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\(_{cat} \)) 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 \(-70 \text{ mV}\).

Mean \( P_o \) for 14 seals (2.57%) peaked at \(-70 \text{ mV}, \) 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\(_{cat} \), bath nifedipine reduced \( P_o \) from 1.03 to 0.15% at \(-63 \text{ mV}\). These patch-clamp data demonstrate a selective, 25-pS, cAMP/PKA-sensitive Ca channel in apical membranes of CNT. \( P_o \) is stimulated by PKA\(_{cat} \) 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 \( \geq 1.9 \text{ kg} \). After decapitation, CNTs were dissected and placed in a solution containing (in mM) KCl 5, NaCl 148, CaCl\(_2\) 1.7, Na\(_2\)HPO\(_4\) 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 M\(\Omega\), made from capillary tubes by a two-stage vertical puller (Narashige USA, Greenvile, 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 (\( \geq 15 \text{ G}\) O) 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 \( \mu \text{M} \) 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-\mu\text{s} rate and filtered at 2 kHz. Currents were also stored in a video cassette 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 \( \geq 500 \mu\text{s} \) and amplitude \( \geq \frac{i_o + (i_i - i_o)/2}{i_o} \), where \( i_o \) denoted closed state and \( i_i \) 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 \( \geq 20 \text{ min} \). 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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All reagent-grade chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) except for: nifedipine, Bay K 8644, and Hapes (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 (Vp) between -10 and 70 mV, spontaneous P0 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 P0 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 (Erere) was -188 mV. Since distal tubule AM is known to be depolarized ~ 42 mV by lumen Ba (from -70 to -28 mV) (10), this Erere corresponds to a transmembrane potential difference (PD) of 160 mV [-28 – (-188)]. Thus, the PD is comparable to the calculated Nernst equilibrium potential (Em) 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 Vp of 0 or 55 mV during the initial 2 and 4 min after 8-Br-cAMP. However, further monitoring at Vp of 0, 25–30, or 55 mV, revealed significant effects on P0 by the 6th min of cAMP exposure (0.60±0.17%) (Fig. 2). Subsequent recordings at 10–11-min intervals pulsed at similar Vp 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 [Ca2+] in the CNT (3, 4, 6). Strictly speaking, it is not possible to rigorously 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 [Ca2+] 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 channel cannot be defined or controlled. To control cytoplasmic solution composition and membrane potential (Vm), subsequent studies were therefore done in excised, inside-out patches. Before excision, an 82-s record was made at zero Vp, although generally no electrical activity was found.

In excised patches, spontaneous activity was absent in all but 1 seal (P0 = 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 (P0 = 0.068±0.057%). When PKA was added to the bath, P0 became 1.25±0.75% (Fig. 3). Conversely, three seals were relatively quiet at -70 mV, with P0 of 0.006% when exposed to only 1 μg/ml of bath PKA and 2 mM of Mg ATP until Bay K was added (P0 = 0.83±0.53%) (Fig. 4). The response of these eight seals, negative with only one agonist but positive with dual agonists (P0 = 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 and Bay K, were subjected to various test potentials (Fig. 6). P0 (2.57%) peaked at -70 mV, the estimated resting Vm for apical membranes of CNT
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 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. $V_m$ 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-BrcAMP 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, 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] 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 (Fig. 8). In two seals studied, substitution of bath nifedipine with bath Bay K restored channel activity.

Bay K 8644

Bay K 8644 & PKA cs

Figure 3. Effect of bath PKAcs (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 PKAcs. 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 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 PKAcs 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

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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 \([\text{Ca}^{2+}]\), 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 and in intact cells by 8-Br-cAMP suggest that this enzyme system potentially mediates the stimulatory effects of PTH and cAMP on \([\text{Ca}^{2+}]\), 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 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 and Bay K 8644 in stimulating \(P_{\text{g}}\) 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 are electrogenic and independent of \([\text{Na}^+]\) or \([\text{H}^+]\) gradients, which are typical of Ca:Na or Ca:H antiporters. Channel conductance and \(E_{\text{rev}}\) 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 \(\cdot\) tubule luminal surface area/patched area \(\cdot\) channel incidence \(\cdot\) channels \(n\) per patch \(\cdot\) \(P_{\text{g}}\)/Faraday constant. Given the measured mean lumen i.d. of 30 \(\mu\)m, 2.3 channels per 1 \(\mu\)m patch diameter, 2.57% for \(P_{\text{g}}\), and 5.18 pA for peak current at resting \(V_{\text{m}}\), 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 \([\text{Ca}^{2+}]\) (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 mM 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 \(P_{\text{g}}\) 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 patchclamp studies were performed, by necessity, with 50 mM \(\text{Ba}^{2+}\), whereas most tubule perfusion experiments employed 1–2 mM perfusate \([\text{Ca}^{2+}]\). Thus, the deduced transmembrane Ca flux could theoretically represent 20–50 underestimate, 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 \(\text{Ba}^{2+}\) 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 \([\text{Ba}^{2+}]\) (18) (0.37 pmol/mm per min), is still

### Figure 4
Effects of bath Bay K 8644 (50 \(\mu\)M) in an excised, inside-out patch pretreated with 18.5 \(\mu\)M pipette Bay K (1 \(\mu\)g/ml of PKA and 2 mM Mg ATP). \(V_{\text{m}}\) = −70 mV. Baseline current levels are denoted as closed. This tracing is representative of three similar patches.
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 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-activated AM Ca channel would provide a potential membrane mechanism for the physiologic function of the PTH and its putative second messen-

senger. 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 P0 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 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 and 2 mM Mg ATP. Before excision, cell was incubated with 8-Br-cAMP, 3.75 mM, for 27 min. Vm = -70 mV. Closed, non-conductive state; open, conductive state showing unitary current.

**Figure 6.** P0 data (mean±SE) are plotted as a function of Vm 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 (1 μg/ml), and Mg ATP (2 mM). At extreme Vm, stable GΩ seals were not always possible, reducing the number of observations.

**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 (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).

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tosolic $[\text{Ca}^{2+}]$ 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 $[\text{Ca}^{2+}]$ 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.

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