Protein Kinase A-independent Activation of ERK and H,K-ATPase by cAMP in Native Kidney Cells

ROLE OF Epac I*

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This study aimed at determining the signaling pathways underlying calcitonin- and isoproterenol-induced stimulation of H,K-ATPase in rat renal collecting duct. H,K-ATPase activity was determined in microdissected collecting ducts preincubated with or without specific inhibitors or antibodies directed against intracellular signaling proteins. Transient cell membrane permeabilization with streptolinin-O allowed intracellular access of antibodies. The stimulation of H,K-ATPase by calcitonin and isoproterenol was mimicked by cAMP analogues and was abolished by adenyl cyclase inhibition. Protein kinase A inhibition abolished isoproterenol but not calcitonin effect on H,K-ATPase. Calcitonin increased the phosphorylation of extracellular signal-regulated kinase (ERK) in a protein kinase A-independent manner, and the inhibition of the ERK phosphorylation prevented the stimulation of H,K-ATPase by calcitonin. Antibodies directed against either the cAMP-activated guanine-nucleotide exchange factor Epac I, the monomeric G protein Rap-1 or the kinase Raf-B, curtailed the stimulation of H,K-ATPase by calcitonin, whereas antibodies against the related monomeric G protein Ras or kinase Raf-1 had no effect. In conclusion, calcitonin stimulates H,K-ATPase through a cAMP/Epac I/Rap-1/Raf-B/ERK cascade.

The rat kidney cortical collecting duct (CCD) consists of three intermingled cell types expressing specific hormone receptors positively coupled to adenyl cyclase via G proteins (1): principal cells with vasopressin V₂ receptors, α-intercalated (Iα) cells with calcitonin receptors, and β-intercalated (Iβ) cells with β-adrenergic receptors (1). Although it is difficult to physically separate pure populations of these different cell types, one may determine the cellular origin of cAMP-mediated responses on the basis of this hormone selectivity. Using this approach, we have previously reported that in rat CCD, the H,K-ATPase is present in both Iα and Iβ cells in which it is stimulated by salmon calcitonin (Sct) and isoproterenol (Iso), respectively (2). The stimulation of H,K-ATPase, along with that of H-ATPase reported previously (3), probably participates in the regulation of proton transport by these two hormones (3, 4).

The signaling mechanisms underlying the regulation of H,K-ATPase in Iα and Iβ cells remain unclear for two reasons. Firstly, besides activating the Gs/adenylyl cyclase/cAMP/protein kinase A (PKA) pathway, calcitonin and isoproterenol also increase intracellular Ca²⁺ concentration ([Ca²⁺)]_i) in rat CCD (5–7). Secondly, several cAMP-binding proteins other than PKA have now been described: the cAMP receptor of Dictyostelium discoideum, which participates in the regulation of the development of this unicellular eukaryote (8), S-adenosyl homocysteine hydrolase, which participates in the regulation protein methylation (9), cyclic nucleotide-gated channels involved in transduction of olfactory and visual signals (10, 11), and cAMP-activated guanine-nucleotide exchange factors (GEF) (Epac I or cAMP-GEF I and CNrasGEF), which activate specifically the monomeric G protein of the Ras family, Rap-1 and Ras, respectively (12–14). Thus, depending on the nature of cAMP-binding proteins expressed in different cell types and on their supramolecular organization, the effects of cAMP may be mediated by PKA-dependent or independent mechanisms. Interestingly, the kidney is one of the tissues displaying the highest levels of expression of mRNAs encoding Epac I (12, 13) and the cyclic nucleotide-gated channel 3 (15), making possible an important role of these two proteins in the signalization of cAMP actions in kidney cells.

Thus, the aim of this study was to determine the signaling pathways underlying the stimulation of H,K-ATPase activity by Set and Iso in the rat CCD, in particular the involvement of the cAMP-binding proteins PKA, Epac I, and cyclic nucleotide-gated channels.

