Regulation of anion exchanger Slc26a6 by protein kinase C

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Submitted 22 August 2006; accepted in final form 27 November 2006

Hassan HA, Mentone S, Karniski LP, Rajendran VM, Aronson PS. Regulation of anion exchanger Slc26a6 by protein kinase C. Am J Physiol Cell Physiol 292: C1485–C1492, 2007. First published December 6, 2006; doi:10.1152/ajpcell.00447.2006.—SLC26A6 (CFEX, PAT1) is a member of the SLC26 gene family of anion exchangers expressed in several tissues including renal proximal tubule, pancreatic duct, small intestine, liver, stomach, and heart. It has recently been reported that PKC activation inhibits A6-mediated Cl/HCO3 exchange by disrupting binding of carbonic anhydrase to A6. However, A6 can operate in HCO3-independent exchange modes of physiological importance, as A6-mediated Cl/oxalate exchange plays important roles in proximal tubule NaCl reabsorption and intestinal oxalate secretion. We therefore examined whether PKC activation affects HCO3-independent exchange modes of Slc26a6 functionally expressed in Xenopus oocytes. We found that PKC activation inhibited Cl/formate exchange mediated by Slc26a6 but failed to inhibit the related anion exchanger pendrin (SLC26A4) under identical conditions. PKC activation inhibited Slc26a6-mediated Cl/formate exchange, Cl/oxalate exchange, and Cl/Cl exchange to a similar extent. The inhibitor sensitivity profile and the finding that PMA-induced inhibition was calcium independent suggested a potential role for PKC-δ. Indeed, the PKC-δ-selective inhibitor rottlerin significantly blocked PMA-induced inhibition of Slc26a6 activity. Localization of Slc26a6 by immunofluorescence microscopy demonstrated that exposure to PKC activation led to redistribution of Slc26a6 from the oocyte plasma membrane to the intracellular compartment immediately below it. We also observed that PMA decreased the pool of Slc26a6 available to surface biotinylation but had no effect on total Slc26a6 expression. The physiological significance of these findings was supported by the observation that PKC activation inhibited mouse duodenal oxalate secretion, an effect blocked by rottlerin. We conclude that multiple modes of anion exchange mediated by Slc26a6 are negatively regulated by PKC-δ activation.

oxalate; formate; chloride; duodenum

SLC26A6 (CFEX, PAT1) is a member of the SLC26 gene family of anion transporters and is expressed in many tissues including renal proximal tubule, pancreatic ducts, small intestine, liver, stomach, and heart (22, 25, 27, 35, 39, 41). Functional expression studies have indicated that A6 can mediate multiple modes of anion exchange including Cl/formate, Cl/oxalate, Cl/HCO3, and Cl/OH exchanges (18, 22, 23, 39, 41). The physiological significance of A6 is indicated by defects in apical membrane Cl/base exchange in the proximal tubule and intestine of Slc26a6-null mice (6, 17, 40).

Recent studies have begun to elucidate the molecular mechanisms regulating A6 transport activity. In particular, Cl/HCO3 exchange activity of A6 is negatively regulated by α-adrenergic stimulation and angiotensin II, effects that are mediated by protein kinase C (PKC) activation (1, 2). Inhibition by PKC was attributed to PKC-mediated displacement of carbonic anhydrase II from binding to SLC26A6 and consequent disruption of the HCO3 transport metabolon (2). Inhibition of A6 by PKC may explain the observation that agonists acting through PKC inhibit HCO3 secretion in the pancreas (13).

However, A6 can operate in HCO3-independent exchange modes of physiological importance. For example, A6-mediated Cl/oxalate exchange plays an important role in proximal tubule NaCl reabsorption as well as a critical role in gastrointestinal oxalate secretion (6, 17, 37, 40). In fact, mice lacking A6 have significant hyperoxaluria and elevated plasma oxalate levels due to enhanced net absorption of ingested oxalate and development of high incidence of calcium oxalate urolithiasis (17). Such HCO3-independent modes of anion exchange would not be dependent on carbonic anhydrase.

