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Physiology

Epithelial K channel expressed in *Xenopus* oocytes is inactivated by protein kinase C

(K balance/expression cloning/intestine/secretion/phorbol ester)

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ABSTRACT **K homeostasis is maintained in higher animals by epithelia of the kidney and intestine. Little is known regarding the molecular regulation of K secretion. We injected *Xenopus* oocytes with mRNA from teleost intestine, a K-secreting epithelium with apical membrane K channels. Oocytes expressed a conductance that displayed whole-cell current properties with the following characteristics: marked selectivity for K over Na and Cl, voltage-independent kinetics, Ca insensitivity, tonic activation, and inward rectification in symmetrical K. Barium, quinine, and tetraethylammonium blocked the conductance, whereas apamin, charybdotoxin, and 4-aminopyridine did not. The K conductance was rapidly ($t_{1/2}$ = 10 min) and completely inactivated by 4β-phorbol 12-myristate 13-acetate but not by 4α-phorbol 12,13-didecanoate. Sucrose density gradient fractionation revealed that mRNA required for expression is in the 1- to 2-kilobase size range, suggesting the possibility that a single subunit encodes the channel. The K conductance expressed from injection of size-fractionated mRNA was identical in all respects to that seen using unfractionated mRNA, including response to 4β-phorbol 12-myristate 13-acetate. The results suggest that protein kinase C regulates K secretion in epithelia by modulation of apical K channels.**

The intestinal epithelium of the winter flounder *Pseudopleuronectes americanus* actively secretes K and absorbs NaCl. This results from the presence of NaK2Cl cotransport and a large K conductance in the apical membrane and Na^+,K^+-ATPase and a Cl channel in the basolateral membrane (1, 2). The thick ascending limb of Henle's loop in mammalian kidney appears to function similarly (3). Other K secretory epithelia, including the kidney cortical collecting tubule (CCT) and the colon, also mediate K secretion through apical K channels (3, 4). In CCT, apical K channels activated by Ca have been described, but their role in K homeostasis is uncertain since they are unlikely to be open under physiologic conditions (5, 6). Recent patch clamp studies on the thick ascending limb of Henle's loop and CCT indicate the presence of another class of apical K channels in epithelia (7, 8). This class of K channels, distinguished by Ca insensitivity and inward rectification, has a relatively small single channel conductance and is expected to be open under physiologic conditions.

Secondary and tertiary messengers involved in regulating K homeostasis have not been completely defined. For example, although aldosterone is known to increase the apical membrane K conductance in rabbit CCT (9), the specific biochemical pathways involved between aldosterone binding and the increase in conductance remain a mystery. Likewise, 4β-phorbol 12-myristate 13-acetate (PMA) blocks K secretion in the rat CCT (10), but the mechanism (e.g., directly by inhibition of apical K channels or indirectly by abolition of the lumen negative transepithelial potential generated by active Na absorption) has not been determined. Here we provide evidence, obtained by expression of mRNA from a K secretory epithelium in frog oocytes, that suggests modulation of apical K channels by protein kinase C (PKC).

METHODS

Total RNA was obtained from fresh or liquid N_2-frozen flounder intestinal mucosal scrapings by extraction with phenol/chloroform (11). In some cases, scrapings were initially homogenized in guanidinium isothiocyanate buffer as described (12). mRNA was isolated by oligo(dT) chromatography (11). *Xenopus laevis* oocytes were isolated and denuded of follicular epithelium by 4- to 8-hr collagenase treatment (Worthington type 2, 2.5 mg/ml) and sorted to obtain Dumont stages V–VI (13). Oocytes were injected with 25–100 ng of mRNA or H_2O in a 50-nl volume and incubated for 1–6 days prior to assay. Injections were performed using a modified Drummond positive displacement pipettor (Broomall, PA) mounted on a micromanipulator. Whole-cell currents were assayed with a two-microelectrode voltage clamp (Dagan, model 8500) controlled by a personal computer using pCLAMP software (Axon Instruments, Burlingame, CA). Microelectrodes (1 MΩ) were pulled on a vertical puller (Kopf 720) and filled with 0.5 M KCl. Tip potentials were <5 mV and were therefore disregarded. Data were output to a chart recorder and an analog/digital converter. Digitized records stored on magnetic disk were analyzed offline (pCLAMP) and exported to graphics software (Sigmaplot, Jandel Scientific, Corte Madera, CA). Current–voltage (*I–V*) plots display values 1.5 s after the initiation of the pulse. All incubations and recordings were performed in Ringer solution at 19°C unless indicated otherwise. The Ringer solution contained (in mM) Na, 90.4; K, 1; Ca, 0.74; Mg, 0.82; Cl, 89.8; HCO_3, 2.4; SO_4, 0.82; NO_3, 0.66; and Hepes acid, 10 (pH 7.4 with NaOH). Also included were penicillin (100 μg/ml), streptomycin (100 μg/ml), and gentamycin (50 μg/ml).

