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# $Ba^{2+}$-Inhibitable $^{86}Rb^+$ Fluxes across Membranes of Vesicles from Toad Urinary Bladder

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**Summary.** $^{86}Rb^+$ fluxes have been measured in suspensions of vesicles prepared from the epithelium of toad urinary bladder. A readily measurable barium-sensitive, ouabain-insensitive component has been identified; the concentration of external $Ba^{2+}$ required for half-maximal inhibition was 0.6 mM. The effects of externally added cations on $^{86}Rb^+$ influx and efflux have established that this pathway is conductive, with a selectivity for $K^+$, $Rb^+$ and $Cs^+$ over $Na^+$ and $Li^+$. The $Rb^+$ uptake is inversely dependent on external pH, but not significantly affected by internal $Ca^{2+}$ or external amiloride, quinine, quinidine or lidocaine. It is likely, albeit not yet certain, that the conductive $Rb^+$ pathway is incorporated in basolateral vesicles oriented right-side-out. It is also not yet clear whether this pathway comprises the principle basolateral $K^+$ channel in vivo, and that its properties have been unchanged during the preparative procedures. Subject to these caveats, the data suggest that the inhibition by quinidine of $Na^+$ transport across toad bladder does not arise primarily from membrane depolarization produced by a direct blockage of the basolateral channels. It now seems more likely that the quinidine-induced elevation of intracellular $Ca^{2+}$ activity directly blocks apical $Na^+$ entry.

**Key Words** $K^+$,$Na^+$ transport · intracellular $Ca^{2+}$ · pH · membrane depolarization · quinine · quinidine · lidocaine

## Introduction

Transcellular sodium transport across tight epithelia is regulated at both the apical and basolateral membranes. However, over time domains of seconds to minutes, this regulation is likely expressed at the apical entry step from the mucosal medium into the cell (Lichtenstein & Leaf, 1965; Lewis, Eaton & Diamond, 1976; Civan, 1986).

In principle, $Na^+$ entry can be modified by altering either the membrane permeability or the electrical and chemical driving forces favoring that entry. A major determinant of the electrical driving force is the basolateral membrane potential largely produced by the $K^+$ gradient between the intracellular and serosal fluids across the $K^+$-selective channels of the basolateral membranes (Koefoed-Johnsen & Ussing, 1958). At least some $K^+$ channels of epithelia are activated by intracellular $Ca^{2+}$ (Petersen & Maruyama, 1984), suggesting that fluctuations in intracellular $Ca^{2+}$ activity ($a^c_{Ca}$) could alter basolateral $K^+$ conductance ($g^{bl}_K$). An increase in $a^c_{Ca}$ could therefore either increase transepithelial $Na^+$ transport ($J^{ms}_{Na}$) by increasing $g^{bl}_K$ (and thereby the electrical potential driving $Na^+$ uptake), or decrease $J^{ms}_{Na}$ by directly or indirectly inhibiting the apical $Na^+$ channels (Chase & Al-Awqati, 1983; Garty & Asher, 1985, 1986; Garty, Asher & Yeger, 1987).

The basolateral $K^+$ channels may also be the major site of action of additional inhibitors of transepithelial $Na^+$ transport. For example, quinidine reduces the short-circuit current across toad (Taylor et al., 1979) and turtle urinary bladders (Arruda & Sabatini, 1980), and inhibits $Na^+$ transport across rabbit proximal (Friedman et al., 1981) and collecting renal tubules (Frindt & Windhager, 1983) and across *Necturus* proximal renal tubules (Lorenzen, Lee & Windhager, 1984). These inhibitions may reflect direct blockage of the apical $Na^+$ channels by an elevation of $a^c_{Ca}$ known to be produced by quinidine in *Necturus* proximal tubule (Lorenzen et al., 1984) and in muscle (Isaacson & Sandow, 1967; Carvalho, 1968; Balzer, 1972; Batra, 1974). However, quinidine, quinine and lidocaine have also been reported to block $K^+$ conductance directly in frog skin (Abramcheck, 1984; Van Driessche & Hillyard, 1985; Van Driessche, 1987), turtle colon (Richards & Dawson, 1985*a*,*b*, 1986; Germann et al., 1986*b*) and molluscan neurons (Hermann & Gorman, 1984).