MATERIALS AND METHODS

Animal Preparation and Tubule Microdissection—Experiments were carried out on male Sprague-Dawley rats anesthetized with sodium pentobarbital (50 mg/kg body weight). Cortical collecting ducts were dissected at 4 °C from collagenase-treated kidneys as described previously (16). After microdissection, the ducts were photographed to determine their length, which served to normalize results. Unless indicated otherwise, microdissection was carried out in a solution containing (in mM) 120 NaCl, 5 KCl, 1 MgSO₄, 4 NaHCO₃, 0.2 NaH₂PO₄, 0.15 Na₃HPO₄, 5 glucose, 0.5 CaCl₂, 0.08 dextran, 2 lactate, 20 Hepes, 4 essential and nonessential amino acids, 0.03 vitamins, and 1 mg/ml bovine serum albumin. The pH was adjusted to 7.4, and osmotic pressure was adjusted to 400 mosmol/kg with mannitol. For RNA extraction, tubule isolation was run under “RNase-free conditions” (17). For Western blotting analysis, antipeptides (1 μg ml⁻¹ leupeptin,

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10 μg ml⁻¹ aprotinin, 10 μg ml⁻¹ aminothiolbenzenesulfonyl fluoride, and 10 μg ml⁻¹ antipain) were added to the dissection solution.

**Pretreatment with Inhibitors and Hormones**—Microdissected CCDs were preincubated with specific inhibitors of different intermediates of signaling pathways or with their solvent before incubation with hormones (23). Each of the phospho-ATPase activities (lys-48, lys-74, and lys-80) was pretreated with or without the inhibitors at 30 °C for 45–120 min before treatment with hormones (10 min at 37 °C). The hormone treatment was stopped by cooling the samples at 4 °C. H89 dihydrochloride, myristoylated protein kinase A peptide inhibitor (Sigma-Aldrich, St. Louis, MO) to each sample and freezing on dry ice. After thawing and pretreatment with Inhibitors and Hormones—Microdissected CCDs were directed against the active portion of the proteins (12, 20, 21), the antibody derived by fusion of spleen cells from a rat immunized with phosphorylated Ser-1859 from Sigma. All inhibitors were prepared from aqueous solutions with the exception of U1026, which was dissolved in dimethyl sulfoxide (Me2SO). The corresponding control groups contained the same concentration of Me2SO (<0.1% v/v).

When specific inhibitors were not commercially available, we evaluated the effect of antibodies directed against several signaling proteins. Intracellular entry of antibodies was made possible by transient per-
Fig. 1. Effect of protein kinase inhibitors on H,K-ATPase. Rat CCDs were preincubated either at 30°C for 45 min (H89, MPKI) without protein kinase inhibitor (bar C) or with 1 μM H89 (H89) (A), or 10 μM myristoylated inhibitory peptide (MPKI) (B), or at 4°C for 80 min (C, Anti PKA) in the absence (bar C) or presence of anti-PKA antibody (dilution 1:100) after streptolysin-O permeabilization. Afterward, samples were incubated at 37°C for 10 min without hormone (solid bars) or with either 10 nM salmon calcitonin (open bars) or 1 μM isoproterenol (hatched bars) before measuring H,K-ATPase activity. N, number of experiments. Statistical comparison between groups was performed by ANOVA with PLSD Fisher. *p < 0.05; **p < 0.001 versus control groups (no hormone).

Activities were quite variable between animals, making it necessary to test the different experimental conditions of a same group in the same animals (paired experiments).

Preincubation with 1 μM H89 (Fig. 1A) did not modify basal and Sct-stimulated H,K-ATPase activity but totally blocked the stimulation by Iso. Preincubation with 10 μM myristoylated inhibitory peptide 14-22 amide, another inhibitor of the PKA catalytic subunit, also blocked Iso-induced but not Sct-induced stimulation of H,K-ATPase (Fig. 1B). The effect of H89 and myristoylated inhibitory peptide 14-22 amide were also mimicked by a polyclonal antibody directed against PKA catalytic subunit (Fig. 1C).