Accordingly, the purpose of the present study was to evaluate whether HCO3-independent modes of A6-mediated transport are similarly regulated by PKC activation. We find that Cl/formate, Cl/oxalate, and Cl/Cl exchange mediated by murine Slc26a6 heterologously expressed in Xenopus oocytes is indeed markedly inhibited by PKC activation. Inhibition of A6 activity due to PKC activation is blocked by rottlerin, a specific inhibitor of PKC-δ. In addition, we show that PKC-mediated inhibition of Slc26a6 transport activity is due to reduction in surface membrane expression of the transporter. The physiological relevance of these findings in Xenopus oocytes is underscored by the observation that PKC activation also inhibits transepithelial oxalate secretion across isolated duodenal tissue, an effect blocked by rottlerin.

MATERIALS AND METHODS

Oocyte preparation and cRNA injection. Mouse Slc26a6 cDNA subcloned into the Xenopus expression plasmid pGHI9 (22) and human pendrin subcloned into pGEMHE (31) were heterologously expressed in Xenopus oocytes as described previously (18, 22). Site-directed mutagenesis of the consensus PKC phosphorylation site (T552) to alanine (T552A) was performed with the Gene Editor in vitro system (Promega) in pGH19 containing full-length Slc26a6. Mutation was confirmed by direct sequencing. Oocytes were injected with 50 nl of sterile water (control) or 50 nl of wild-type Slc26a6, mutant Slc26a6 (T552A), or pendrin cRNA solution (25 ng) and incubated in ND96 buffer (mM: 96 NaCl, 2 KCl, 1.8 CaCl2, 1.0 MgCl2, 5 HEPES, pH 7.5; supplemented with 5 mM sodium pyruvate and 50 U/ml penicillin-streptomycin) at 16°C for 48–72 h before transport studies.

Measurements of radiolabeled solute fluxes. Oocytes were washed twice at room temperature in 1 ml of Cl-free buffer (mM: 98...
potassium-gluconate, 1.8 hemi-calcium-gluconate, 1 hemi-magnesium-gluconate, 5 Tris-HEPES, pH 7.5) and then incubated in 500 μl of uptake medium (nM: 100 potassium-gluconate, 5 Tris, pH adjusted to 7.5 with HEPES) containing the radiolabeled solutes to be tested (71.4 μM [14C]formate, 20 μM [14C]oxalate or 3.4 mM 36Cl) for 10 min. Oocytes were then washed three times in ice-cold CI-free buffer to remove the external isotope. For Cl efflux measurements, oocytes were first preloaded with radioisotope by incubating for 60 min in K-gluconate buffer containing 3.4 mM 36Cl. After three washes in the same buffer, the radioisotope content of oocytes was measured both initially and after 15 min of reincubation in 36Cl-free K-gluconate medium without (control) or with isotopic replacement of gluconate by 10 mM CI. Net efflux was calculated as the difference between the initial oocyte 36Cl content and that remaining after 15-min reincubation. Oocytes were lysed individually in 200 μl of 10% SDS, and the radioisotope content of each individual oocyte was measured by scintillation spectrometry after addition of 3 ml of scintillation fluid (Opti-Fluor, Packard).

**SDS-PAGE and Western blotting.** Oocyte yolk-free protein lysate was prepared with a modification of the procedure of Forster et al. (5). In brief, oocytes were homogenized by pipetting (20–40 μl per oocyte) in a homogenization buffer (100 mM NaCl, 1% Triton X-100, 20 mM Tris·HCl, pH 7.6) containing protease inhibitor cocktail (0.7 mg/ml pepstatin A, 0.5 mg/ml leupeptin, 40 mg/ml phenylmethylsulfonyl fluoride, and 1 mM Na2 EDTA). Oocytes were incubated for 5 min on ice, the homogenate was then centrifuged (15,000 g, 4°C, 3 min), and the supernatant was retained for gel electrophoresis. Proteins were separated by SDS-PAGE using 7.5% polyacrylamide gels, with subsequent electrotransfer to polyvinylidene difluoride (PVDF; Immobilon-P, Millipore). For Western blotting, PVDF strips were incubated first in Blotto (5% nonfat dry milk and 0.1% Tween 20 in PBS) for 1 h to block nonspecific binding, followed by overnight incubation in primary antibody (anti-Slc26a6 antibody generated to a peptide corresponding to the COOH-terminal 29 amino acids of the protein, 1:1,000; Ref. 22). The strips were then washed in Blotto and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG, Zymed; 1:2,000). Antibody reactivity in oocyte membranes was detected with an enhanced chemiluminescence system (Amersham) according to the manufacturer’s protocol. Surface-expressed proteins were biotinylated by a modification of the procedure of Forster et al. (5). Oocytes were washed five times in ND96 solution at 4°C and then incubated in the same solution containing the biotinylation reagent Sulfo-NHS-LC-biotin (2.2 mM; Pierce) at 4°C twice for 30 min. The oocytes were then incubated for 10 min in ND96 solution containing 5 mM glycine (to quench excess biotin) and subsequently washed twice for 5 min in ND96 solution. The yolk-free protein lysates were prepared as described above. Streptavidin precipitation was then performed as described by Forster et al. (5). Biotinylated oocyte proteins were dissociated from the beads with sample buffer (10% SDS, 2% β-mercaptoethanol, 20% glycerol, 5 mM Tris·HCl, pH 6.8) containing 100 mM dithiothreitol. After separation by SDS-PAGE, proteins were transferred to immunoblots and probed with the anti-Slc26a6 antibody as above.

