remaining significant transport activity. Therefore, the residual transport activity after treatment with either PTH-(1–34) or 8-BrcAMP is related to a Na-Pi cotransporter different from the type II-transporter protein, which is not under the control of this hormone. Consequently, β-TPA led only to a partial transport inhibition and membrane retrieval of the type II Na-Pi cotransporter under the conditions used, e.g., 200 nM β-TPA for 2 h (Fig. 1).

The F-actin staining in cells treated with β-TPA was markedly different from the corresponding F-actin stainings seen in cells treated either with PTH-(1–34) or 8-BrcAMP, respectively (Fig. 2). These observations suggested that treating cells with β-TPA had profound effects on cytoskeletal structures, including actin filaments, thereby also affecting the expression of the type II Na-Pi cotransporter. This was directly tested by

![Fig. 2. PTH leads to a markedly reduced expression of type II Na-Pi cotransporter (NaPi-4) at apical membrane of OK cells, an effect that is mimicked by treatment of OK cells with pharmacological protein kinase A (PKA) activator 8-BrcAMP. OK cells were grown to confluency on glass coverslips. Immunofluorescence stainings for NaPi-4 (left), as well as for F-actin (stained by use of phalloidin rhodamine; right), were performed as described in MATERIALS AND METHODS. Immunofluorescence pictures were obtained by confocal microscopy. Depicted focal planes represent sections through apical membrane of OK cells that were treated for 4 h with 10^{-8} M PTH-(1–34) (dissolved in 10 mM acetic acid), 4 h with 10^{-4} M 8-BrcAMP (dissolved in water), 2 h with 200 nM β-TPA (dissolved in DMSO), or with corresponding vehicle. Because treating OK cells with different vehicles did not lead to any differences in immunofluorescence stainings, only 1 control experiment is shown.](http://ajprenal.physiology.org/DownloadedFrom/10.220.32.247)
Fig. 3. Treating OK cells with β-TPA (2 h, 200 nM) leads to disappearance of distinct bundles of clustered microvilli at apical membrane, as well as to disappearance of stress fibers at basal membrane of OK cells. OK cells grown to confluency on glass coverslips were treated with or without β-TPA and further processed for immunofluorescence. Each set of pictures (control and β-TPA) consists of apical section, representing focal plane through apical membrane of OK cells, and basal section, representing focal plane through basal membrane of OK cells. Arrowheads, locations of depicted apical and basal focal planes.

Fig. 4. Treating OK cells with β-TPA (2 h, 200 nM) resulted in a markedly reduced expression of microvilli and had severe effect on tight junctions. OK cells grown to confluency on glass coverslips were treated with or without β-TPA and further processed for scanning electron microscopy, as described in MATERIALS AND METHODS. A, control; B, with β-TPA.

Fig. 5. PTH-(3–34) leads to an inhibition of Na-P_i cotransport activity (A) in absence of an increase in intracellular cAMP level (B). To determine effect of PTH-(3–34) and PTH-(1–34) on Na-P_i cotransport activity, we treated OK cells grown to confluency for 4 h with 10^{-8} M PTH-(1–34) and 10^{-6} M PTH-(3–34), respectively. To determine intracellular cAMP levels, we treated cells grown to confluency for 5 min with 10^{-8} M PTH-(1–34) or 10^{-6} M PTH-(3–34) in presence of phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX).
confocal microscopy. Each set of pictures in Fig. 3 showing staining for F-actin (control and β-TPA) consists of an apical section, representing a focal plane at the apical membrane, and a basal section, representing a focal plane at the basal membrane. Under control conditions, the apical section reveals distinct clusters related to clustered microvilli (15). The corresponding basal section reveals the stress fibers. In cells treated with 200 nM β-TPA, virtually no specific staining for F-actin can be detected at the apical membrane. On the basolateral side, an amorphous F-actin staining is observed. Stress fibers are no more detectable after treatment with β-TPA. As β-TPA had no effect (data not shown), these effects of β-TPA were likely due to pharmacological activation of PKC. Therefore, the observed β-TPA effects on transporter activity and expression (Figs. 1 and 2) could be secondary to the cytoskeletal alterations, e.g., to a reduced expression of microvilli. Indeed, a β-TPA-dependent reduction in the number of microvilli could be documented by the use of scanning electron microscopy (Fig. 4). Moreover, Fig. 4 reveals that β-TPA-treated OK cells have a spherical shape.

