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Extracellular Ca^{2+} Controls Outward Rectification by Apical Cation Channels in Toad Urinary Bladder: Patch-Clamp and Whole-Bladder Studies

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Summary. Outward rectifying, cation channels were observed in the epithelial cells of the urinary bladder of the toad, *Bufo marinus*. As studied in isolated cells using the patch-clamp technique, the channel has an average conductance of 24 and 157 pS for pipette potentials between 0 and +60 mV and −60 to −100 mV, respectively, when the major cation in both bath and pipette solutions is K^+. The conductance of the channel decreases with increasing dehydration energy of the permeant monovalent cation in the order $Rb^+ = K^+ > Na^+ > Li^+$. Reversal potentials near zero under biionic conditions imply that the permeabilities for all four of these cations are similar. The channel is sensitive to quinidine sulfate but not to amiloride. It shares several pharmacological and biophysical properties with an outwardly-rectifying, vasopressin-sensitive apical K^+ conductive pathway described previously for the toad urinary bladder. We demonstrate, in both single-channel and whole-bladder studies, that the outward rectification is a consequence of interaction of the channel with extracellular divalent cations, particularly Ca^{2+}, which blocks inward but not outward current. Various divalent cations impart different degrees of outward rectification to the conductive pathway. Concentrations of Mg^{2+} and Ca^{2+} required for half-maximal effect are 3×10^{-4} and 10^{-4} M, respectively. For Co^{2+} the values are 10^{-6} M at +50 mV and a 10^{-4} M at +200 mV. The mechanism of blockade by divalent cations is not established, but does not seem to involve a voltage-dependent interaction in which the blocker penetrates the transmembrane electric field. In the absence of divalent cations in the mucosal solution, the magnitudes of inward current carried by Rb^+, K^+, Na^+ and Li^+ through the apical K^+ pathway at any transepithelial voltage, are in the same order as in the single-channel studies. We propose that the cation channel observed by us in isolated epithelial cells is the single-channel correlate of the vasopressin-sensitive apical K^+ conductive pathway in the toad urinary bladder and is also related to the oxytocin- and divalent cation-sensitive apical conductivity observed in frog skin and urinary bladder.

Key Words patch clamp · epithelial ion transport · quinidine · divalent cation blockade · ion channels

Introduction

Toad urinary bladder has been widely used as a model of the mammalian distal nephron due to its ability to reabsorb sodium and water from the urine by hormonally regulatable processes. Studies of transepithelial fluxes and electrical properties have yielded a great deal of information on Na^+ and water transport and on the action of hormones that affect these processes. The presence of hormone-sensitive, amiloride-blockable Na^+ channels in the apical membrane of the epithelium has been well documented (Garty & Benos, 1988). Relatively little is known about the other conductive pathways in this membrane. Palmer (1986) described an outwardly rectifying, amiloride-insensitive K^+ conductance in the apical membrane which was stimulated by ADH and cholinergic agonists. In addition, a nonselective cation channel blockable by mucosal Ca^{2+} and stimulated by oxytocin has been reported (Van Driessche, Aelvoet & Erlij, 1987; Aelvoet, Erlij & Van Driessche, 1988). No information is available on the single-channel properties of these pathways. In this paper we report the characterization of a nonspecific, outwardly rectifying ion channel in isolated toad urinary bladder epithelial cells using patch-clamp techniques. We propose that this channel is responsible for the oxytocin-sensitive, Ca^{2+}-blockable cation conductance and for the vasopressin-sensitive outwardly rectifying apical K^+ conductance reported earlier.

Materials and Methods

Chemicals and Materials

Amphibian culture medium, fetal bovine serum, antibiotic-antimycotic mixture (10×), lactalbumin hydrolysate, vitamin solution (10×) and kanamycin sulfate were obtained from GIBCO (Grand Island, NY). Soybean trypsin inhibitor was obtained from Worthington Biochemical Corp. (Freehold, NJ). CellTak was obtained from Biopolymers (Farmington, CT). All other chemicals were obtained from Sigma Chemical (St. Louis, MO). Toads (*Bufo marinus*) were obtained from NASCO (Wisconsin) and were maintained in plastic tanks with access to tap water.

Solutions and Culture Medium

For isolation of toad urinary bladder epithelial cells, two Ringer solutions were prepared, designated *A* and *B*. Ringer *A* was primarily used for perfusion and contained (in mM): NaCl 90, $NaHCO_3$ 25, KCl 3.5, KH_2PO_4 0.5, $CaCl_2$ 1, $MgSO_4$ 0.5, glucose 6 and HEPES 10 at pH 7.8. Ringer *B* was used for isolation, washing and plating and contained (in mM): NaCl 30, $NaHCO_3$ 25, Na pyruvate 10, KCl 4.5, KH_2PO_4 0.5, $CaCl_2$ 1, $MgSO_4$ 0.5, glucose 15, sucrose 50 and HEPES 10 at pH 7.8.

The amphibian culture medium (GIBCO) did not support the maintenance of the epithelial cells isolated from the toad urinary bladder. Greater success in maintaining the cells was achieved when the supplied amphibian culture medium was modified to contain (in mM): NaCl 30, Na-pyruvate 10, glucose 35, sucrose 12.5, HEPES 5 as well as fetal bovine serum 10 ml/liter, toad serum 2 ml/100 ml, lactalbumin hydrolysate 1 g/liter and antibiotic-antimycotic mixture 10 ml/liter, final pH adjusted to 7.8. All solutions were sterilized by filtration.

For whole bladder studies the serosal solution contained (in mM): KCl 85, sucrose 50, $CaCl_2$ 1, $MgCl_2$ 0.5, glucose 5, HEPES 5. Final pH was adjusted to 7.8 using KOH. The ionic composition of the mucosal solution was changed depending on the experiment, but the concentration of monovalent metal chloride was 115 mM (except where specifically mentioned to be otherwise) and HEPES 5 mM, final pH adjusted at 7.8. The mucosal solutions containing Na^+ and Li^+ as principal cations also contained 100 μM amiloride. For mucosal solutions containing Cu^{2+} and Mn^{2+}, MES (5 mM) was used to buffer the solution to pH 5.0. Controls used in those experiments also contained 5 mM MES at final pH of 5.0.

K^+ Ringer solutions with well-defined free Mg^{2+} and Ca^{2+} values at a given pH were prepared by using the dissociation constants of Mg^{2+} and Ca^{2+} with EDTA or EGTA according to Fabiato and Fabiato (1979).