**Immunocytochemistry.** Oocytes were fixed for 1 h in a solution containing 1% paraformaldehyde in 100 mM sodium phosphate, pH 7.4, and subsequently placed in holding buffer (100 mM phosphate with azide, 0.5% paraformaldehyde). Immunocytochemistry was then performed after tissue embedding and antigen retrieval as described previously (32). In brief, oocytes were embedded in Embed 812, cut into 1-μm sections, etched for 5 min in a solution containing KOH (2 g), methanol (10 ml), and propylene oxide (5 ml), washed in methanol, and subjected to microwave antigen retrieval in citrate buffer (10 mM, pH 6.0). For immunofluorescence staining, sections were quenched for 15 min in Tris-buffered saline (TBS) containing 0.5 M ammonium chloride and 0.1% BSA, rinsed in TBS, incubated in 1% SDS in TBS for 5 min, and washed in TBS. After blocking with 0.1% BSA and 10% goat serum in TBS for 1 h at room temperature, sections were washed in TBS and incubated overnight with primary antibody (S126a6 anti-peptide antibody; 1:50 dilution in TBS-0.1% BSA-10% goat serum). Sections were washed in TBS and incubated for 1 h with goat anti-rabbit IgG-conjugated fluorescein isothiocyanate (Molecular Probes) (1:200 dilution in TBS-0.1% BSA-10% goat serum). Finally, sections were washed, mounted in VectaShield (Vector Laboratories, Burlingame, CA), and then visualized by immunofluorescence microscopy.

**Measurement of duodenal oxalate secretion.** Mouse (BALB/c, males, 10–12 wk of age) duodenal segments were opened longitudinally along the mesenteric border and mounted as an intact sheet in a modified Ussing chamber that had an exposed surface area of 0.11 cm2. The mucosal and serosal surfaces of the duodenal segments were bathed with 10 ml of warmed (37°C) Ringer buffer (mM: 139.4 Na+, 132 Cl−, 5.4 K+, 1.2 Mg2+, 21 HCO3−, 0.6 HPO42−, 2.4 mM H2PO4−, 10 mM glucose, pH 7.4, gassed with 100% O2-5% CO2). Transepithelial short-circuit current and total tissue conductance were measured as described previously (34). We added 2 μM [36]Cloxalate to the serosal bath, and [14C]oxalate secretion (serosa to mucosa) was measured by previously described methods (11). After a 30-min equilibration, samples of the mucosal solution were collected before and after a 60-min period for calculation of unidirectional [14C]oxalate secretory flux. All flux studies were performed under voltage-clamp conditions with a DVC 1000 (World Precision Instruments). All animal protocols were approved by the Institutional Animal Care and Use Committee of Yale University.

**Protein kinase activators and inhibitors.** Phorbol 12-myristate 13-acetate (PMA), 1,2-dioctanoyl-sn-glycerol (DOG), G6-6983, and G6-6976 were obtained from Calbiochem. Rottlerin and 4a-PMA were obtained from Sigma. All of these agents were dissolved in DMSO and stored at −20°C. They were added to the incubation medium just before use. Equivalent volumes of DMSO (0.1–0.4%) were added to control media.