In summary, these results demonstrate that Iso stimulates H,K-ATPase activity through the canonical PKA signaling pathway, whereas the effect of calcitonin is independent of PKA. Therefore, we attempted next to determine the signaling mechanism of calcitonin.

Role of cAMP in Sct-induced Stimulation of H,K-ATPase—To investigate whether or not cAMP is the second messenger that underlies Sct-induced stimulation of H,K-ATPase in Ia cells, the requirement for adenyl cyclase and cAMP was determined by three approaches. Firstly, CCDs were pretreated with 2.5 mM 2',5'-dideoxyadenosine, an inhibitor of adenyl cyclase. Results show that 2',5'-dideoxyadenosine did not change basal H,K-ATPase activity but abolished its stimulation by Sct (Fig. 2A). This finding demonstrates that adenyl cyclase activity is essential for the stimulation of H,K-ATPase by Sct. Secondly, to further investigate the role of cAMP in Sct-induced enzyme activation, we determined the effects of the permeant cAMP analogue 8-Br-cAMP (100 μM) and of forskolin (1 μM). Because the results obtained with the two compounds were similar, they were pooled (Fig. 2B). The addition of 8-Br-cAMP or forskolin stimulated H,K-ATPase activity over 8-fold. The fact that this stimulatory factor (×8) equals the sum of those observed in response to Iso (×4) and Sct (×4) suggests that 8-Br-cAMP and forskolin mimic the effects of the two hormones. This observation is further demonstrated by the finding that stimulation by 8-Br-cAMP or forskolin was inhibited by 50% with H89 (as expected from inhibition of β-intercalated cells). Finally, Sct-induced stimulation of H,K-ATPase was also mimicked by (R)-cAMP (Fig. 2C), a diastereomer of cAMP that inhibits PKA (24).

These findings demonstrate that in Ia cells, cAMP interacts with a cAMP-binding protein, different from PKA that mediates the stimulation of H,K-ATPase activity. Interestingly, this cAMP-binding protein is equally well activated by cAMP and its analogue (R)-cAMPs conversely to PKA regulatory subunit, which is inhibited by (R)-cAMPs.

Lack of Implication of Cyclic Nucleotide-gated Channels in Sct-induced Stimulation of H,K-ATPase—Because cyclic nucleotide-gated channels 3 are expressed at a high level in the kidney (15) and because Sct increases [Ca2+]i in rat CCD (5), cyclic nucleotide-gated channels and [Ca2+]i, might possibly mediate the stimulation of H,K-ATPase activity. However, Sct-induced increase in [Ca2+]i, was markedly reduced by pretreating CCDs with 1 μM PKA-specific inhibitor H89 (Fig. 3). This finding demonstrates that these increases in [Ca2+]i, resulted from the activation of the canonical cAMP/PKA cascade. This
equilibration period necessary to reach a stable [Ca$^{2+}$]

CCDs were preincubated at 30°C for 45 min in microdissection medium containing Fura-2/AM (10 μM) without or with the protein kinase A inhibitor H89 (1 μM). Afterward, samples were superfused at 37°C without or with H89 with a solution containing 1 mM Ca$^{2+}$ (instead of 0.5 mM for H,K-ATPase measurements) to amplify the magnitude of Ca$^{2+}$ signals secondary to Ca$^{2+}$ entry. After a 10-min equilibration period necessary to reach a stable [Ca$^{2+}$], level, tubules were stimulated successively with Sct (10 nM) and ATP (100 μM), a nucleotide known to stimulate phospholipase C in this segment through extracellular P$_{i}$/Y receptors (25). A and B, representative traces obtained in the same rat from a control and a H89-treated tubule respectively. C, mean [Ca$^{2+}$] increases (peak minus basal) calculated for each agonist from seven controls (solid bars) and seven H89-treated tubules (open bars) isolated from four rats. *, p < 0.001 versus control (no H89) by unpaired Student’s t test.