Cell Isolation

Epithelial cells were isolated using the method described by Rodriguez et al. (1980), with minor modifications. Two toads were killed by double pithing and perfused by cardiac puncture for 15–20 min using 250 ml of Ringer solution *A* also containing gentamycin sulfate (25 mg/liter) and kanamycin sulfate (100 mg/liter). Urinary bladders were then excised and pooled. After collection of the bladders, all procedures were carried out aseptically. The bladders were chopped and washed thoroughly in Ringer *A* and were treated for 5 min in a mixture containing 1 mg/ml each of collagenase, soybean trypsin inhibitor and bovine serum albumin in Ringer *B*. The tissue was first transferred to Ringer *B* containing 0.5 mM EGTA and without $CaCl_2$ or $MgCl_2$ for 5 min and then to Ringer *B* with 2 mM EGTA for 10 min. After this the tissue was transferred back to the previous enzyme mixture now also containing 1 mg/ml DNase I. After 30 min in this solution, the tissue was passed through a polyethylene tubing (i.d. 2.15 mm) fitted onto a metal needle (14 ga) several times using a 30-ml glass syringe. This procedure was repeated every 10 min. The vial containing the mixture was continuously flushed with 95% O_2/5% CO_2. Following enzyme treatment, the cell suspension was filtered through a nylon mesh, diluted tenfold using Ringer *B* containing 0.5 ml EGTA and centrifuged at 4°C for 10 min at 800 × *g*, refiltered through two layers of cheese cloth and washed again by centrifugation. 1 ml of the suspension was pipetted aseptically on each of ten plastic petri dishes (65 mm dia., Falcon) precoated with polylysine or with CellTak. 40 min after plating, the supernatant was aspirated off and 5 ml of modified amphibian medium was poured into each petri dish. Petri dishes were then maintained overnight in a dessicator flushed with 95% oxygen/5% CO_2 at room temperature. All apparatus and solutions used for isolation and maintainance were presterilized and procedures were carried out aseptically. The cells were used for patch-clamp studies within 1–2 days after plating.

Patch Clamping

Standard patch-clamp recording techniques were followed using fire-polished pipettes having open pipette resistances of 2–8 MΩ to form patches with seal resistances of 1–10 GΩ which were stable on excision. The presence of Ca^{2+} in the pipette solution and a hypertonic bath were found to favor seal formation. Currents were amplified using the amplifier EPC-7 (List), digitized through an analog-to-digital converter (PCM-1, Medical Systems Corp.) and recorded in unfiltered form on video tapes using a video cassette recorder (Panasonic) which was modified to accept and record external audio signals. For analysis, records were played back through a variable frequency 8 pole Bessel filter (902LPF, Frequency Devices) onto a storage oscilloscope (3091, Nicolet) or a laboratory computer (PDP 11-23).

Whole Bladder Studies

Urinary bladders were excised from double-pithed toads and mounted in Lucite chambers with minimal edge damage and maintained in a short-circuited state as described previously (Palmer, 1986). Current-voltage relationships were obtained by applying voltage steps of varying amplitude and 20 msec duration to the command port of the voltage clamp as described previously (Palmer, 1985). ΔI was calculated by measuring the difference in transepithelial current at transepithelial clamping potentials of +50, +100 and +200 mV in the presence and absence of mucosal divalent cations as shown in Fig. 6. When several *I-V* relationships were obtained from the same preparation under different conditions, ΔI values were normalized by dividing by a control value of ΔI. The normalized values are designated by $\Delta I\%$.

Results

Single-Channel Studies

Single-Channel Characteristics

Figure 1 shows single-channel activity at various pipette potentials (V_p) of the most commonly observed channel in the isolated epithelial cells. The records are from an inside-out patch with a pipette solution containing K^+ as the major cation and 1 mM Ca^{2+}. At positive V_p, with positive current flowing from pipette to bath, single-channel current (i) were small and the channels opened relatively briefly and infrequently. At negative V_p single-channel currents were significantly larger and the channels opened in relatively long-lived bursts contain-

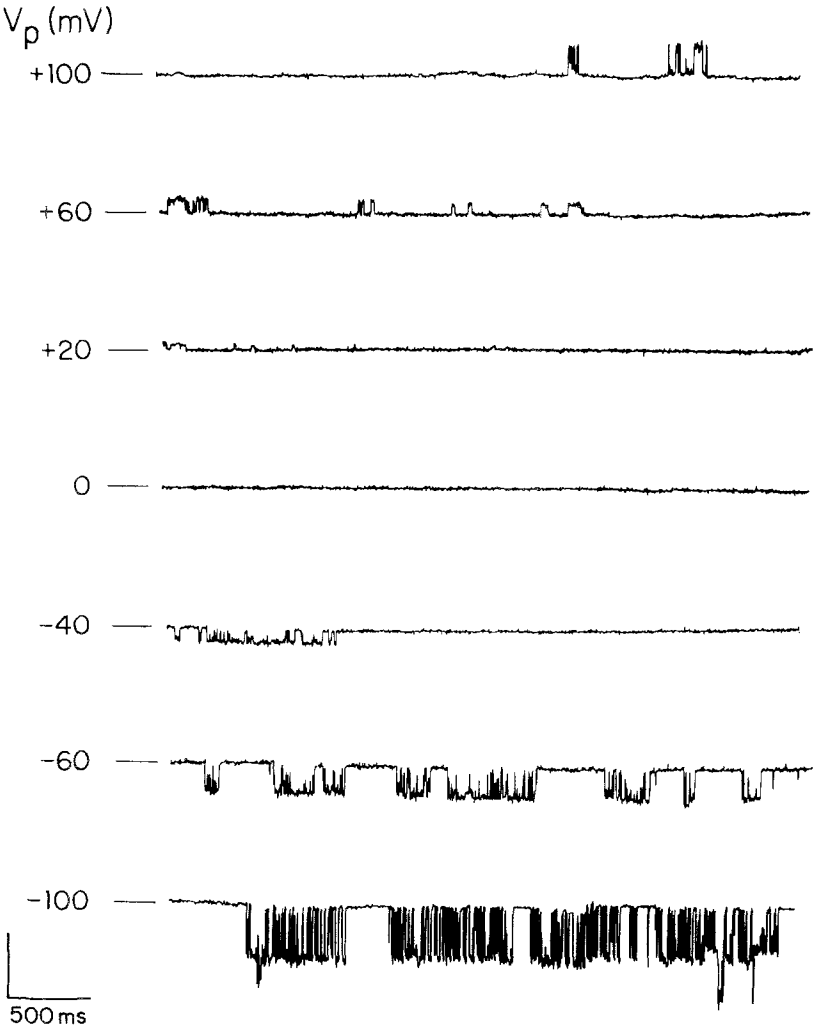

Fig. 1. A cation channel in the epithelial cells isolated from the urinary bladder of *Bufo marinus*. Channel activities at various pipette potentials in an excised inside-out patch are shown. V_p indicates the holding potential of the pipette (in mV). The pipette solution contained (in mM): KCl 90, $CaCl_2$ 1, $MgCl_2$ 0.5 and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) 5, at pH 7.8. The bath solution contained (in mM): NaCl 90, KCl 0 and dextrose 25, while the concentration of the other components were same as in the pipette. The horizontal bar beside each tracing indicates the closed state of the channel. An upward deflection represents flow of positive charge from the pipette to the bath. The vertical bar represents 12.5 pA for $V_p = -100$ mV and 5 pA for all other V_p. Note the difference in channel kinetics and magnitude of single-channel current between +100 and −100 mV. Records were filtered at 300 Hz and sampled at 2 kHz

ing rapid flickers to the closed state. At all negative voltages the channels showed long segments of high activity occasionally interspersed with inactive periods. The i-V_p curve of the channel was nonlinear, showing strong outward rectification with symmetric salt solutions (Fig. 2*a*). Average conductances for all patches studied ($n = 12$) with Na^+ and K^+ as the major cations, were 26 pS (0 mV $< V_p <$ +60 mV) and 145 pS (−60 mV $> V_p >$ −100 mV). The reversal potential remained near zero mV with K^+ as the major cation in the pipette whether the bath contained Na^+, K^+, Rb^+ or Li^+ as the major conducting cation (Fig. 2*a*), thus showing roughly equal permeabilities for these monovalent cations. However, the conductance of the channel at negative V_p with Li^+ in the bath was smaller than that with the other monovalent cations. These features of the channel remained unaltered when Cl^- ions in the bath were replaced with the impermeant anion gluconate, indicating that the channel is cation selective. Upon replacing the bath Na^+ with a presumably impermeant cation $NMDG^+$ (N-methyl-D-glucamine), the channel activity disappeared at negative pipette potentials (Fig 2*a* and *b*) but remained at positive V_p, confirming the cation specificity of the channel.

