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Patch-clamp evidence for calcium channels in apical membranes of rabbit kidney connecting tubules.

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Research Article

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Patch-Clamp Evidence for Calcium Channels in Apical Membranes of Rabbit Kidney Connecting Tubules

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Abstract

To test the hypothesis that Ca channel plays a role in renal epithelial Ca transport, we exposed and patched apical membranes of freshly microdissected rabbit connecting tubules (CNTs). Single channel Ca currents were recorded with Ba as the charge carrier. In the cell-attached mode, 8-Br-cAMP increased the open-state probability (P_o) to 0.6%. In excised, inside-out patches, P_o was low spontaneously and remained low during either bath protein kinase A catalytic subunit (PKA_{cs}) or Bay K 8644. Exposure to both agonists, however, unmasked Ca channels previously latent with only one, raising P_o by 1.05% at membrane potential of −70 mV.

Mean P_o for 14 seals (2.57%) peaked at −70 mV, declining with either hyperpolarization or depolarization. The slope conductance was 25 pS. The extrapolated reversal potential (138 mV) agrees with the calculated equilibrium potential for Ca (158 mV). The Ca to Na permeability ratio exceeded 2,800. In four patches stimulated by Bay K 8644 and PKA_{cs}, bath nifedipine reduced P_o from 1.03 to 0.15% at −63 mV. These patch-clamp data demonstrate a selective, 25-pS, cAMP/PKA_{cs}-sensitive Ca channel in apical membranes of CNT. P_o is stimulated by PKA_{cs} and dihydropyridine (DHP) agonist, but inhibited by DHP antagonist and by depolarization. The data are consistent with the potential role of apical membrane Ca channel in epithelial Ca transport. (*J. Clin. Invest.* 1993. 92:2731–2736.) Key words: apical membrane • calcium • channels • patch-clamp • tubules

Introduction

Earlier studies indicate that PTH and cAMP stimulate Ca transport in rabbit kidney connecting tubules (CNT)[1] (1, 2). Our recent fura-2 studies suggest that PTH and cAMP both increase Ca entry across the apical membrane (AM) (3, 4). Together, these results raise the hypothesis that apical membrane Ca channels are the initial and PTH-regulated step in lumen-to-bath Ca transport. However, to date, there exists no direct or definitive evidence other than changes in $[Ca^{2+}]_i$ consistent with the expected response to dihydropyridine (5) and/or PTH (3–5). But PTH also activates basolateral membrane (BLM) Ca influx (3), a process reproducible by cAMP (4) and suppressible by protein kinase A (PKA) inhibitors (6). Thus, whole-cell $[Ca^{2+}]_i$ response does not necessarily identify the specific site of actions in a polarized epithelial cell, let alone defining the pathway as an apical Ca channel.

Recently, the techniques of tubule dissection, lumen exposure, and patch-clamp electrophysiology have been combined to fruitfully study AM Na and K channels in cortical collecting tubules (7, 8). We used a similar strategy to test the Ca channel hypothesis in the rabbit CNT. Our results are herein reported.

Methods

Albino rabbits were fed regular laboratory diet until they weighed ≥ 1.9 kg. After decapitation, CNTs were dissected as before (3, 4, 6), at 10°C in a solution containing (in mM) KCl 5, NaCl 148, $CaCl_2$ 1.7, Na_2HPO_4 2, $MgCl_2$ 1.2, D-glucose 5.6, L-alanine 6, and pH to 7.4 with NaOH. Placed on a 0.5-cm^2 plastic plate and following the method of Palmer and Frindt (7), the CNT was split lengthwise to expose the AM and immobilized by tacking down at both ends. The plate was transferred into a superfusion chamber (0.35 ml) over an inverted microscope. Separate power supply and grounding were used to minimize noise. All patch-clamp equipment was housed, if possible, inside an aluminum Faraday cage on a vibration-free table. Direct current battery illuminated the microscope. Till excision, the CNT was bathed in a solution containing (in mM) Na acetate 138, K acetate 5, Mg acetate 1.5, Ca acetate 1.8, Hepes 10, D-glucose 5, and pH to 7.4 with NaOH. Patch electrodes, 8–10 MΩ, made from capillary tubes by a two-stage vertical puller (Narashige USA, Greenvale, NY), were filled with a solution containing (in mM) Ba acetate 50, Ca acetate 0 or 1.8, D-mannitol 143–148, and in some experiments, amiloride 0.2 mM and/or Bay K 8644. Mounted on the integrated head stage of an Axopatch 1-D amplifier (Axon Instruments, Inc., Foster City, CA), the electrode was lowered into the bath. After offsetting liquid junction potential, the electrode was deposited on the AM to establish a high resistance seal (≥ 15 GΩ) by gentle suction. Both on-cell and excised, inside-out patches were studied at 22±1°C. After excision, bath contained 0 or 20 mM Na acetate, 0.23 or 0.5 mM Mg acetate, 10 μM EGTA, and enough D-mannitol (231–273 mM) to match the osmolality of pipette solution. The 0.23 mM Mg bath was used to accommodate the extra free Mg due to 2 mM Mg ATP. CsOH was used to adjust pH of Na-free solutions to 7.4. Cations leaving pipette were recorded as upward deflections on line to a personal computer interfaced with analog to digital/digital to analog converter at a 500-μs rate and filtered at 2 kHz. Currents were also stored in a videocassette recorder (Toshiba, Elmont, NY) via a digital recorder. Data were analyzed by pCLAMP software program (Axon Instruments, Inc.) at 2 kHz by a computer digital Gaussian filter. A channel was considered open if duration ≥ 500 μs and amplitude ≥ $i_0 + (i_1 - i_0)/2$, where i_0 denoted closed state and i_1 open-state currents. P_o is calculated as the ratio of fractional open time to the maximal number of unitary current amplitudes in a given seal recorded ≥ 20 min. Fractional open time, derived by the pCLAMP software, is defined as the sum total of time duration of all open events divided by the total recording time.