**RESULTS**

As a first approach to examine whether HCO3-dependent transport mediated by Slc26a6 is regulated by PKC activation, we examined the effect of PKC activation on CI/formate exchange measured as [14C]formate influx in the presence of an outward CI gradient. To this end, Slc26a6-expressing Xenopus oocytes were preincubated with the PKC activator PMA before solute transport was measured. As shown in Fig. 1A, formate uptake was minimal in water-injected oocytes but was greatly stimulated after Slc26a6 cRNA injection, confirming previous findings (18, 22, 41). Preincubation with PMA caused marked inhibition of Slc26a6-mediated formate uptake. In contrast, preincubation with 4α-PMA, a structurally similar analog that does not activate PKC, failed to inhibit formate uptake. Another PKC activator, DOG, also significantly inhibited Slc26a6-mediated transport activity. Importantly, PMA had no effect on formate transport mediated by another member of the SLC26 anion transporter family, pendrin (SLC26A4), expressed in oocytes under identical conditions (Fig. 1B). These findings indicate that the observed PKC regulation of Slc26a6 is selective and not due to a general inhibition of membrane protein function or expression.

We next examined the effects of PMA preincubation on additional modes of anion transport, namely Cl/oxalate exchange and CI/CI exchange (18, 22, 41). As illustrated in Fig. 2, the rates of influx of [14C]formate, [14C]oxalate, and 36Cl measured in the presence of an outward CI gradient were each inhibited by PMA to a similar extent. These data indicate that multiple modes of HCO3-independent transport mediated...
by Slc26a6 are inhibited to an equivalent degree by PKC activation.

The most straightforward interpretation of the observation that PKC activation inhibits Cl/formate, Cl/oxalate, and Cl/Cl exchange is that this inhibition results from a direct effect on expression or function of Slc26a6 itself. However, one must consider the alternative possibility that PKC activation affects endogenous ion transport pathways in the oocyte leading to increased dissipation of the outwardly directed Cl gradient driving uptake of [14C]formate, [14C]oxalate, and 36Cl. To rule out this possibility, we conducted 36Cl efflux studies, as indicated in Fig. 3. Slc26a6-expressing oocytes were preincubated and thereby preloaded with 36Cl for 60 min and then washed and reincubated in a 36Cl-free medium. The oocyte content of 36Cl was measured at the end of the preloading period and after 15-min reincubation in the absence or presence of 10 mM Cl. As seen in Fig. 3, there was no detectable 36Cl efflux in the absence of Cl in the external medium. The addition of external Cl markedly stimulated 36Cl efflux, reflecting Cl/Cl exchange. PMA failed to increase the efflux of 36Cl measured in the absence of Cl in the external medium, indicating that PKC activation does not enhance leak of intracellular Cl from the oocytes. However, the ability of external Cl to stimulate 36Cl efflux was strongly inhibited by PMA, indicating inhibition of Slc26a6-mediated Cl/Cl exchange. These results strongly argue that PKC activation directly inhibits Slc26a6-mediated transport activity.

PKC inhibition of Cl/HCO3 exchange activity of human SLC26A6 is abolished by mutation of the PKC phosphorylation site (S553A) located near the binding site for carbonic anhydrase II (547DVDF550) on SLC26A6 (2). Since both of these sites are conserved in mouse Slc26a6 (T552 and 546DVDR549, respectively), we mutated the corresponding PKC phosphorylation site in mouse Slc26a6 (T552A) to test whether it is involved in the observed PKC inhibition of anion transport. As shown in Fig. 4, the T552A mutant was fully functional and similarly sensitive to inhibition by PMA compared with wild-type Slc26a6. This finding indicates that the observed PKC inhibition of mouse Slc26a6 does not result from phosphorylation of residue T552. This finding is consistent with the fact that the HCO3-independent modes of anion exchange assayed in these experiments would not be expected to be dependent on binding of carbonic anhydrase II.