When the cytoplasmic side of the patch was exposed to 1 mM quinidine sulfate, the channel kinetics at negative V_p were altered (Fig. 3). Instead of rapid flickers within the open state, the channel exhibits only brief openings at −80 mV and other negative V_p values. The transitions from open to closed state were so fast that the unitary events were not fully resolved even with a bandwidth of 2 kHz. The action of quinidine sulfate on channel kinetics could not be reversed even by extensive washing of the cytoplasmic side of the patch.

Modulation of Channel Conductance by Ca^{2+}

Voltage-dependent block of single-channel conductance by intracellular Mg^{2+} is known to cause in-

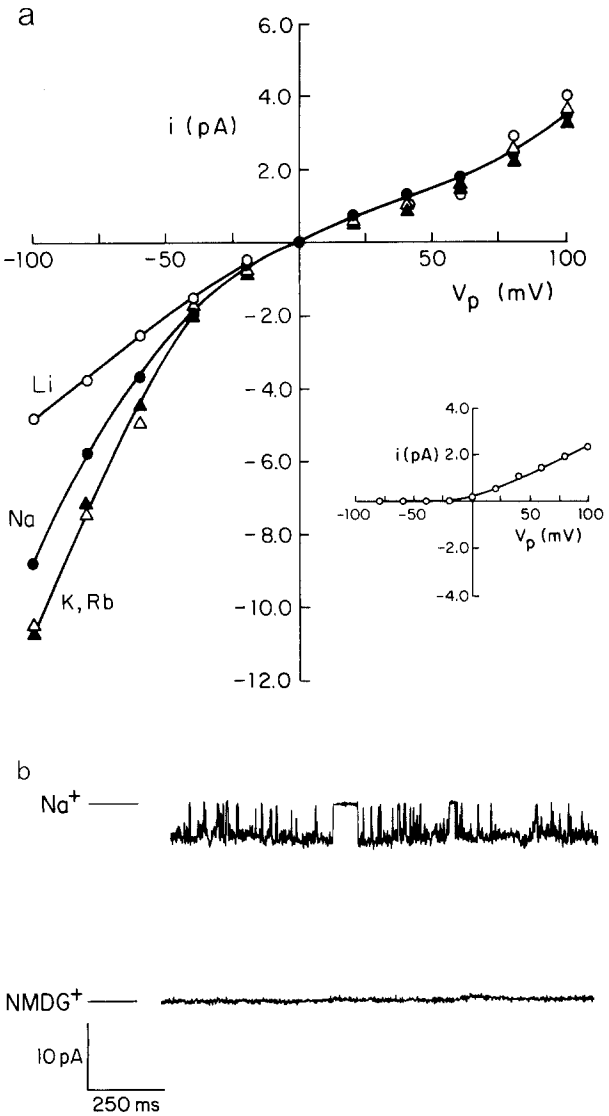

Fig. 2. Single channel current-voltage relationship and ionic selectivity of the channel. (*a*) Initially bath and pipette solutions were as in Fig. 1, the curve with filled circles representing the outward rectifying i-V_p characteristics of the channel. The conductances of this channel are 27 and 126 pS with V_p between 0 to +60 mV and −60 to −100 mV, respectively. NaCl (●) in the bath solution was replaced sequentially by KCl (▲), RbCl (△) and LiCl (○). The inset shows the cation specificity of the channel. The pipette solution contained (in mM): NaCl 90, N-methyl-D-glucamine hydrochloride (NMDGCl) 10, KCl 10, $CaCl_2$ 1, $MgCl_2$ 0.5 and HEPES 5 at pH 7.8. The bath initially contained the same solution as in the pipette and then was exchanged with a solution where NaCl was replaced by NMDGCl. For each data point single-channel currents were obtained by averaging at least 10 transitions. (*b*) Absence of channel activity with NMDGCl as the major cation in the bath solution at $V_p = -80$ mV. Experimental conditions were as in *a*. Single-channel currents were undetectably small with NMDGCl as the major cation in the bath solution. The current records were filtered at 300 Hz and sampled at 2 kHz. The horizontal bar beside each tracing indicates the closed state of the channel

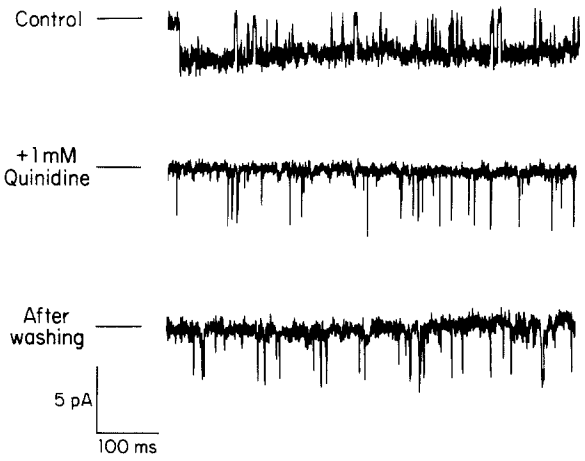

Fig. 3. Effect of quinidine sulfate on channel activity. The channel activities of an inside-out excised patch at $V_p = -80$ mV are shown before (top panel) and after (middle panel) flushing the bath with solution containing 1 mM quinidine sulfate. Both bath and pipette solutions contained (in mM): KCl 90, NaCl 10, $CaCl_2$ 1, $MgCl_2$ 0.5 and HEPES 5 at pH 7.8. Addition of quinidine sulfate to a final concentration of 1 mM to the bath changed the kinetics of the open state significantly, while the single channel conductance remained unaltered. The horizontal bar beside each tracing indicates the closed state of the channel. The effect of quinidine sulfate could not be removed by extensive washing with quinidine-free solution (bottom panel). Data were filtered at 2 kHz and sampled at 10 kHz

ward rectification of K channels in heart cells (Matsuda, Saigusa & Irisawa, 1987). Extracellular Mg^{2+} has also been reported to bring about rectification of glutamate-activated cation channels in mouse spinal chord neurones by changing the gating properties of the channel (Nowak et al., 1984; Mayer, Westbrook & Gathrie, 1984). To investigate whether the rectification shown by this channel is an inherent property or dependent upon divalent cations present in the medium, we studied excised inside-out patches with no divalent cation present in the pipette solution. With 0.1 mM EGTA and no Ca^{2+} in the pipette solution, the outward rectifying property of the channel disappeared and the i-V_p curve became symmetric (Fig. 4) due to an increase of single-channel conductance at positive V_p. This apparently indicates a voltage-dependent block of the channel from apical side by extracellular Ca^{2+}, although we will present data below demonstrating that this interpretation is over-simplified. The control i-V_p relationship in Fig. 4 was obtained with 1 mM $CaCl_2$ as the sole divalent cation in the pipette solution. It showed the usual outward rectification characteristic of this channel, implying that Ca^{2+} alone was sufficient to bring about this effect.