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1. *Abbreviations used in this paper:* AM, apical membrane; BLM, basolateral membrane; CNT, connecting tubule; E_{eq}, equilibrium potential; E_{rev}, reversal potential; P_o, open-state probability; PD, potential difference; PKA_{cs}, protein kinase A catalytic subunit; PKC, protein kinase C; V_m, membrane potential; V_p, pipette potential.

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All reagent-grade chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) except for: nifedipine, Bay K 8644, and Hepes (Ultra grade) from Calbiochem Corp. (La Jolla, CA); fura-2 free acid from Molecular Probes Inc. (Eugene, OR), CsOH from Aldrich Chemical Co. (Milwaukee, WI). The composition and osmolality of all solutions were verified by published methods (3, 4, 6). Data were subjected to appropriate Student's t tests (9) and presented as mean±SE. A P value ≥ 0.05 is considered significant.

Results

For pipette potentials (V_p) between −10 and 70 mV, spontaneous P_o for cell-attached patches was 0.026±0.023% (n = 7). Activity was very infrequent in the absence of agonists. 11.5±2.3 min after incubation with bath 8-Br-cAMP, 1–3.75 mM, channel recordings were resumed in the continued presence of cAMP. The midpoint of this cAMP period corresponded to 17.8±2.2 min after introducing 8-Br-cAMP to the bath. Mean P_o was raised to 0.54±0.13%, with an increment of 0.51±0.15, $P < 0.02$ (Fig. 1, *top*). From the current–voltage relationship (Fig. 1, *bottom*), the slope conductance was calculated to be 24.2 pS and the extrapolated pipette reversal potential (E_{rev}) was −188 mV. Since distal tubule AM is known to be depolarized ~ 42 mV by lumen Ba (from −70 to −28 mV) (10), this E_{rev} corresponds to a transmembrane potential difference (PD) of 160 mV [−28 − (−188)]. Thus, the PD is comparable to the calculated Nernst equilibrium potential (E_{eq}) of 162 mV.

To better define the time course of cAMP action, recordings were made at 2, 4, and 6 min after administration of 8-Br-cAMP in four of these seven cell-attached patches. No stimulation was detectable at an applied V_p of 0 or 55 mV during the initial 2 and 4 min after 8-Br-cAMP. However, further monitoring at V_p of 0, 25–30, or 55 mV, revealed significant effects on P_o by the 6th min of cAMP exposure (0.60±0.17%) (Fig. 2). Subsequent recordings at 10–11-min intervals pulsed at similar V_p demonstrated sustained and similar action during the 11th min (0.50%), 22nd min (0.46%), 33rd min (0.85%), 43rd min (0.78%), 53rd min (0.40%), and 93rd min (0.40%) of 8-Br-cAMP treatment. These results suggest that at the latest, significant and full effects of 8-Br-cAMP are demonstrable between 5 and 7 min after bath administration, resembling the time course of stimulation of $[Ca^{2+}]_i$ in the CNT (3, 4, 6). Strictly speaking, it is not possible to vigorously compare these two effects of 8-Br-cAMP because of several technical and intrinsic biological reasons. First, by definition, the apical membrane patch clamp approach precludes the contribution of Ca influx across the basolateral membrane, which is known to be a definite and major source for the rise in $[Ca^{2+}]_i$ as revealed by prior epifluorescence studies (4). Second, the change in fura-2 signals represents the total summation of all Ca influxed across a number of cells in a 0.3–0.5-mm length of CNT continually monitored, as opposed to the periodic measurements of ion current across 1 μm of apical membrane pulsed at intervals. Third, in our previous epifluorescence studies, the chamber temperature was 37°C, as opposed to 22°C in the present patch-clamp experiments. Fourth, the sensitivity limits may be quite different between these two vastly different experimental techniques.