![Fig. 1. Effect of PKC activation on Slc26a6- and pendrin-mediated uptake of [14C]formate. A: A6-expressing oocytes were incubated in ND96 containing phorbol 12-myristate 13-acetate (PMA) (50 nM for 20 min), 1,2-dioctanoyl-sn-glycerol (DOG) (5 μM for 60 min), or 4α-PMA (50 nM for 20 min), and then [14C]formate uptake was measured as described in MATERIALS AND METHODS. Values are means ± SE of 3 different experiments each of which was normalized to the Control value (7–15 oocytes per group in each experiment). B: pendrin-expressing oocytes were incubated in ND96 containing PMA (50 nM for 20 min), and then [14C]formate uptake was similarly measured. Values are means ± SE of 5 different experiments each of which was normalized to the Control value (7–15 oocytes per group in each experiment). Inhibition by PMA and DOG of A6-mediated formate uptake was significantly different from the control value (P < 0.001 and P < 0.02, respectively; 2-tailed t-test).](https://www.ajpcell.org/pdfs/AJP-CellPhysiol989-999.pdf)
mM 36Cl (Initial) and after 15-min reincubation in 36Cl-free medium without expressing oocytes was assayed after an initial 60-min preincubation with 3.4 mM 36Cl-containing PMA (9, 19). Go 6983 has highest affinity for the classic calcium-dependent PKC isozymes (cPKC) and the novel isoform PKC-ε (7, 29). Thus our observation that Go 6976 suggests a potential role for PKC-δ since PKC-ε is an atypical PKC that is not activated by phorbol esters (16).

PKC-δ, a novel calcium-independent PKC isoform (14, 16). As an additional confirmation for the involvement of a calcium-independent PKC isoform in the observed regulation of Slc26a6 activity, we examined whether the effect of PMA to inhibit Slc26a6 activity would still occur in a calcium-free medium containing the calcium chelator EGTA at 0.5 mM. It should be noted that PMA-induced inhibition of a dopamine transporter was completely prevented under these conditions in Xenopus oocytes, supporting the involvement of cPKC isoforms in its regulation (4). As shown in Fig. 6, PMA-induced inhibition of Slc26a6-mediated formate influx was unaffected by the use of a calcium-free medium, providing additional evidence that Slc26a6 inhibition is mediated by a calcium-independent PKC isoform like PKC-δ.

To confirm the specific role of PKC-δ in mediating inhibition of Slc26a6 activity, we tested the effect of the selective PKC-δ inhibitor rottlerin (3, 8, 15, 28, 33). As illustrated in Fig. 7, rottlerin greatly reduced the suppressive effect of PMA on Slc26a6-mediated transport of formate, whereas it had no significant effect on baseline transport measured in the absence of PMA. These findings provide strong evidence that the effect of PMA to inhibit Slc26a6 transport activity is primarily mediated by PKC-δ.

PKC-δ-mediated inhibition of Slc26a6 transport activity could result from reduced function of individual transporters or by reduced abundance of transporters in the plasma membrane. As a first approach to evaluate the plasma membrane expression of Slc26a6, we performed immunofluorescence microscopy on semithin sections of Slc26a6-expressing oocytes treated without or with PMA. As shown in Fig. 8, PMA treatment led to significant redistribution of Slc26a6 protein from the plasma membrane to the intracellular space.
immediately below it, suggesting that PKC activation suppresses Slc26a6 transport activity by reducing surface expression of the transporter.

To further confirm that net redistribution of Slc26a6 is the molecular mechanism underlying PKC-mediated inhibition of Slc26a6 transport activity, we performed surface biotinylation studies. Control or PMA-treated Slc26a6-expressing oocytes were exposed to the surface biotinylation agent Sulfo-NHS-LC-biotin, biotinylated proteins were precipitated with streptavidin, and then immunoblots were prepared and probed with an anti-Slc26a6 antibody to assess surface Slc26a6 expression. In addition, immunoblots of oocyte lysates were prepared and probed to assess total Slc26a6 expression. A representative immunoblot is shown in Fig. 9A, and the scanned data from four experiments are illustrated in Fig. 9B. As indicated in Fig. 9, PMA caused significant reduction of biotin-labeled Slc26a6. However, total Slc26a6 abundance in oocyte lysate was unaffected by PMA treatment. Together, these findings confirm redistribution from the surface membrane without a change in total protein expression as the mechanism by which PKC activation regulates Slc26a6 activity.