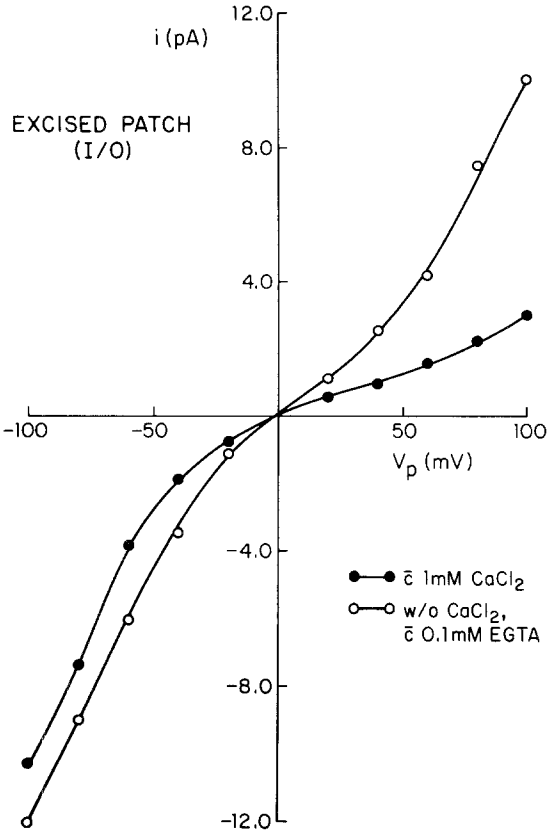

Fig. 4. Control of outward rectification by extracellular Ca^{2+} in excised inside-out patches. Bath solution was as in Fig. 3 with K^+ as the major cation. The control pipette solution contained (in mM): KCl 90, NaCl 10, $CaCl_2$ 1 and HEPES 5 at pH 7.8, yielding an i-V_p curve (●) showing rectification. When Ca^{2+} was ommitted from the pipette solution and 0.1 mM EGTA was added, the outward rectification disappeared and the curve became symmetric (○). Each curve represents data from three patches. Data were filtered at 300 Hz and sampled at 2 kHz

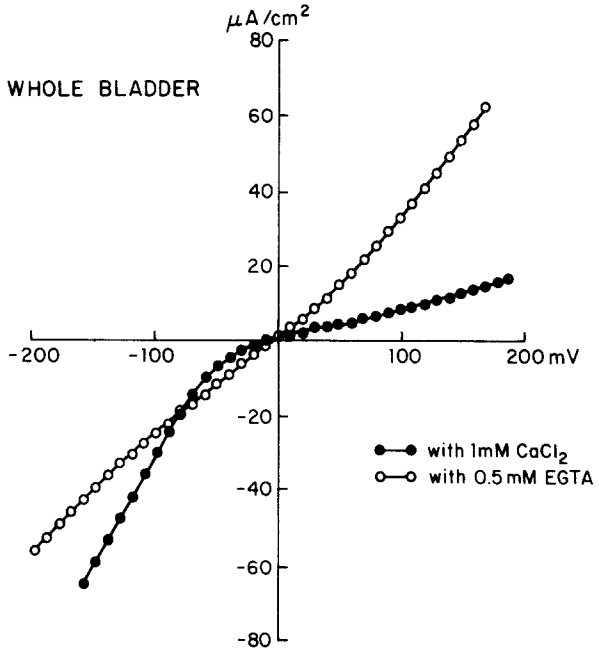

Fig. 5. Control of outward rectification of the apical K^+ conductance by mucosal Ca^{2+} in intact toad urinary bladder. Current-voltage relationships were obtained under voltage-clamp conditions. Control mucosal solutions contained (in mM): KCl 115, $CaCl_2$ 1 and HEPES 5 at pH 7.8. With this mucosal solution (●), the conductive pathway showed outward rectification. When mucosal $CaCl_2$ was replaced by 1 mM EGTA (○), the rectification disappeared and there was significant inward ion flow. Identical results were obtained when mucosal chloride was replaced by an impermeant anion gluconate

Whole-Bladder Studies

Interaction of Divalent Cations with the Apical K^+-Conductive Pathway

Several characteristics of the channel, including outward rectification and sensitivity to quinidine sulfate, resemble those of the apical membrane K^+ conductance in the intact bladder reported previously by Palmer (1986). To test whether the channels described here are responsible for this conductance, the apical membrane of the intact toad urinary bladder was studied under voltage-clamp conditions in the absence of Na^+ and both in the absence and presence of $CaCl_2$ (1 mM) on the luminal or mucosal side. For all following experiments, except selectivity experiments with the whole bladder, K^+ was the principal cation carrying current in the direction of mucosa-to-serosa at positive clamping voltages. When Ca^{2+} was present in the mucosal solution, the I-V curve showed outward rectification (Fig. 5) as previously described and comparable to that in Figs. 2 and 4. When the mucosal solution was exchanged with a solution of otherwise identical ionic composition but lacking Ca^{2+} and containing 0.5 mM EGTA or EDTA, the outward rectifying property of the conductive pathway disappeared within 1 min and the I-V curve became symmetric, due to an increase in the inward conductance. This effect was completely reversible. Thus the K^+ permeability of the apical membrane in the intact bladder shows Ca^{2+}-dependent outward rectification similar to that of the channels studied in isolated cells. The increased conductance was not due to the opening of parallel shunt pathways in the absence of Ca^{2+}. If this were the case, the outward conductance would also have increased in the absence of Ca^{2+}. As seen in Fig. 5, the outward conductance actually decreased. Thus it appears that a specific interaction of extracellular Ca^{2+} with