In the cell-attached mode, cytosolic conditions facing the interior of the patched membrane cannot be defined or controlled. To control cytoplasmic solution composition and membrane potential (V_m), subsequent studies were therefore done in excised, inside-out patches. Before excision, an 82-s record was made at zero V_p, although generally no electrical activity was found.

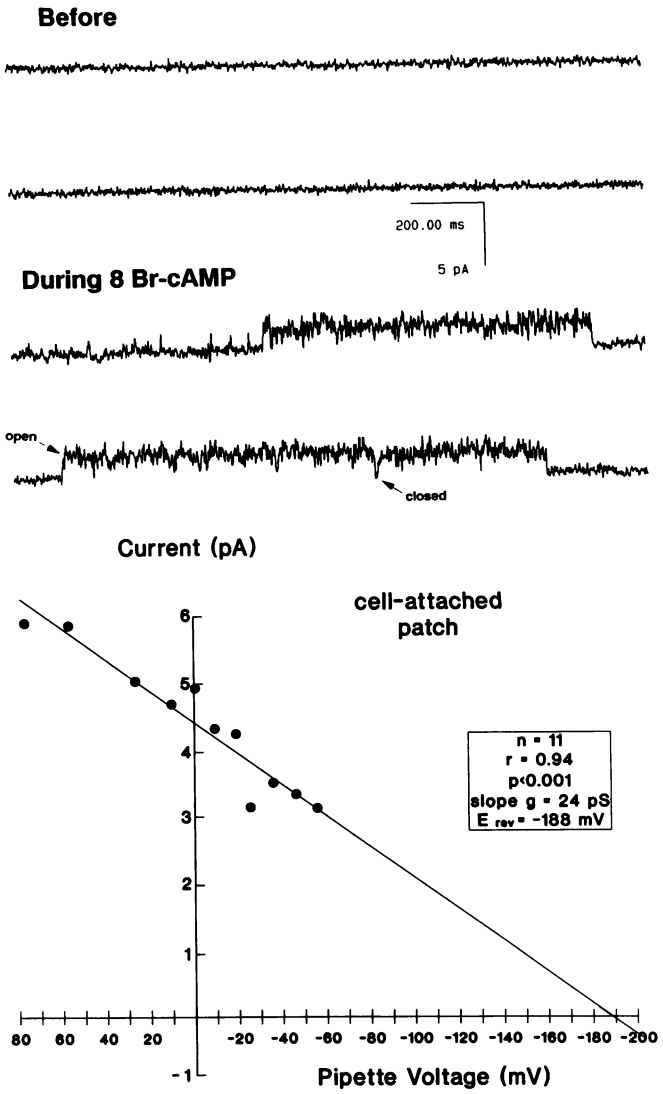

Figure 1. (*Top*) Response of a cell-attached patch before (upper two tracings) and during 1 mM 8-Br-cAMP (lower two tracings). Pipette contained 0.2 mM amiloride. V_p = 55 mV. Channel closure is indicated by "closed." (*Bottom*) Corresponding current-voltage curve.

In excised patches, spontaneous activity was absent in all but 1 seal (P_o = 0.052% at −60 mV), under the conditions of our experiment. Overall, 36 of 60 excised patches displayed Ca channel if stimulated by agonists. Five seals were relatively quiet at −70 mV, with only bath Bay K, 20±8 μM (P_o = 0.068±0.057%). When PKA_{cs} was added to the bath, P_o became 1.25±0.75% (Fig. 3). Conversely, three seals were relatively quiet at −70 mV, with P_o of 0.006% when exposed to only 1 μg/ml of bath PKA_{cs} and 2 mM of Mg ATP until Bay K was added (P_o = 0.83±0.53%) (Fig. 4). The response of these eight seals, negative with only one agonist but positive with dual agonists (P_o = 1.09±0.45, $P < 0.05$), suggests cooperativity. On the average, 2.3 channels were found per patch (mean tip diameter, 1 μm). A single channel recorded for over 20 min with unambiguous unitary current is shown in Fig. 5.