The pretreatment with CCDs with a polyclonal antibody directed against an epitope close to the C terminus of Rap-1 abolished the stimulatory effect of Sct on H,K-ATPase activity (Fig. 6B). Similarly, the pretreatment with an antibody directed against the C terminus of B-Raf abolished the stimulatory effect of Sct on H,K-ATPase, whereas a monoclonal antibody mapping a C terminus epitope of the related kinase Raf-1 had no effect (Fig. 6, C and D). Western blot analysis using an antibody against phospho-ERKs p42 and p44 demonstrates that calcitonin increased the phosphorylation of ERK1/2 by ≥50% and that this effect was insensitive to the PKA inhibitor H89 (Fig. 7A). (p)-cAMPs mimicked the effect of calcitonin on ERK1/2 phosphorylation (Fig. 7B). These findings are compatible with the involvement of ERK activation in the stimulation of H,K-ATPase by Sct. Indeed, the preincubation with 5 μM U0126, an inhibitor of the ERK kinase MEK (27), abolished Sct-induced stimulation of H,K-ATPase (Fig. 7C).

**DISCUSSION**

This study shows that calcitonin-induced stimulation of H,K-ATPase in Iα cells of rat collecting duct is mediated by cAMP but is independent of PKA. Instead, it involves Epac I, the guanine-nucleotide exchange factor of the monomeric G protein Rap-1, whose activation triggers B-Raf and the MAPK kinase ERKs.

**Study of Signaling Pathways in Native Cells**—A myriad of molecules participates in the intracellular control of cell functions. These molecules can constitute different signaling networks according to their level of expression, the presence of their various isoforms and their subcellular compartmentalization. Thus, the signaling pathways characterized in cultured and/or transfected cells may not be relevant in native cells under physiological conditions, especially in highly differentiated cells such as kidney epithelial cells. However, deciphering signaling pathways in native cells remains difficult because we lack specific pharmacological inhibitors for most intracellular signaling molecules.

In this study, we tentatively used antibodies directed against signaling proteins as specific inhibitors based on the hypothesis that the binding of an immunoglobulin on or close to the active site of a protein should alter its activity and/or its interaction with other proteins located upstream or downstream of the signalization cascade. Although an inhibitory activity was previously demonstrated only for the anti-Ras antibody (22), the five antibodies, which were tested in this study, proved to display a biological effect. Furthermore, the inhibitory effect of an antibody may be as potent as that of a pharmacological inhibitor as illustrated in Fig. 1 for the anti PKA antibody. In addition, the biological effects of the antibodies were specific. (a) As shown on Fig. 1C, the anti-PKA antibody inhibited the effect of isoproterenol in Iβ cells but not that of calcitonin in Iα cells, whereas two different anti Epac I antibodies curtailed calcitonin but not isoproterenol action (Fig. 5). (b) Antibodies directed against Rap-1 and B-Raf abolished calcitonin action, whereas antibodies directed against Ras and Raf-1, two related proteins of the same families, had no effect (Fig. 6).

Finally, transient permeabilization of cell membranes with streptolysin-O is a convenient method for integrating macro-molecules into native epithelial cells, because it allows to maintain a cell integrity sufficient to preserve the activity of H,K-
Fig. 4. Expression of Epac 1 and Rap-1 mRNAs along the nephron. mRNAs from one glomerulus or 1 mm of different segments of the nephron were reverse-transcribed, and the cDNA was amplified by PCR using primers specific for either Epac 1 (A) or Rap-1 (B). The DNA fragments were separated on 2% agarose gels and visualized using a PhosphorImager. The upper panels show representative gels from structures microdissected from a same animal. In each experiment, values were expressed as the percent of the PCT value, and the bottom panels show the means ± S.D. from four animals (with the exception of ATL for which n = 3). G, glomerulus; PCT, proximal convoluted tubule; PST, proximal straight tubule; TDL, thin descending limbs of Henle’s loop; ATL, ascending limbs of Henle’s loop; MTAL, medullary thick ascending limbs of Henle’s loop; CTAL, cortical thick ascending limbs of Henle’s loop; CTD, cortical duct; OMCD, outer medullary duct; and IMCD, inner medullary collecting duct.