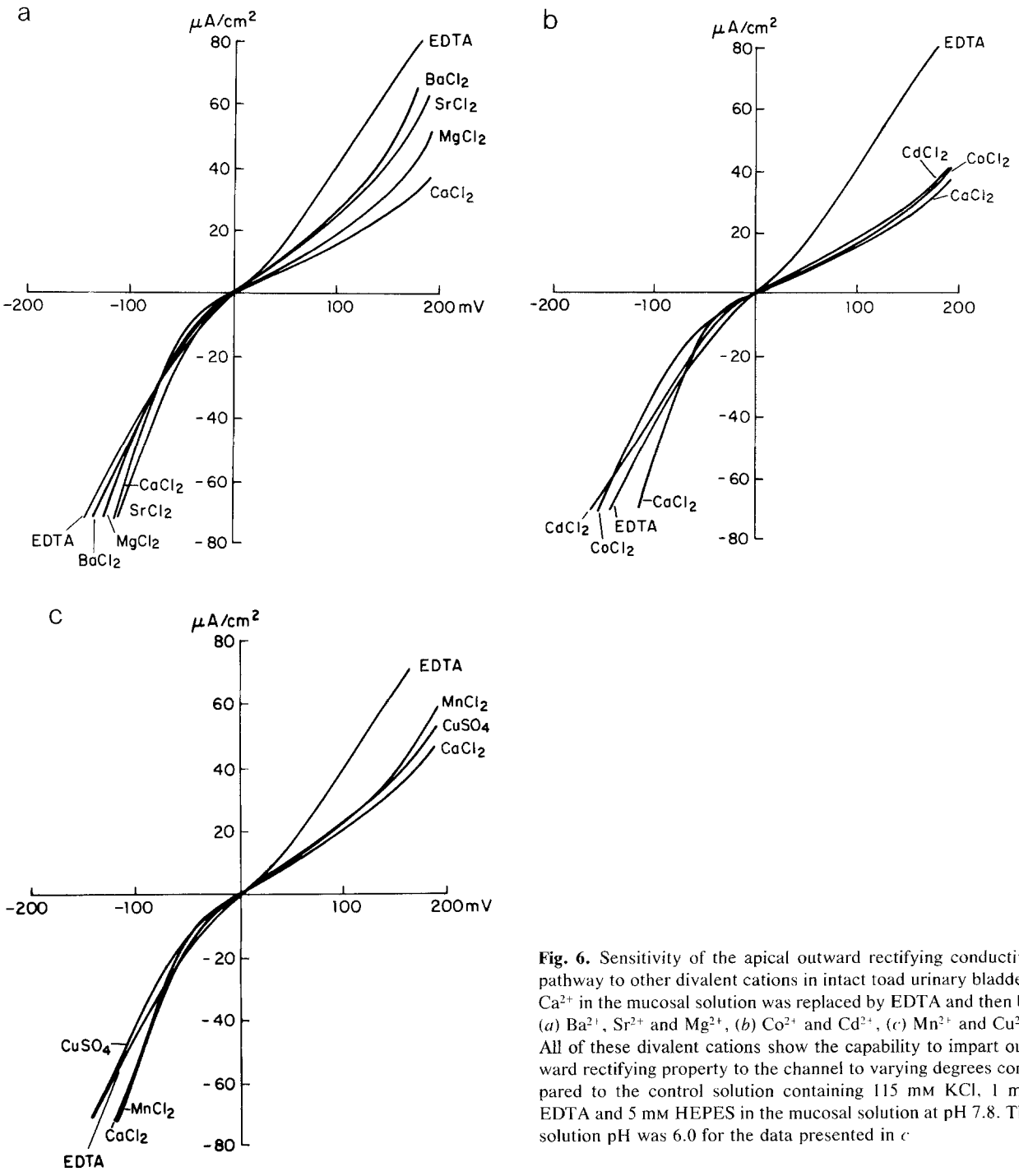

Fig. 6. Sensitivity of the apical outward rectifying conductive pathway to other divalent cations in intact toad urinary bladder. Ca^{2+} in the mucosal solution was replaced by EDTA and then by (*a*) Ba^{2+}, Sr^{2+} and Mg^{2+}, (*b*) Co^{2+} and Cd^{2+}, (*c*) Mn^{2+} and Cu^{2+}. All of these divalent cations show the capability to impart outward rectifying property to the channel to varying degrees compared to the control solution containing 115 mM KCl, 1 mM EDTA and 5 mM HEPES in the mucosal solution at pH 7.8. The solution pH was 6.0 for the data presented in *c*

an apical membrane channel results in a decreased conductance at mucosa positive voltages.

The degree of rectification was often more pronounced in the whole bladder *I-V* relationships than in the single-channel *i-V* relationships, as seen in Figs. 4 and 5. In the intact tissue, the presence of Ca^{2+} in the mucosal solution stimulated outward currents while inhibiting the inward currents. The stimulatory effect was not maximal until 3 to 5 min after addition of Ca^{+} to the mucosal side, whereas the inhibitory effect was completely immediately after changing solutions. Both effects were fully reversible.

Various degrees of rectification could be observed with a number of divalent cations at equimolar concentrations (Fig. 6*a–c*). The divalent cations could be roughly divided into two groups, depending on their ability to modulate the apical K^{+}

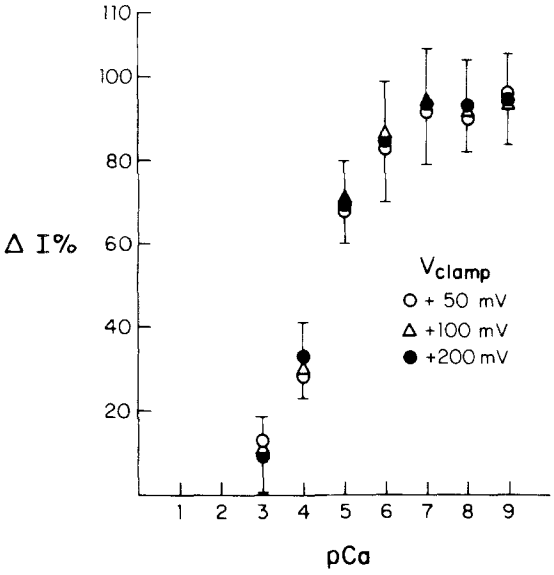

Fig. 7. Dependence of apical inward K^+ current on Ca^{2+} concentration in intact toad urinary bladder. $\Delta I\%$ is plotted against pCa at three different transepithelial clamping potentials, +50 mV (○), +100 mV (△) and +200 mV (●). Points represent means of data from five different hemibladders. The bars indicate standard deviations of the means

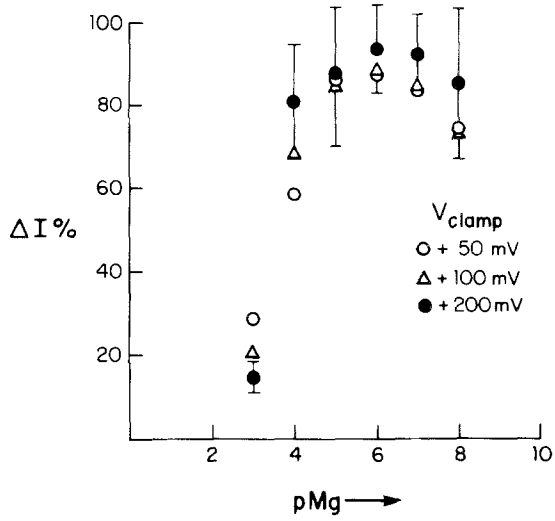

Fig. 8. Dependence of apical inward K^+ current on Mg^{2+} concentration in intact toad urinary bladder. $\Delta I\%$ is plotted against pMg at three different transepithelial clamping potentials, +50 mV (○), +100 mV (△) and +200 mV (●). Points represent means of data from five different hemibladders. The bars indicate standard deviations of the means

conductance. Co^{2+}, Cd^{2+}, Mn^{2+} and Cu^{2+} were as effective as Ca^{2+} (Fig. 6*b* and *c*) while Mg^{2+}, Ba^{2+} or Sr^{2+} had weaker effects (Fig. 6*a*). The rapid and complete reversal of the rectification by washing the mucosal side with 0.5 mM EDTA-K^+ Ringer suggests an apical site of interaction of these divalent cations. Rectification and its reversal by removal of divalent cations were unaffected by replacement of mucosal Cl^- with gluconate, implying that this pathway is cation selective and that anion transport pathways do not contribute to this phenomenon.