14 seals, all treated with PKA_{cs} and Bay K, were subjected to various test potentials (Fig. 6). P_o (2.57%) peaked at −70 mV, the estimated resting V_m for apical membranes of CNT

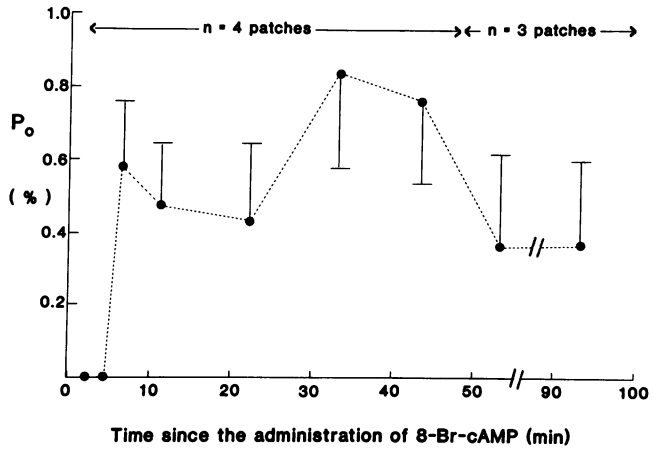

Figure 2. Time course of the stimulatory effects of 8-Br-cAMP on P_o of apical Ca channels in four cell-attached patches. Recordings were made at 2, 4, and 6 min after introducing 1–3.75 mM 8-Br-cAMP into the bath and repeated at 11, 23, 33, 63, 53, and 93 min. V_p was 0, 25 or 30, and 55 mV at each time point.

(11), declining to 0.57% at −50 mV and to < 0.3% as the V_m deviated from the resting value. Depolarization beyond −20 mV essentially abolished all openings, which were restored by repolarization.

Peak current was plotted against V_m for 12 inside-out patches (Fig. 7). E_{eq} was calculated by the Goldman equation. Pipette Ba^{2+} (50 mM) and Ca^{2+} (1.8 mM) were assumed to be 100% dissociated, based on the measured osmolality. Bath [Ca^{2+}], buffered by 10 μM EGTA, was 200 nM, as analyzed by fluorometric titration with fura-2 free acid. The calculated E_{eq} was 158 mV, in good agreement with the E_{rev} extrapolated from the current-voltage curve (138 mV) (Fig. 7), although more vigorous determinations of E_{rev} will depend on additional data obtained over V_m in the more positive range. In contrast, the calculated E_{eq} for Na was 34.9 mV, using pipette [Na] of 80 μM and bath [Na] of 20 μM, as measured by atomic absorptionmetry from four bath aliquots. In eight seals, pipette contained 0.2 mM amiloride and bath [Na] was 20 mM. The calculated Na E_{eq} was −139 mV. The current-voltage curve for these eight seals yielded similar results (r = 0.93, P < 0.01, slope g = 26 pS, and E_{rev} = 132 mV). Calculated as published (12), the Ca/Na permeability ratio was 2,855, indicating a high selectivity of this channel for Ca over Na.

Effects of nifedipine, 25 μM to 1 mM, were studied in four seals (Fig. 8). Before excision, CNTs were incubated in 3.75 mM 8-Br-cAMP for 21±5 min. Pipette contained 24±10 μM Bay K throughout all phases of the experiments. After excision, bath contained 1 μg/ml PKA_{cs}, 2 mM Mg ATP, and ≤ 0.05% DMSO. In two seals, bath also contained 50 μM Bay K, but this additional source of Bay K produced similar results. These agonists raised peak P_o to 1.03±0.12%. When nifedipine was added to the bath with identical [PKA_{cs}] and [Mg ATP], replacing any previous bath BAY K, P_o fell by 0.88±0.09% to 0.15±0.10%, P < 0.005, despite the continued presence of pipette Bay K and bath PKA_{cs} (Fig. 8). In two seals studied, substitution of bath nifedipine with bath Bay K restored channel activity.

Discussion

Our data document Ca channels in apical membranes of rabbit CNT, with a 60% incidence. With 50 mM Ba, the slope g is 25