A critical question is whether the observed PMA-induced inhibition of Slc26a6 heterologously expressed in Xenopus oocytes actually reflects the behavior of the transporter in mammalian tissues in which it is endogenously expressed. It was recently shown that oxalate secretion across isolated duodenal segments is largely mediated by A6, as it was reduced by 70% in Slc26a6-null mice (17). Moreover, Slc26a6 expression in mouse intestinal tissue is highest in duodenum (39). We therefore used the same model of duodenal oxalate secretion to examine whether PKC activation with PMA affects endogenous Slc26a6 activity in a mammalian tissue under physiological conditions. As seen in Fig. 10, PMA caused 50% inhibition of the secretory flux of [14C]oxalate across mouse duodenal segments mounted in Ussing chambers. Importantly, just as in the case of Slc26a6 expressed in Xenopus oocytes, the selective PKC-δ inhibitor rottlerin completely blocked the PMA-induced inhibition of duodenal [14C]oxalate secretion, whereas it had no significant effect on [14C]oxalate secretion measured in the absence of PMA. Of note is that no significant change in transepithelial resistance or short-circuit current (compared to control) was observed during the 90-min incubation with PMA or PMA plus rottlerin (not shown). These findings confirm that activation of PKC inhibits endogenous Slc26a6 activity in a mammalian tissue and that this effect is blocked by the PKC-δ inhibitor rottlerin.
and after a 60-min period for calculation of unidirectional [14C]oxalate secretion in mucosal solutions, and samples of the mucosal solutions were collected before (100 nM) PMA (or was not (Control) added both to the serosal and was added to the serosal solution. After a 30-min equilibration period, PMA availability to surface biotinylation was assessed. Experiments using PKC inhibitors strongly suggest that PKC-δ is the PKC isoform mediating this inhibitory regulation. Moreover, assessment of Slc26a6 localization by immunocytochemistry and biotinylation indicates that reduction in surface membrane expression of the transporter is the molecular mechanism underlying PKC-mediated inhibition of its transport activity. Supporting the physiological significance of these findings in the Xenopus oocyte expression system, we show that duodenal oxalate secretion, a process largely mediated by Slc26a6 (17), is also inhibited by PKC activation and that this effect is similarly blocked by the PKC-δ inhibitor rottlerin.

The effect of PKC activation to reduce surface expression of Slc26a6 is similar to the previously described effect of PKC activation to inhibit NaPi-2 transport activity by reducing its surface membrane expression when heterologously expressed in Xenopus oocytes (5). However, specificity of the effect of PKC activation to inhibit anion exchange mediated by Slc26a6 is indicated by the observation that PKC activation has no effect on transport activity of the closely related anion exchanger pendrin (SLC26A4) when expressed under identical conditions in Xenopus oocytes. The differential effects of PKC activation on the activities of these two anion exchangers may possibly be explained by the fact that Slc26a6 is predicted to have several PKC phosphorylation sites that are conserved among A6 orthologs, whereas pendrin is predicted to have only one conserved site by the same prediction program (http://scansite.mit.edu/cgi-bin/motifscan).

Studies in Slc26a6-null mice have revealed that intestinal oxalate secretion mediated by A6 plays a major role in limiting net absorption of ingested oxalate, thereby preventing hyperoxaluria and calcium oxalate urolithiasis (17). Experiments using Slc26a6-null mice have also suggested that intestinal oxalate secretion results from apical membrane Cl/oxalate exchange activity mediated by A6 (6). Thus PKC regulation of A6-mediated Cl/oxalate exchange activity is a potential factor that may affect overall oxalate homeostasis. Of note in this context is that regulation of oxalate transport in response to epinephrine and angiotensin II has been described in rabbit and rat colon, respectively (10, 12). Although the role of A6 in mediating oxalate transport in the colon is not yet known, these studies establish that intestinal oxalate transport is hormone regulated, and it is therefore possible that PKC-mediated modulation of A6 activity participates in these responses.

Another epithelium in which HCO3-independent modes of Cl/base exchange mediated by A6 are important physiologically is the proximal tubule of the kidney. Studies in isolated membrane vesicles and microperfused tubules have supported a model in which NaCl reabsorption across the apical mem-

**DISCUSSION**

In the present study we used the Xenopus oocyte expression system to demonstrate that multiple modes of anion exchange mediated by Slc26a6—namely Cl/formate exchange, Cl/oxalate exchange, and Cl/Cl exchange—are similarly inhibited by PKC activation. Experiments using PKC inhibitors strongly suggest that PKC-δ is the PKC isoform mediating this inhibitory regulation. Moreover, assessment of Slc26a6 localization by immunocytochemistry and biotinylation indicates that reduction in surface membrane expression of the transporter is the molecular mechanism underlying PKC-mediated inhibition of its transport activity. Supporting the physiological significance of these findings in the Xenopus oocyte expression system, we show that duodenal oxalate secretion, a process largely mediated by Slc26a6 (17), is also inhibited by PKC activation and that this effect is similarly blocked by the PKC-δ inhibitor rottlerin.