Dose Dependence of the Divalent Cation Block

Inward current through this pathway depends on the concentration of Ca^{2+} present in the mucosal solution (Fig. 7). At 10^{-6} M, Ca^{2+} does not block the conductive pathway significantly, whereas the block is appreciable at 10^{-5} M and almost complete at 10^{-4} M. Decrease in the inward current (mucosa-to-serosa) at three clamping voltages (+50, +100 and +200 mV) were plotted against pCa (Fig. 7). The plot reveals two features of the blocking interaction of Ca^{2+}: (i) half-maximal blockage occurs at a pCa of about 4.5 and (ii) the percent decrease of the inward current does not depend on the transepithelial voltage.

Mg^{2+} was less effective than Ca^{2+} in decreasing the inward flow of K^+. A plot of the decrease in inward current at three clamping potentials against pMg (Fig. 8) shows that half-maximal blockage occurs at around pMg 3.5, a concentration one order of magnitude larger than that in case of Ca^{2+}. Thus the site(s) of interaction shows higher affinity for Ca^{2+}. At a pMg of 8.0, an apparent small decrease in the apical inward current was observed at all transepithelial voltages. The Mg^{2+} block of inward current also appears to be voltage independent.

The dependence of the decrease in inward current on pCo at three different transepithelial voltages (+50, +100 and +200 mV) is shown in Fig. 9. Block by Co^{2+} is more potent at +50 mV than at more positive voltages. Half-maximal decrease in current flow occurs at pCo of 3.5 to 4 at +200 mV and at pCO of 6 to 7 at +50 mV. This is opposite to what would be expected in case of a voltage-dependent block in which Co^{2+} is driven into the channel by the transmembrane electric field.

Dependence of Ca^{2+}-Sensitive Inward Current on Mucosal K^+ Concentration

The inward current depends on the K^+ concentration in the mucosal solution (Fig. 10). In these experiments KCl was replaced by sucrose at a constant osmolarity. However, the quantitative relationship depends on the transepithelial clamping

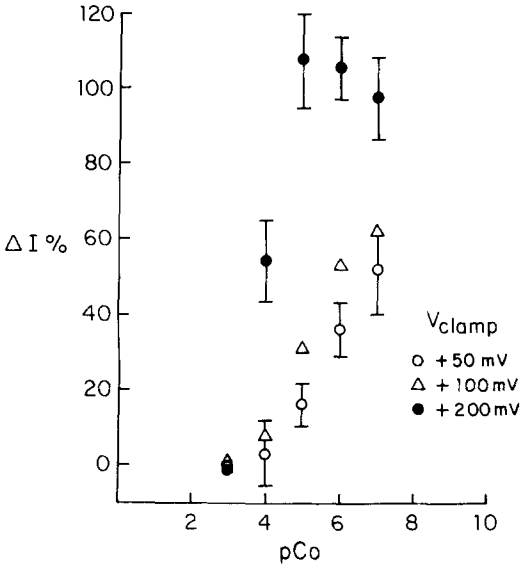

Fig. 9. Dependence of apical inward K^+ current on Co^{2+} concentration in intact toad urinary bladder. $\Delta I\%$ is plotted against pCo at three different transepithelial clamping potentials, +50 mV (○), +100 mV (△) and +200 mV (●). Points represent means of data from five different hemibladders. The bars indicate standard deviations of the means

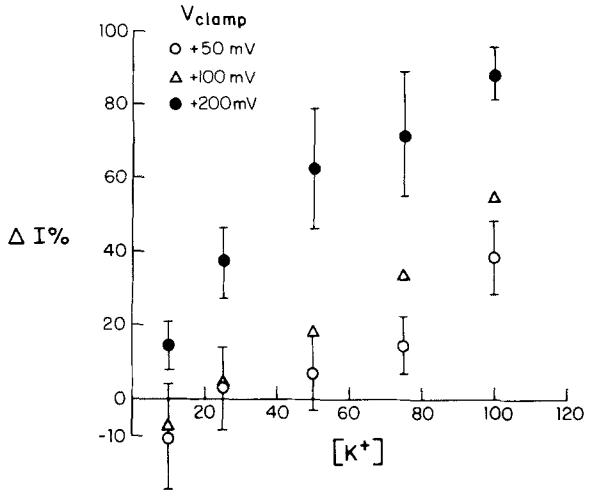

Fig. 10. Dependence of Ca^{2+}-sensitive apical inward current on K^+ concentration in intact toad urinary bladder. $\Delta I\%$ is plotted as a function of the mucosal K^+ concentration up to $[K^+] = 100$ mM, KCl being replaced isosmotically with sucrose. $\Delta I\%$ at three different transepithelial clamping potentials are shown. Values of ΔI with $[K^+] = 115$ mM were taken as 100%. Each data point represents the mean of data from five different hemibladders. The bars indicate standard deviations of the means

potential. At +50 and +100 mV the current through this pathway increased less steeply with K^+ concentration compared to that at +200 mV.

Ionic Selectivity of the Ca^{2+}-Sensitive Apical Conductance

Principal cation carrying the current from mucosa to serosa at positive clamping voltages was the principal cation present in the mucosal solution. K^+ in the mucosal solution was sequentially replaced totally by choline$^+$, Rb^+, Na^+ and Li^+ for this experiment. The mucosal solutions containing Na^+ and Li^+ as principal cations also contained 100 μM amiloride. The Ca^{2+}-sensitive conductances of five different monovalent cations were studied (Fig. 11 and Table 2). Here the inward currents, at positive clamping voltages, are carried through the outward rectifying pathway by the major cations in the mucosal bath, as indicated in the figure. In all cases, the outward currents are carried by K^+, the major monovalent cation in the cell (Palmer, 1986). We assume that the inward currents in excess of these seen with choline$^+$, which is presumably impermeant, are mediated by the outward rectifying pathway. Note that the inward currents in the presence of 1 mM Ca^{2+}, which blocks the channels, are similar to those in the absence of a permeant cation, i.e., with choline chloride. The relative conductivity of this pathway at mucosa-positive clamping voltage is $Rb^+ = K^+ > Na^+ > Li^+ >$ choline$^+$. This sequence is the same as that of the outward rectifying channel (Fig. 2 and Table 1). Permeabilities of K^+, Rb^+, Na^+ and Li^+ are similar as the reversal potentials are near zero for all of these ions.

Action of Various Inhibitors

Several known inhibitors of ion channels were tested including amiloride (100 μM), quinidine sulfate (100 μM), diltiazem (1 mM) and nifedipine (10 μM). Of these only quinidine sulfate blocked the outward rectifying Ca^{2+}-sensitive conductive pathway.