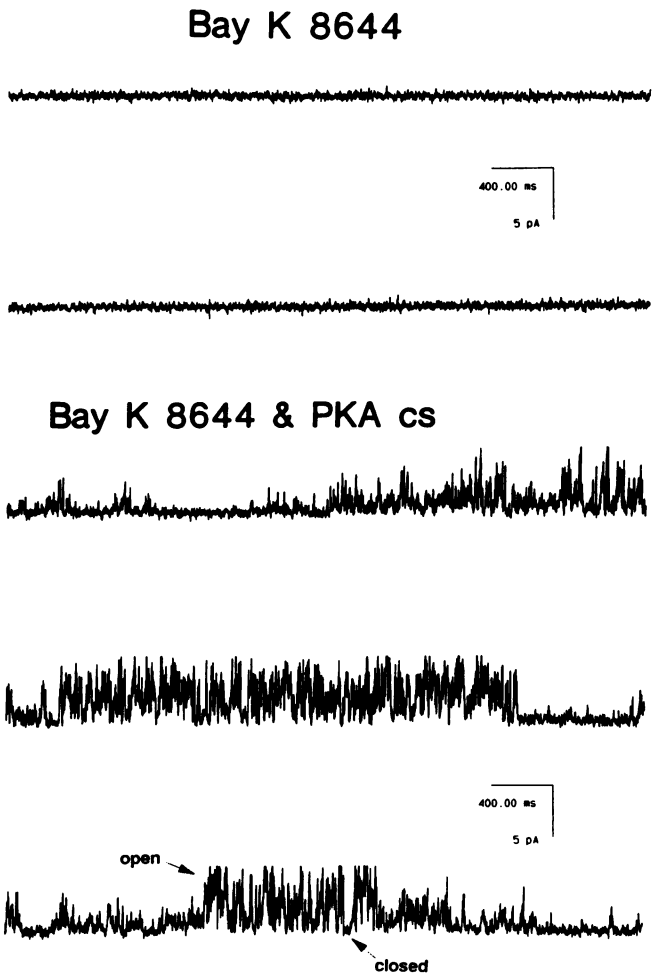

Figure 3. Effect of bath PKA_{cs} (1 μg/ml) and Mg ATP (2 mM) in an excised, inside-out seal pretreated with 18.5 μM bath Bay K 8644. V_m = −90 mV. Upper two tracings, Bay K alone; bottom three tracings, during Bay K and PKA_{cs}. Baseline current levels are denoted as closed. These tracings are representative of five similar seals.

pS. Ca/Na permeability ratio was ~ 2,800. Spontaneous P_o is negligible, but it is stimulated to ~ 2% by agonists. The channels are activated by either cAMP/PKA_{cs} or DHP Ca agonist, with the most consistent stimulation if exposed to both. Channel activity is reversibly inhibited by dihydropyridine Ca antagonist. These features are shared by voltage-activated L-type Ca channels in excitable tissues (12–15).

There are, however, notable differences. First, renal cell Ca channels are not activated by membrane depolarization (Fig. 6), which largely though reversibly abolished all openings. Activity seemed to peak around −70 mV, the reported resting V_m (10). Second, the run-down phenomenon found in excitable cells (11, 13, 14) is absent in cell-free membrane patches of CNT if treated with PKA_{cs} and ATP.

Several inferences can be made from our results. First, the patch clamp data are consistent with the notion that the PTH-activated, cAMP-stimulated apical membrane Ca influx step is a Ca channel. Second, between the BLM and AM across which PTH and cAMP enhance Ca entry (3, 4), our data suggest the AM as at least one target site, also consistent with the known stimulation of transepithelial Ca absorption. Third, the precise cellular mechanism(s) for PTH action have been controversial, given the evidence separately for the second messenger role of

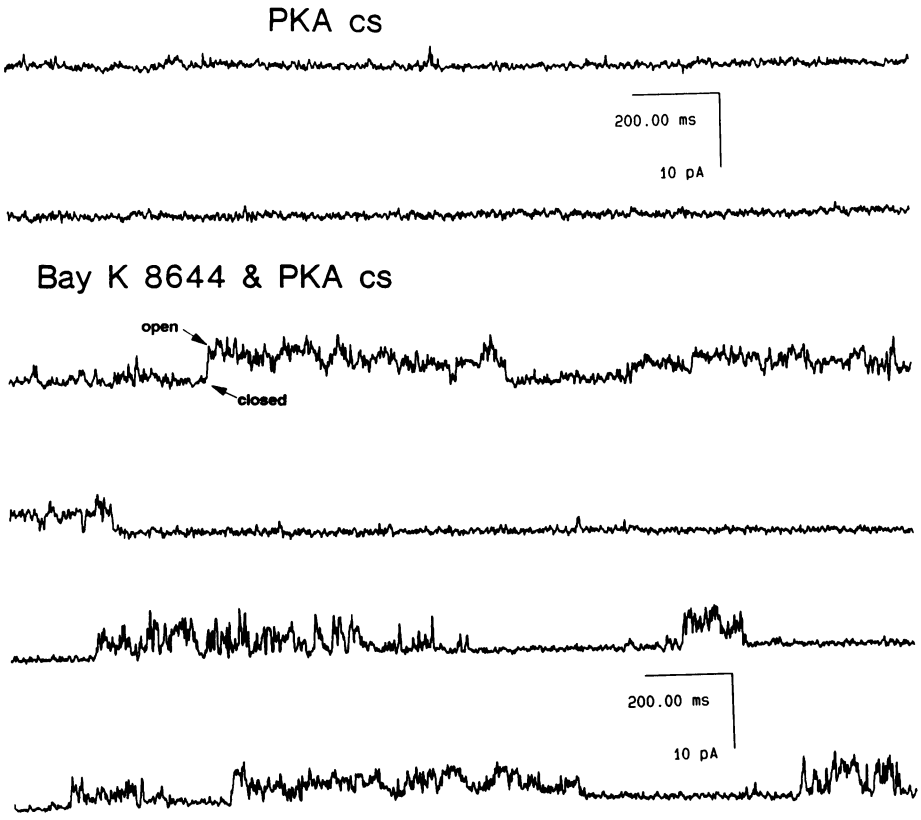

Figure 4. Effects of bath Bay K 8644 (50 μM) in an excised, inside-out patch pretreated with 18.5 μM pipette Bay K (1 μg/ml of PKA_{cs} and 2 mM Mg ATP). V_m = −70 mV. Baseline current levels are denoted as closed. This tracing is representative of three similar patches.