Sucrose density gradient fractionation of flounder intestinal mRNA was performed essentially as described (14). Fractions (2 μg per lane) were electrophoresed on an agarose gel (1%) and visualized under UV light by ethidium bromide fluorescence. An RNA ladder (BRL) was used for molecular size determination. All fractions were prescreened for translational activity by ability to incorporate trichloroacetic acid-precipitable [^{35}S]methionine into protein in an *in vitro* translation (IVT) assay. Kits for IVT were obtained from Promega or BRL and used according to the manufacturer's recommendations. mRNA fractions were screened for K channel activity by ability to hyperpolarize oocyte resting membrane potential.

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Abbreviations: PMA, 4β-phorbol 12-myristate 13-acetate; PKC, protein kinase C; TEA, tetraethylammonium; CCT, cortical collecting tubule; *I–V*, current–voltage.

This approach, though insensitive to the number of K channels expressed, allowed the individual screening of a large number of oocytes from the same frog on the same day, thus controlling for possible artifacts due to time or animal variance.

RESULTS

Expression of Epithelial K Channel. When we injected 25–100 ng of mRNA from flounder intestinal mucosa into *Xenopus* oocytes, a conductance was expressed that persisted for 1–6 days, diminishing in magnitude with time. The expressed conductance was easily distinguished from endogenous oocyte conductances by its characteristic inward rectification (compare Fig. 1 *a* and *b*). It shifted the reversal potential of whole-oocyte current to near the predicted equilibrium potential for K (E_K) (15) and did not display voltage activation or inactivation, being tonically open at all membrane potentials examined (Fig. 1*c*). The expressed conductance was highly selective for K over Na and Cl (Fig. 1*d*): replacing external Cl with gluconate did not alter the *I–V* relation; substituting external Na with K shifted the reversal potential to 0 mV, indicating a membrane dominated by K-selective channels (slope = 47 ± 2 mV per decade [K] in unfractionated mRNA-injected cells, n = 8; slope = 43 ± 4 mV per decade [K] in fraction 3 mRNA-injected cells, n = 4). At approximately equal inside and outside K concentrations, the slope conductances were 27 μS in the inward direction and 9 μS in the outward direction (Fig. 1*d*).

Pharmacology. The K conductance was blocked by external Ba (Fig. 1*b*). The concentration of drug required for 50% inhibition (IC_{50}) by Ba was 160 μM (Fig. 2). The Ba block was always complete in a few seconds and could be reversed upon washing. The K conductance was also reduced by external quinine (IC_{50} = 300 μM) and TEA (IC_{50} = 10 mM (Fig. 2), effects that were only partially reversed by prolonged washing. Quinidine and lidocaine inhibited the conductance with IC_{50} values of 350 μM and 1 mM, respectively (not shown, n = 2). No inhibition was detected with the following compounds applied externally (n = 4 for each compound): apamin

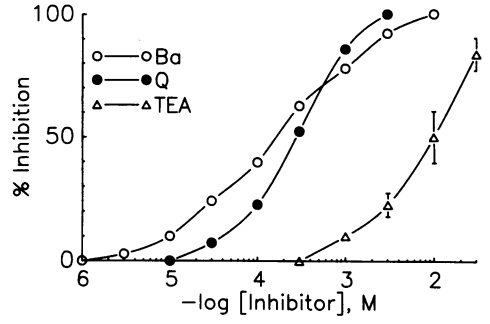

FIG. 2. K conductance is blocked by K channel inhibitors. Dose–inhibition curves for barium (Ba, n = 5), quinine (Q, n = 4), and tetraethylammonium (TEA, n = 3) are shown. Values are means ± 1 SE. The data displayed are derived from fraction 3 mRNA-injected cells (see Fig. 4). Identical results were obtained for unfractionated mRNA-injected cells. Data points without error bars had SE smaller than the symbol. Effects of inhibitors on currents in H_2O-injected oocytes were subtracted from results in mRNA-injected oocytes. TEA had no consistent effect on endogenous currents (measured in H_2O-injected controls). For TEA, 100% inhibition was taken as that produced by 3 mM barium.