To test for the role of a cAMP-independent pathway in the PTH control of the type II Na-Pi cotransporter, without using phorbol esters (β-TPA), we used the PTH analog PTH-(3–34). We treated OK cells for 4 h with 10^{-6} M PTH-(3–34). In accordance with previous reports (3, 4, 8, 9), our results show that treating OK cells with PTH-(3–34) (10^{-6} M, 4 h) led to a half-maximal inhibition of Na-Pi cotransport activity (Fig. 5A), in the absence of an increase in the total cellular cAMP level (Fig. 5B). In parallel experiments, PTH-(1–34) (10^{-6} M, 4 h) lead to a maximal inhibition of Na-Pi cotransport activity (Fig. 5A), in the presence of a marked increase in the cellular cAMP level (Fig. 5B). Treating OK cells with PTH-(3–34) (10^{-6} M, 4 h) led to a downregulation of the number of type II Na-Pi cotransporters by ~50% (Fig. 6), whereas treating OK cells with PTH-(1–34) (10^{-8} M, 4 h) led to the almost complete downregulation of the number of transporters. Also, immunofluorescence pictures, shown in Fig. 7, demonstrate that treating OK cells with PTH-(3–34) (10^{-6} M, 4 h) leads to a downregulation of the number of type II Na-Pi cotransporters. Results presented in Figs. 5–7 provide evidence for a cAMP-independent signal transduction pathway that can account for the inhibition of Na-Pi cotransport activity, as well as for the downregulation of the number of type II Na-Pi cotransporters.

We conclude that PTH signal transduction via cAMP-dependent, as well as cAMP-independent, pathways leads to a membrane retrieval and degradation of the number of type II Na-Pi cotransporters and, thereby, to the inhibition of Na-Pi cotransport activity. The cAMP-independent pathway itself leads only to a partial inhibition and related membrane retrieval of the Na-Pi cotransporter. This might be explained by the previously suggested interdependence of PKA and PKC.

Fig. 6. Treating OK cells with PTH-(3–34) (10^{-6} M, 4 h) leads to a downregulation of number of type II Na-Pi cotransporters. OK cells grown to confluency were treated for 4 h with 10^{-6} M PTH-(1–34) and 10^{-6} M PTH-(3–34), respectively. Total cellular homogenates were prepared and analyzed by SDS-PAGE and immunoblotting, as described in MATERIALS AND METHODS. To assess changes in expression level of type II Na-Pi cotransporter, we have included a dilution series.

Fig. 7. Treating OK cells with PTH-(3–34) (10^{-6} M, 4 h) leads to a markedly reduced expression of type II Na-Pi cotransporter, as revealed by immunocytochemistry. Experiment was performed as described in legend to Fig. 2. A, control for NaPi-4; B, NaPi-4 in OK cells treated for 4 h with 10^{-6} M PTH-(3–34); C, control for F-actin; D, F-actin in OK cells treated for 4 h with 10^{-6} M PTH-(3–34).
regulatory pathways in PTH-induced control of OK cell apical Na-Pi cotransporter (10, 18, 23).

We thank C. Gasser for assistance in preparing the figures for this paper.

This work was financially supported by Swiss National Science Foundation Grant 31.46523 (to H. Murer). Antibodies against OK cell Na-Pi cotransporter protein were a generous gift from E. Lederer.

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Received 9 September 1998; accepted in final form 12 February 1999.

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