Discussion

The purpose of our patch-clamp studies of ion channels in isolated epithelial cells was to identify the single-channel correlates of the conductive pathways in the toad urinary bladder. Because we found it very difficult to establish gigohm seals between the patch-clamp pipette and the apical membrane of the intact bladder, we chose instead to study channels in the isolated epithelial cells. We used a method of preparing isolated cells previously devised by Rodriguez et al. (1980). This preparation consisted mostly of single cells, more than 95% of

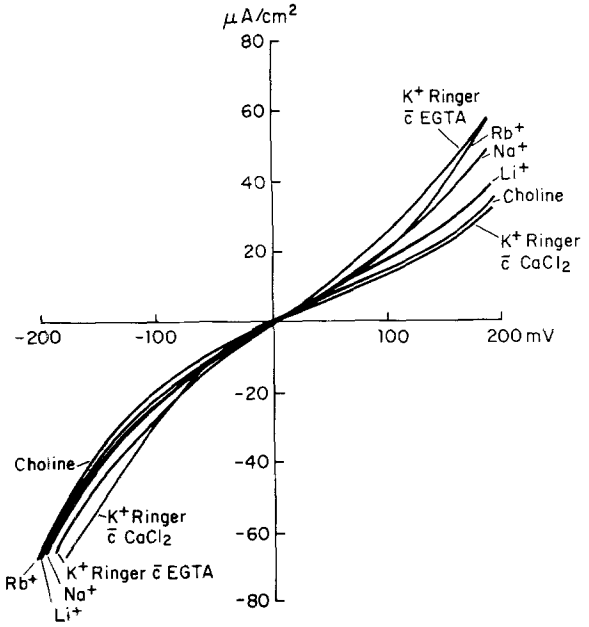

Fig. 11. Permeability of various monovalent cations through the apical Ca^{2+}-sensitive, K^+-conductive pathway in the absence of divalent cations in intact toad urinary bladder. KCl was replaced in the mucosal solution by NaCl, RbCl, LiCl and choline Cl at equimolar concentration (115 mM). The solutions contained 1 mM EGTA and no added Ca. The mucosal solutions containing Na^+ and Li^+ as principal cations also contained 100 μM amiloride. The control solution contained (in mM): KCl 115, $CaCl_2$ 1 and HEPES 5 at pH 7.8. The same permeability sequence was obtained in eight different hemibladders with these cations

Table 1. Conductance of the channel with various cations in bath and pipette

Major cation in		Conductance, g (pS) at			
Bath	Pipette	$0 < V_p < +60$ mV		-60 mV $> V_p > -100$ mV	
K	K	24	(n = 4)	157	(n = 4)
Na	K	24	(n = 4)	126	(n = 4)
K	Na	34	(n = 2)	184	(n = 2)
Na	Na	30	(n = 2)	118	(n = 2)
Li	K	26	(n = 2)	64	(n = 2)
Rb	K	23	(n = 2)	149	(n = 2)

Both the pipette and bath solutions contained (in mM): MCl 90, $CaCl_2$ 1, $MgCl_2$ 0.5 and HEPES 5 at pH 7.8, where M = major monovalent cation. Channel conductances are given as the means of the number of independent observations quoted within parentheses

which excluded the supravital dyes erythrosin B and trypan blue. Rodriguez et al. (1980) showed that these cells have intact machinery for protein synthesis and that they respond to vasopressin and carbachol, two hormones which affect transepithelial transport, with increases in intracellular cAMP and cGMP, respectively. The use of isolated cells has the disadvantage that it is difficult to establish whether the channels being observed were originally on the apical or basolateral membrane. The extent to which the apical and basolateral membrane constituents are retained or maintained in a segregated state in the membrane of the isolated cell preparation is unknown. This must be determined indirectly, by comparing the biophysical and pharmacological properties of the single-channel currents with those of the intact epithelium.

In this paper we have characterized one type of channel. It shares several characteristics with the apical K^+ conductance, described earlier (Palmer, 1986) in intact toad urinary bladder, including sensitivity to quinidine sulfate and outward rectification. Here we have demonstrated that in both the single channel and the apical K^+-conductive pathway of the intact bladder, the outward rectification is a result of interaction of the channel with extracellular Ca^{2+}. Furthermore, the ion selectivities of the single channels in the isolated cells and the Ca^{2+}-sensitive conductance of the apical membrane of the intact urinary bladder are similar. We therefore propose that the channel described in this paper is the single-channel equivalent of the apical K^+ conductive pathway. However, neither the patch-clamp nor the whole bladder studies address the question of whether these channels might also be found in the basolateral membrane.

A recent noise analysis study has revealed a cation channel in the apical membrane of frog skin epithelium which is permeable to all the alkali metal cations and can be blocked by a number of divalent cations (Van Driessche & Zeiske, 1985). A similar amiloride-insensitive conductive pathway was observed in the apical membrane of toad urinary bladder which could be stimulated by oxytocin and cAMP and was blockable by divalent cations (Van Driessche et al., 1987). We propose that these conductances are closely related if not identical to the outward rectifying apical membrane conductance described here both at the whole bladder and single-channel level and also reported earlier (Palmer, 1986). The stimulation by oxytocin and vasopressin of both the Ca^{2+}-dependent conductance (Van Driessche et al., 1987) and the outward rectifying apical K conductance (Palmer, 1986), as well as their blockade by divalent cations, is consistent with this idea. In addition, the cationic selectivity of the oxytocin-stimulated, Ca^{2+}-sensitive pathway in the toad bladder (Aelvoet et al., 1988) is the same as the apical K^+ conductive pathway described here. One caveat to this conclusion is that Van Driessche

Table 2. Percent Ca^{2+}-sensitive current ($\Delta I\%$) carried by various monovalent cations through the cation-selective apical pathway

Cation	$\Delta I\%$ at a transepithelial voltage of[a]		
	+50 mV	+100 mV	+200 mV
Li^+	46.1 ± 12.2 (4)	39.3 ± 9.2 (4)	57.7 ± 2.8 (4)
Na^+	68.1 ± 18.1 (3)	68.3 ± 13.4 (3)	75.7 ± 3.0 (3)
K^+	100 (5)	100 (5)	100 (5)
Rb^+	36.5 ± 17.2 (4)	56.8 ± 15.8 (4)	105.6 ± 13 (4)
$Choline^+$	0 ± 9.9 (5)	1.6 ± 4.8 (5)	16.5 ± 10.1 (5)

[a] The difference in apical inward current carried by K^+ in absence of Ca^{2+} with 1 mM EGTA and in presence of Ca^{2+} (1 mM) in the mucosal solution was taken as 100% at indicated transepithelial clamping potentials. Similarly, $\Delta I\%$ for a cation was calculated by measuring the Ca^{2+}-sensitive apical inward current carried by it and normalizing it with respect to that carried by K^+ ions. For all the experiments, the concentration of monovalent cations in the mucosal solution was 115 mM which also contained 5 mM HEPES at pH 7.8 besides 1 mM EGTA or $CaCl_2$ as required. The serosal solution was same as that in Fig. 5. The data represent mean of indicated number of observations quoted within parentheses ± SDM

(1987) and Aelvoet et al. (1988) reported that the apical Ca^{2+}-sensitive channels are blocked by nicardipine and nitrendipine, two dihydropyridine Ca^{2+} channel blockers. However, we have found no effect of nifedipine on the outward rectifying currents in our preparation. Van Driessche and Zeiske (1985) also reported no significant effect of nicardipine and diltiazem on the Ca^{2+}-sensitive apical cation channels in adult frog skin epithelium.