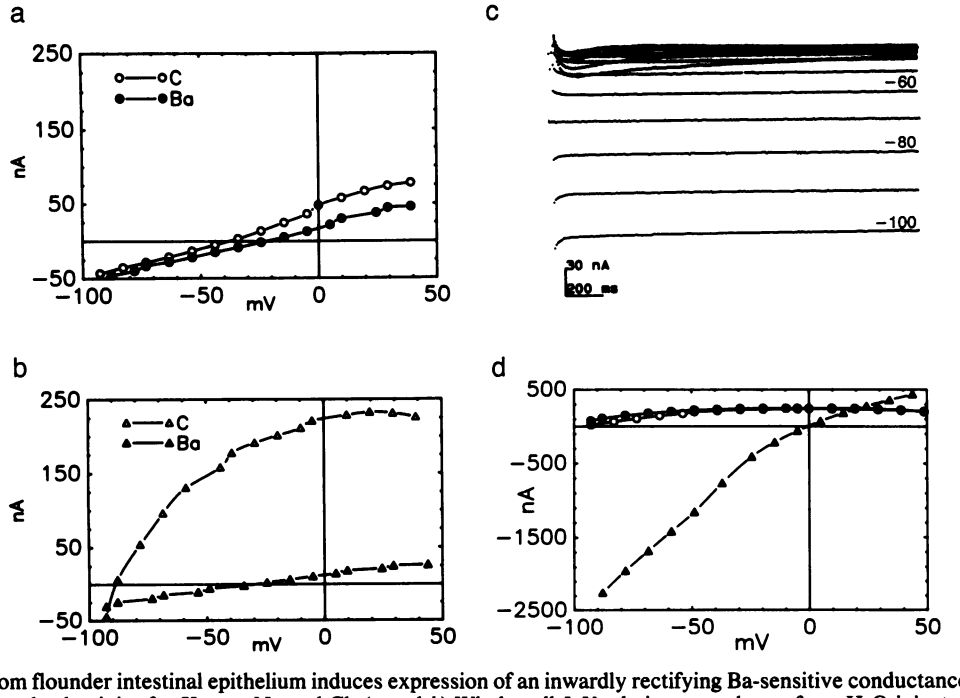

FIG. 1. mRNA from flounder intestinal epithelium induces expression of an inwardly rectifying Ba-sensitive conductance that has a reversal potential close to E_k and selectivity for K over Na and Cl. (*a* and *b*) Whole-cell *I–V* relations are shown for a H_2O-injected oocyte (*a*) and an oocyte injected with unfractionated mRNA (*b*) in the absence (C) or presence (Ba) of barium (1 mM). Outward currents (cations leaving the cell) are depicted as positive. The reversal potential is shifted from −40 mV in the control oocyte to −90 mV in the mRNA-injected cell. Plots are representative of 33 and 52 oocytes in each group, where the average currents (at 0 mV) were 44 ± 1 nA and 265 ± 22 nA, in controls and mRNA-injected cells, respectively. The majority of recordings were made 1–2 days after injection. On these days, >80% of mRNA-injected cells that survived the injection expressed the conductance. For fraction 3 mRNA (see Fig. 4), the average current (at 0 mV) was 251 ± 25 nA (53 cells). (*c*) Family of Ba-sensitive currents for the cell depicted in *b*. Membrane potential was held at −70 mV and stepped to potentials between −100 and +40 mV in 10-mV increments. Pulse duration was 2 s and the interpulse interval was 2 s or 10 s. Ba-sensitive currents from the H_2O-injected cell in *a* were subtracted. The current traces at −100, −80, and −60 mV are labeled accordingly. The small components of current activation with time at potentials positive to −30 mV were not a consistent observation. (*d*) The expressed conductance is highly selective for K. Representative plot depicting the *I–V* relation of whole-cell current in the standard Ringer (○), Ringer containing gluconate for all but 3 mM Cl (●), and Ringer containing K for all but 5 mM Na (▲). Replacement of external chloride with gluconate had no effect on the shape of the *I–V* relation or on the reversal potential either in unfractionated mRNA-injected cells (n = 5) or in fraction 3 mRNA-injected cells (n = 10).