diacylglycerol-PKC (16), inositol 1,3,4 trisphosphate (16) and PKA (6). For example, in intact tubules, reproduction by cAMP of the stimulatory effects of PTH on $[Ca^{2+}]_i$ could be due to increased luminal Na entry and consequently enhanced BLM Ca influx via the Ca:Na antiporter. Likewise, the suppression of these effects by nonspecific protein kinase inhibitors (6) does not exclude the role of PKC. Activation of Ca channels in isolated AM by PKA_{cs} and in intact cells by 8-Br-cAMP suggest that this enzyme system potentially mediates the stimulatory effects of PTH and cAMP on $[Ca^{2+}]_i$ and on lumen-to-bath Ca transport. It must however be emphasized that these implications do not necessarily establish the role of apical Ca channel in Ca absorption or exclude other functions (see below).

Fourth, nifedipine suppression despite prior and continued exposure to PKA_{cs} and ATP suggests that the phosphorylated substrate(s) or protein(s) must be integral in the membrane and associated with the dihydropyridine-binding site, analogous to excitable cells L-type Ca channels (14, 15). The cooperativity between PKA_{cs} and Bay K 8644 in stimulating P_o reinforces this notion.

Fifth, with full control and definition of the electrochemical milieu on both sides of the membrane, it can be inferred that the effects of PKA_{cs} are electrogenic and independent of $[Na^+]$ or $[H^+]$ gradients, which are typical of Ca:Na or Ca:H antiporters. Channel conductance and E_{rev} are essentially unaltered by 20 mM bath Na and pipette amiloride. The high Ca/Na permeability ratio argues against a nonspecific cation channel.

Sixth, the AM Ca flux rate (pmol/mm per min) can be calculated by Ca current · tubule luminal surface area/patched area · channel incidence · channels n per patch · P_o/Faraday constant. Given the measured mean lumen i.d. of 30 μm, 2.3 channels per 1 μm patch diameter, 2.57% for P_o, and 5.18 pA for peak current at resting V_m, estimates yield a transmembrane Ca flux rate of 6.9 pmol/mm per min. This is comparable to the net transepithelial Ca absorption rates recently reported for microperfused CNT after treatment with cAMP (5.83±0.37), or with 0.1 or 1.0 nM of synthetic PTH-(1-34) (3.93±0.79 or 9.44±1.13 pmol/mm per min, respectively) (2). With a slower perfusion rate (2.4 vs. 6.5 nl/min) and a lower perfusate $[Ca^{2+}]$ (1 vs. 1.8 mM), a smaller rate (1.08±0.26 pmol/mm per min) was found by Shareghi and Stoner (17), despite treatment with 30 mU/ml of PTH extracts. Before PTH, the basal rate (0.38 pmol/mm per min) was also lower in their hands (17), which is compatible with the lower flux rate of 0.31 pmol/mm per min we calculated from the spontaneous P_o of 0.052%.

We must emphasize the inherent uncertainties and potential errors associated with the above correlation attempt. There are many differences in experimental conditions, preparations, techniques, and methodologies, as well as many unproven assumptions involved in the estimates. For example, our patch-clamp studies were performed, by necessity, with 50 mM Ba^{2+}, whereas most tubule perfusion experiments employed 1–2 mM perfusate [Ca]. Thus, the deduced transmembrane Ca flux could theoretically represent 20–50 overestimate, although the impact of divalent cation concentration on current is not linear, with apparent dissociation constant of 14 and 28 mM for Ca and Ba respectively (18). In practice, the effect of 50 mM Ba^{++} on current amplitude could be calculated to be 18.5 fold higher than that obtained with 1 mM (18).

Relative to the Ca absorption rate reported by Shareghi and Stoner (1.08±0.26 pmol/mm per min), our transmembrane Ca flux estimate, after adjustment for the 18.5-fold increase due to high $[Ba^{2+}]$ (18) (0.37 pmol/mm per min), is still