As shown in Table 2, the conductances of K^+ and Rb^+ through the channel are comparable, that for Na^+ being slightly less. The conductance of Li^+ is less than half of other monovalent cations. In these experiments with inside-out patches, the cations on the cytoplasmic side of the membrane were varied and the relative conductances in the outward direction were compared. Due to inaccessibility of the cytoplasmic face of the apical membrane for experimental maneuvers, this protocol could not be repeated in the whole bladder preparation. However, in the absence of mucosal Ca^{2+} we could study the specificity of the conductive pathway in the inward direction (Fig. 11). The same relative order of conductances were observed, i.e., $Rb^+ = K^+ > Na^+ > Li^+$. The sequence of conductance is that of the Eisenmann sequences for weak field strength sites (Eisenman, 1962). Thus the more easily dehydrated ion would be more favored for passage through the channel.

The conductive properties of the channel can be used to make some inferences about the length of the pore. Using the following equation (Hille, 1984), the ratio of length to conductive pore area of the channel may be calculated:

$$l/d^2 = \pi z c \lambda_o / 4g$$

where l = length of the conductive pathway, d = diameter of the conductive pathway, z = valence of the cation, c = equivalent concentration of the cation, λ_o = equivalent conductivity of the cation, and g = single-channel conductance for the cation.

Using values of λ_o obtained in bulk solution (Robinson & Stokes, 1965) and a value of l = 100 Å yields unacceptably high values of d (14.5 Å for Li^+ and 15.6 Å for Rb^+). Such a large pore should readily conduct ions as large as choline. If, as seems likely, the conductivity of the pore is less than that of bulk solutions, the calculated diameters would be even larger. This could indicate that the length of the narrow, rate-limiting segment of the pore is smaller than the width of the lipid bilayer, implying the presence of a wide mouth near either end of the conductive pathway. Such a model has been proposed for the Ca^{2+}-activated K^+ channel from sarcoplasmic reticulum (Latorre & Miller, 1983).

The channel does not seem to possess any inherent asymmetry of the conductive pathway other than that in the location of the binding site(s) of divalent cations. Upon removal of divalent cations from the mucosal solution or the pipette solution the current-voltage characteristic of the channel becomes symmetric. In addition, as mentioned in the results sections, the sequence of monovalent cationic conductivities of the channel in either direction is the same. Thus the binding of divalent cations, in particular Ca^{2+}, to the channel confers asymmetry to the conduction pathway. Furthermore, to induce outward rectification Ca^{2+} must be present in the solution bathing the external side of the membrane. No rectification is observed when Ca^{2+} is present only on the cytoplasmic side of the membrane (Fig. 4).

We have studied the specificity of the interaction with various divalent cations. Of group IIA metals, Ca^{2+} is the strongest blocker. Other divalent cations tested which block the channel nearly as effectively as Ca^{2+} include Co^{2+}, Cd^{2+}, Mn^{2+} and Cu^{2+} (Fig. 6). The specificity is also clear from the concentrations of divalent cations required to bring about half-maximal blockade. The values for Co^{2+} and Ca^{2+} differ by an order of magnitude with that for Mg^{2+} at a transepithelial clamping potential of +200 mV (Figs. 7–9). Thus the interaction of the channel with divalent cations exhibits a specificity not correlated with any simple physical characteristic such as the size or hydration energy of the ion (Hille, 1984).

The mechanism of block by divalent cations does not appear to involve a voltage-dependent binding with the blocker being driven into or out of the pore by the electric field across the membrane (*see* Woodhull, 1973). This is illustrated in Figs. 7–9, which show that the block of inward current by mucosal Ca^{2+} and Mg^{2+} is not detectably voltage-dependent as would be expected of such a mechanism. Block of inward current by Co^{2+} was voltage dependent but in the opposite direction to that predicted if the blocker were being driven into the pore by a mucosa-positive voltage. It is also unlikely that the divalent cations interact specifically with the gating mechanism that governs the transitions between open and closed states. A change in channel gating would reduce the open probability of the channel rather than the single-channel conductance.

Rectification by external divalent cations might be explained if fixed negative charges near the outer surface of the membrane were being screened. This effect could reduce the actual concentration of permeant cations near the mouth of the channel. Since in the concentration range tested the conductance does not saturate with increasing concentrations of K^+ (Fig. 10), this would result in a reduction of the single-channel conductance for inward current flow. We do not favor this idea in light of the specificity of block by the divalent cations. Thus it is more likely that the divalent cations are interacting more specifically with a component of the channel.

One such possibility would be a conformational change of the channel between states of higher and lower conductances. Such an effect could explain the observation if the shift to a lower conductance state required both the presence of external Ca^{2+} and mucosa-positive voltages. This mechanism would involve an allosteric interaction of Ca^{2+} with the pore.

Another possibility is that the divalent cations interact with a binding site in the conduction pathway but very near the outer end of the pore, such that they would sense a very small portion of the electric field across the membrane. The relief of block at negative mucosal or pipette potentials could then arise from the movement of permeant ions through the channel, knocking the blocker off its binding site when the current is flowing in the outward direction. Such a mechanism was proposed to describe the interaction between K^+ and quaternary ammonium blockers in the delayed rectifier K^+ channel in the squid axon (Armstrong, 1971). According to this scheme, relief of block should be correlated best with the occupancy of the channel by permeant cations, rather than by the transmembrane voltage *per se*.

In summary, we have characterized an ion channel both in isolated epithelial cells of the toad urinary bladder and in the apical membrane of the intact tissue. The channel is cation selective but shows little specificity for different alkali metal cations. The channel is outward-rectifying and this property is a consequence of its interaction with divalent cations, particularly Ca^{2+}. The physiological role of this channel is uncertain. Van Driessche (1987) suggested that a similar channel in the frog skin might be involved in Ca^{2+} transport across the apical membrane. Ca^{2+} conductance of the pathway was demonstrated, but only in the presence of Ag^+ ions in the mucosal solution. The channel reported here could also mediate significant fluxes of monovalent cations under some conditions, for example, if the concentration of Ca^{2+} in the urine were 0.1 mM or below. We are unaware of any measurements of Ca^{2+} concentration in toad urine in the literature. It is possible that with low Ca^{2+} levels in the urine this channel may aid in the reabsorption of Na^+ from the urine. We do not know under what physiological circumstances this might occur. The channels would also allow significant outward flow of monovalent cations, particularly K^+, when the mucosal potential is very negative, a condition which may arise under high rates of transepithelial Na^+ transport. In this case the nonselective channels may play an important role in K^+ and Na^+ homeostasis by secreting or absorbing these ions.

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