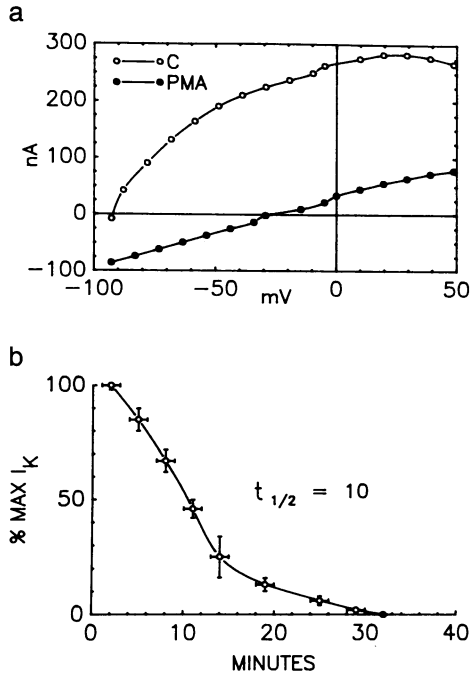

FIG. 3. K conductance is inactivated by PKC. (*a*) *I–V* relations of an oocyte injected with unfractionated mRNA determined immediately before (○) and 30 min after (●) addition of 100 nM PMA to the bath. Identical results were obtained from fraction 3 (see Fig. 4) mRNA-injected cells. (*b*) The time required for 50% inactivation ($t_{1/2}$) of the expressed conductance is 10 min. Currents were measured at −30 mV and are expressed as the percentage of the total current inhibited by PMA (% MAX I_K), which averaged 154 ± 22 nA (n = 12) in unfractionated mRNA-injected cells and 178 ± 53 nA (n = 9) in fraction 3 mRNA-injected cells. Phorbol esters were dissolved in dimethyl sulfoxide, which had a final concentration in the bath of 0.1% (vol/vol).

(500 μM), capsaicin (100 μM), 4-aminopyridine (3 mM), cesium ion (10 mM), scorpion venom (from *Leiurus quinquestriatus*; charybdotoxin up to 500 nM assuming it is 0.1% of the venom).

Regulation. Treatment of mRNA-injected oocytes with 0.5–1 mM 8-bromoadenosine 3′,5′-cyclic monophosphate or 1–10 μM A23187 for up to 1 hr had no effect. Similarly, lowering Ca_i by treatment with 50–250 μM bis(2-amino-5-methylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetraacetoxymethyl ester for 5–22 hr had no effect. In contrast, the PKC activator PMA (100 nM) inactivated the K conductance with a $t_{1/2}$ of 10 min (Fig. 3 *a* and *b*). The effect of PMA was consistent with inactivation of only the expressed K conductance since a Ba-sensitive outward current of 25–35 nA (measured at V_m = 0) persisted after PMA treatment of mRNA-injected cells (n = 5). This residual current was identical in magnitude to that found in control oocytes (Fig. 1*a*). The phorbol ester 4α-phorbol 12,13-didecanoate, which does not activate PKC, was without effect (n = 6). PMA had no effect on control cells over the time course examined.

Fractionation of mRNA. Sucrose density gradient fractionation defined an mRNA molecular size window, 1–2 kilobases (kb), required for hyperpolarization of the oocyte membrane (Fig. 4). Complete electrophysiologic characterization (pharmacology, selectivity, and regulation) utilizing fraction 3 gave identical results to experiments using unfractionated mRNA (see above and figure legends).

DISCUSSION

In this report we have demonstrated the expression of an epithelial K channel in frog oocytes and its regulation by

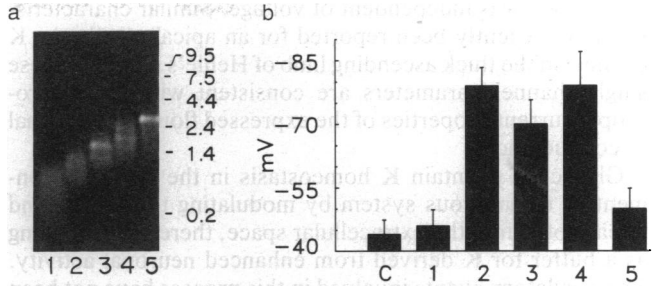

FIG. 4. mRNA encoding the expressed K conductance is 1–2 kb. (*a*) Molecular size distribution of mRNA fractions 1–5 (ordinate, kb). (*b*) Resting membrane potential in control H_2O-injected oocytes (C) and in oocytes injected with mRNA fractions 1–5 (25 ng per oocyte). Cells receiving fractions 2–4 were hyperpolarized, whereas cells receiving fractions 1 and 5 were not different than controls (n = 10–15 oocytes per group, $P < 0.001$, analysis of variance).