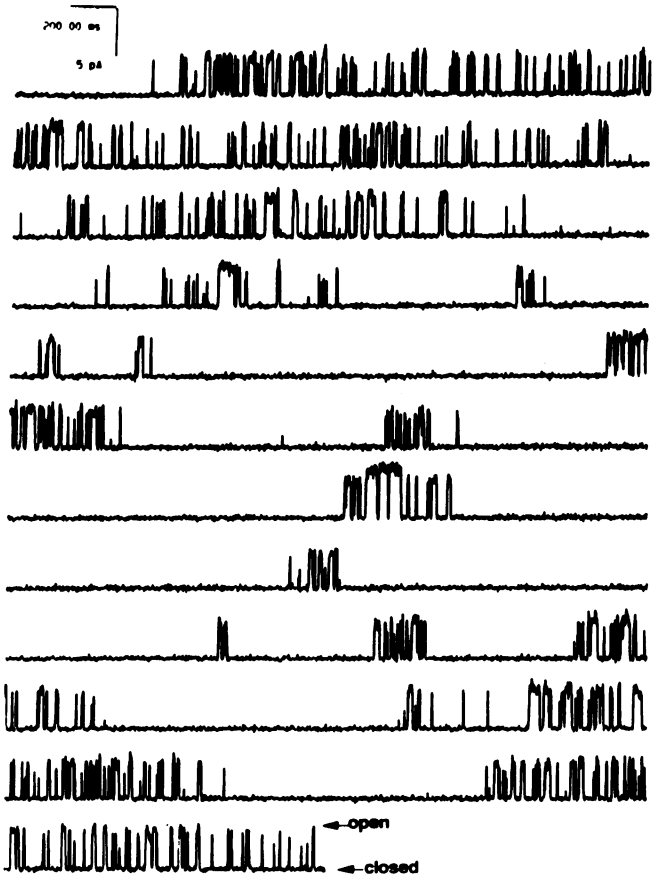

Figure 5. Single Ca channel recording from an excised, inside-out patch, activated by pipette and bath Bay K (50 μM); and by bath 1 μg/ml PKA_{cs} and 2 mM Mg ATP. Before excision, cell was incubated with 8-Br-cAMP, 3.75 mM, for 27 min. V_m = −70 mV. Closed, nonconductive state; open, conductive state showing unitary current.

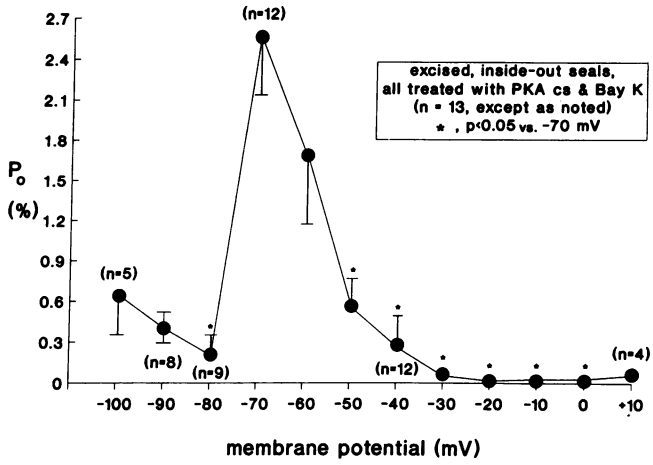

Figure 6. P_o data (mean±SE) are plotted as a function of V_m from 14 excised, inside-out patches. Before excision, 10 were treated with 8-Br-cAMP (2.7±0.4 mM) for 36±9 min. Nine were exposed to pipette Bay K (43±4 μM). After excision, all seals were exposed to bath BAY K (37±5 μM), PKA_{cs} (1 μg/ml), and Mg ATP (2 mM). At extreme V_m, stable GΩ seals were not always possible, reducing the number of observations.

somewhat lower. However, this might be the consequence of the room temperature condition of our experiment, which is known to depress Ca transport (19) and channel activity (20) as compared to 37–38°C. In addition, transcellular Ca transport is modulated by other cytosolic events and BLM extrusion mechanisms (2, 21–23), contribution of which is not available in isolated apical membrane preparation. Given these inherent differences, some discrepancies from measurements obtained in intact tubules are likely, if not inevitable. Nevertheless, the above flux estimates must be viewed as speculative till more vigorous and systematic observations.

The current view that Ca diffuses across the AM down electrochemical gradients (24) may be open to questions, given the negligible spontaneous channel activity in most seals, despite a transmembrane potential gradient of −70 mV and chemical concentration difference of 50 mM. Passive Ca influx was absent until PKA_{cs} was introduced, consistent with the proposed notion of a regulated Ca influx pathway.

The precise function of apical Ca channels identified here is presently undefined. We speculate a role in transcellular Ca transport, because in the CNT, PTH and cAMP are known to not only stimulate Ca absorption (1, 2, 17, 22) but also enhance apical membrane Ca influx from across the lumen (3, 4, 6). The presently documented PKA_{cs}-activated AM Ca channel would provide a potential membrane mechanism for the physiologic function of the PTH and its putative second messenger. The CNT is the most important rabbit nephron segment in regulating final urine Ca (23, 24), due to its unique sensitivity to 10^{-10} M PTH (2, 3, 4), thiazide, amiloride (22, 25), and lumen Na depletion (21, 22), features not shared by other nephron segments.