Fig. 5. Role of Epac 1 and Rap-1 on hormone-induced stimulation of H,K-ATPase. CCDs were transiently permeabilized with streptolydin-O (0.4 units ml−1 for 8 min at 37 °C) in the absence (Control) or the presence of the two antibodies (dilution 1:100) directed against the C terminus (A) or the N terminus of Epac 1 (B) and incubated at 4 °C for 90 min. Afterward, samples were incubated at 37 °C for 10 min without hormone (solid bars) or with either 10 nM salmon calcitonin (open bars) or 1 μM isoproterenol (hatched bars) before measuring H,K-ATPase activity. N, number of experiments. Statistical comparison between groups was performed by ANOVA with PLSD Fisher. *, p < 0.05; **, p < 0.005; and ***, p < 0.001 versus control groups (no hormone).

Fig. 6. Role of Rap-1 and B-Raf on hormone-induced stimulation of H,K-ATPase. CCDs were transiently permeabilized with streptolydin-O (0.4 units ml−1 for 8 min at 37 °C) in the absence (Control) or the presence of two antibodies (dilution 1:100) directed against Rap-1 (A), related G protein Ras (B), B-Raf (C), or the kinase Ras (D). Afterward, samples were incubated at 37 °C for 10 min without hormone (solid bars) or with 10 nM salmon calcitonin (open bars) before measuring H,K-ATPase activity. N, number of experiments. Statistical comparison between groups was performed by ANOVA with PLSD Fisher. *, p < 0.05; **, p < 0.005; and ***, p < 0.001 versus control groups (no Sct).
Calcitonin increased the phosphorylation of ERKs (Fig. 7A (a)). The regulation of ERK and H,K-ATPase by calcitonin were beyond ERK has not been investigated. In most cells, the activation of ERK triggers its translocation into the nucleus where it activates transcription factors through direct phosphorylation (Elk-1, Myc, cAMP-response element-binding protein, Sap-1) or through phosphorylation of kinases such as p90RSK. In turn, these activated transcription factors control the expression of specific genes. The rapid time course of calcitonin-induced activation of H,K-ATPase activity (~10 min) rules out a possible mediation through de novo synthesis of its subunits and suggests an activation of preexisting ATPase units. Several observations support that calcitonin may stimulate H,K-ATPase activity through exocytotic insertion into the plasma membrane of H,K-ATPase units present in the membrane of cytoplasmic vesicles. (a) H,K-ATPase colocalizes with H-ATPase in native kidney cells. (b) It outlines the important role of Epac I in mediating some effects of cAMP in collecting duct cells. (c) It demonstrates an intracellular compartmentalization of cAMP effects. (c) It indicates that ion transporters such as H,K-ATPase can be extranuclear effectors of ERK signalization. The expression of Epac I in other nephron segments (Fig. 4c) suggests that many other effects of cAMP-producing hormones, usually attributed to the activation of PKA, may be mediated in fact through the activation of Epac I and ERKs.

In conclusion, this study provides original data regarding several aspects of protein G-coupled receptor signalization in native kidney cells. (a) It outlines the important role of Epac I in mediating some effects of cAMP in collecting duct cells. (b) It demonstrates an intracellular compartmentalization of cAMP effects. (c) It indicates that ion transporters such as H,K-ATPase can be extranuclear effectors of ERK signalization. The expression of Epac I in other nephron segments (Fig. 4A) suggests that many other effects of cAMP-producing hormones, usually attributed to the activation of PKA, may be mediated in fact through the activation of Epac I and ERKs.

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