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**Fig. 9.** Effect of PKC activation on Slc26a6 protein expression assayed by immunoblotting. **A:** representative Western blot analysis of total and surface biotinylated Slc26a6. Oocytes were incubated with 50 nM PMA for 20 min, and then Slc26a6 protein expression was evaluated in oocyte lysate (lanes 1 and 2, 50 μg protein/lane) and after streptavidin precipitation of surface biotinylated proteins from 500 μg of initial cell lysate (lanes 3 and 4) performed as described in MATERIALS AND METHODS. **B:** densitometry of immunoblot results. Western blot band density was quantified with NIH Scion Image software. Values are means ± SE for 4 different experiments each of which was normalized to the respective Control value. PMA significantly reduced the amount of Slc26a6 protein available to surface biotinylation (P < 0.02, 2-tailed t-test).

**Fig. 10.** Effect of PKC activation on duodenal oxalate secretion. Mouse duodenal sheets were mounted in Ussing chambers, and 2 μM [14C]oxalate was added to the serosal solution. After a 30-min equilibration period, PMA (100 nM) was (PMA) or was not (Control) added both to the serosal and added to the serosal solution. After a 60-min period for calculation of unidirectional [14C]oxalate secretion in mucosal solutions, and samples of the mucosal solutions were collected before and after a 60-min period for calculation of unidirectional [14C]oxalate secretion activity. These measurements were also performed in the presence of rottlerin (10 μM) added to the serosal and mucosal solutions during both the 30-min equilibration period and the 60-min flux period in which PMA (100 nM) was (PMA + Rottlerin) or was not (Rottlerin) also present. Values are means ± SE for 12–16 different experiments. Rottlerin significantly reduced the inhibition induced by PMA (P < 0.02, 2-tailed t-test).
brane of proximal tubule cells can occur by Cl/formate exchange in parallel with Na/H exchanger isoform 3 (NHE3)-mediated Na/H exchange and H-coupled formate recycling (21, 30, 36–38) and by Cl/oxalate exchange in parallel with Na-sulfate cotransport and sulfate/oxalate exchange (20, 24, 36, 37). Tubule microperfusion studies in Slc26a6-null mice have shown that Slc26a6 is responsible for all oxalate-dependent NaCl transport and possibly a component of formate-dependent NaCl transport as well (40). Similarly, studies of renal brush border vesicles isolated from Slc26a6-null mice have demonstrated that A6 mediates all Cl/oxalate exchange activity and possibly a component of Cl/formate exchange activity (17). Thus the results of the present study support the possibility that signaling via PKC may permit regulation of A6-mediated NaCl transport in the proximal tubule.

Previous work using transfected HEK293 cells has attributed PKC inhibition of Cl/HCO3 exchange mediated by human Slc26A6 to phosphorylation at a specific site that disrupts binding of carbonic anhydrase II to the transporter, thereby disrupting a HCO3 transport metabolon (2). However, in the present study using Xenopus oocytes we find that mutation of the corresponding phosphorylation site of mouse Slc26a6 has no effect on inhibition in response to PKC activation. Moreover, we demonstrate that regulation of Slc26a6 activity by PKC results from reduced surface expression of the transporter, thereby inhibiting all tested modes of transport. It will therefore be important in future studies to determine whether one or both of these mechanisms of regulation of A6 by PKC operate(s) under physiological conditions in different native tissues.

In summary, we have demonstrated that multiple modes of anion exchange mediated by Slc26a6 are negatively regulated by PKC activation. PKC regulation of A6-mediated Cl/oxalate exchange may play important roles in modulating intestinal oxalate secretion and proximal tubule NaCl reabsorption.

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This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants K08-DK-067245 (H. A. Hassan), P01-DK-17433 (P. S. Aronson) and R01-DK-33793 (P. S. Aronson).