However, other roles must be considered. For example, future experiments performed at 38°C may reveal no significant stimulation of P_o by any known agonists to approximate the in vitro Ca absorption rate. There is increasing evidence to support the concept that Ca transients can serve to activate renal epithelial cell processes (26–28). For instance, increases in cy-

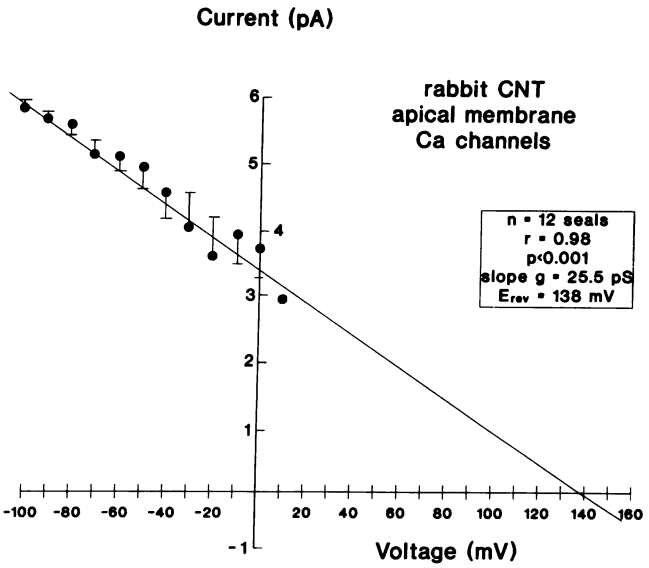

Figure 7. Current-voltage curve from 12 excised, inside-out patches. Current data are presented as mean±SE. Before excision, eight were exposed to 8-Br-cAMP (1–3.75 mM). After excision, all were bathed by PKA_{cs} (1 μg/ml), and Mg ATP (2 mM). Bay K (5–50 μM), was present in the pipette (n = 3), bath (n = 6), or both (n = 1).

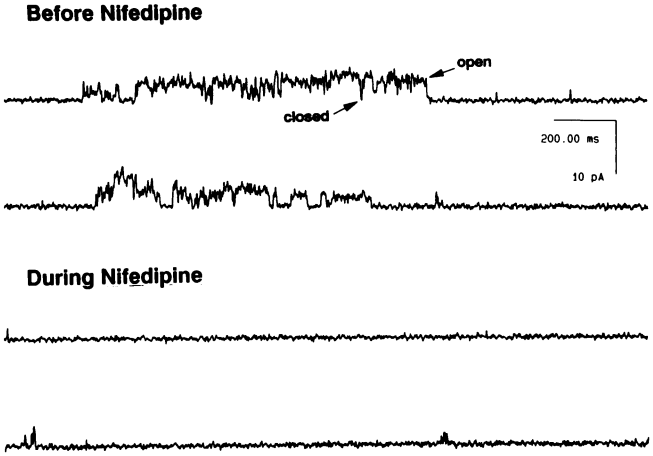

Figure 8. Effect of nifedipine on Ca channel activity in an excised, inside-out patch treated with bath 1 μg/ml PKA_{cs}, 2 mM Mg ATP, and < 0.05% DMSO. Pipette contained 18.5 μM Bay K throughout. Upper two tracings were obtained before nifedipine, when bath contained 18.5 μM Bay K 8644. Lower two tracings were obtained during nifedipine when bath Bay K 8644 was replaced by 25 μM of nifedipine. $V_m = -50$ mV. These recordings are representative of three similar seals.

tosolic [Ca^{2+}] have been found to stimulate distal nephron AM K channels (26, 27) and to inhibit AM Na channels (28). Interestingly, PTH and cAMP elicit biphasic changes in CNT AM potential, consistent with the proposal of a dual modulation of AM Na channels (29): a direct stimulation by cAMP, but an indirect inhibition via the known increases in cytosolic [Ca^{2+}] secondary to PTH or cAMP (3, 4). Specific studies will be needed to directly test these mutually nonexclusive hypotheses.

We did not focus on cell-attached patches for several reasons. One, V_m cannot be precisely controlled. Ba, amiloride, PTH, and cAMP are known to alter V_m (10, 29). Two, cytoplasmic (ions) and enzyme compositions could not be measured or controlled, rendering uncertain any estimates on E_{eq}. Three, the ease, certainty, and timing of completely delivering and/or withdrawing agonists/antagonists are better achieved in cell-free membrane patches. Finally, in our hands, on-cell patches are less stable than excised patches, especially during prolonged superfusion.

In summary, our results provide the first direct evidence for Ca channels in connecting tubule cells, consistent with the hypothesis of a possible functional role in transepithelial Ca transport. The properties, regulation, and function of these channels are yet to be defined.

Acknowledgments

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