PKC. The conductance displays high selectivity for K over Na and Cl, inward rectification, and voltage-independent kinetics of whole-cell currents; it is open under physiologic conditions. Barium, quinine, and TEA inhibit the conductance, whereas apamin, 4-aminopyridine, and charybdotoxin do not. It is not modulated by cAMP or Ca but is inactivated by PMA. The mRNA encoding the channel is in a 1- to 2-kb molecular size class.

The most likely explanation for the present results is that injection of exogenous epithelial mRNA results in the direct expression of epithelial K channels. Definitive proof of this will require reconstitution of the protein product in phospholipid vesicles in the absence of other proteins, a difficult task that has yet to be achieved for any K channel.

Recently, Takumi *et al.* (16) reported that a 585-nucleotide cDNA clone isolated from a rat kidney library induced expression of a K conductance when *in vitro* mRNA transcripts were injected into oocytes. This conductance displayed inward rectification and was reduced by TEA and Ba, similar to the conductance reported here. However, the kidney-derived K conductance was only activated at depolarizing potentials (positive to −40 mV). The voltage activation (and inactivation) had a slow time course that did not appear complete at 100 s (16). In contrast, the enterocyte-derived K conductance did not display voltage activation but was tonically open at all membrane potentials in the physiologic range for epithelia.

In the intact flounder intestinal epithelium, treatment of the basolateral membrane with 5 mM Ba had no effect on transepithelial conductance, short-circuit current, or K transport (17). Moreover, K substitution experiments performed while monitoring basolateral membrane potential indicated that this membrane was essentially nonconductive to K (18). In contrast, similar experiments targeted at the apical membrane revealed that net K secretion was reversed to net absorption by 5 mM Ba (17) and was partially inhibited by 10 mM TEA (19). The apical membrane potential was a near-Nernstian function of bath [K], and the estimated apical membrane K conductance was unaffected by A23187, indicating Ca insensitivity (20). Therefore, it is likely that the channel expressed in oocytes has an apical location in the intact epithelium and is involved in the active secretion of K.

In *in vitro* microperfusion studies, PKC has been shown to inhibit K secretion in the mammalian CCT (10), the primary site for control of whole body K balance. A K channel with characteristics similar to the K conductance reported here, including insensitivity to Ca, was found in the apical membrane of the CCT (7). Cell-attached patch clamp experiments indicated that this channel had a conductance of 9 pS (NaCl pipet, potassium gluconate bath), an inwardly rectifying single-channel *I–V* relation, and high open probability (P_o >

0.9), which was independent of voltage. Similar characteristics have recently been reported for an apical membrane K channel in the thick ascending limb of Henle's loop (8). These single-channel parameters are consistent with the macroscopic current properties of the expressed flounder intestinal K conductance.

Glial cells maintain K homeostasis in the microenvironment of the nervous system by modulating the uptake and release of K into the extracellular space, thereby functioning as a buffer for K derived from enhanced neuronal activity. The regulatory events involved in this process have not been completely elucidated, however (21, 22). The present data, together with the recent observation that PMA inactivates a K conductance in oligodendrocytes (23), raise the possibility that PKC functions as a component of the K homeostatic regulatory cascade in the nervous system as well as in epithelia.

Effects of phorbol esters on ionic currents independent of PKC have been reported (24). Since the concentration of phorbol esters employed in this study was in the nanomolar range, and since the 4α derivative had no effect on expressed K current, we conclude that K channel inactivation is mediated by PKC. Whether or not PKC phosphorylates the K channel directly or an intermediate regulatory protein remains to be determined.

PKC modulation of K conductance has been reported in *Hermissenda* photoreceptor cells (25, 26), insulinoma cells (27–29), cardiac ventricular myocytes (30, 31), hippocampal pyramidal neurons (32, 33), and hippocampal and striatal synaptosomes (34). The present study suggests that PKC regulation of K conductance also occurs in K-secreting epithelia. This modulation may be an integral component of the regulatory events contributing to epithelial control of whole body K homeostasis.

Fractionation of mRNA suggests the message required for K channel expression is 1–2 kb. Moreover, the data are consistent with the epithelial K conductance resulting from the expression of a single polypeptide. This is in agreement with studies employing transcripts derived from the Shaker locus of *Drosophila* (35, 36) and mammalian homologs (37), a delayed rectifier K channel cDNA (38), and a cDNA isolated from a kidney library (16); in all cases a single transcript was sufficient for expression of K conductance. The possibility that cotranslation of more than one mRNA species of similar size is required for epithelial K channel expression cannot be ruled out, however.

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