Despite the potential importance of the $K^+$ channels in regulating apical $Na^+$ entry, they are

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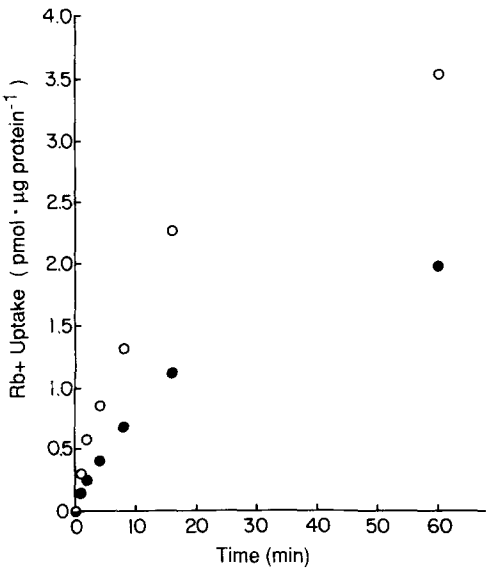


**Fig. 1.** Time dependence of $^{86}Rb^+$ uptake by vesicles. The open and closed circles refer to data points obtained in the absence and presence, respectively, of 5.7 mM external $Ba^{2+}$. No ouabain was present in the media

largely uncharacterized in toad bladder and frog skin. Furthermore, given the range of $K^+$ channels found in other biological membranes, their characteristics are not readily apparent from considerations of other systems. Latorre and Miller (1983) consider six different types of $K^+$ channels, while Rae (1985) reports the existence of 9 to 11 $K^+$ channels in ocular epithelial membranes alone. The channels vary in a number of ways, including their sensitivities to intracellular $Ca^{2+}$ and their selectivities for $K^+$ over $Rb^+$ (Petersen & Maruyama, 1984).

Progress in characterizing the basolateral $K^+$ channels of frog skin and toad bladder has been slowed by the complexity of the whole epithelial preparations. Recently, new approaches have been applied to the problem. Patch clamping has been reported to be feasible for analysis of the basolateral membrane of frog skin (Yantorno & Civan, 1986). Fluctuation analysis has also been applied to the basolateral $K^+$ channels of nystatin-treated urinary bladders from the frog (Van Driessche, 1987). In the present work, we report that membrane vesicles can now be used to study rheogenic, $Ba^{2+}$-blockable $Rb^+$ fluxes by toad urinary bladder. As in the case of the patch clamping of excised membrane patches, the great advantage of this approach is to uncouple the channels from undefined cytoplasmic factors, and to perturb the channels selectively with single, well-defined perturbations.

In this initial study, we have used $^{86}Rb^+$ to probe the $K^+$ channels of toad bladder vesicles. With this approach, we have observed conductive ion-selective, $^{86}Rb^+$ uptake by these vesicles, which is inhibited by external barium. The $^{86}Rb^+$ fluxes are little affected by altering intravesicular $Ca^{2+}$ activity or by administration of external quinine, quinidine or lidocaine.

## Materials and Methods

### Preparation of Vesicles

Vesicles were prepared largely as previously described (Garty, Civan & Civan, 1985). Toads of Mexican origin and of either sex (Lemberger, Oshkosh, Wis.) were doubly pithed and deblooded by transventricular perfusion with approximately 500 ml of a Ringer's solution containing (in mM): 110.0 NaCl, 1.0 $CaCl_2$, 0.5 $MgCl_2$, 3.5 total $KH_2PO_4$ and $K_2HPO_4$, at a pH of 7.5 U. The excised urinary bladders were rinsed several times at 0°C in a homogenizing medium consisting of (in mM): 90.0 KCl, 44 sucrose, 5.0 $MgCl_2$, 1.0 or 10.0 EGTA [ethyleneglycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid], and 10.0 Tris-HCl, at a pH of 7.8.

The epithelial cells were scraped off the whole bladders with glass slides, and then suspended in the homogenizing medium by rapid triturations with Pasteur pipettes. After being twice washed in the ice-cold homogenizing medium, the cells were (unless otherwise stated) incubated for 30 to 45 min at 25°C in the same medium. This incubation has been shown to markedly increase the amiloride-blockable $Na^+$ uptake (Garty & Asher, 1985), but did not seem to affect $^{86}Rb^+$ fluxes in this preparation. The cells were finally fragmented by the application of shear forces with a polytron homogenizer (Ystral GmbH, Gottingen, FRG) for 6 sec at top speed. Intact cells and nuclei were separated into a pellet by centrifuging the homogenates at 1,000 × *g* for 5 min; the supernatant suspensions were subsequently centrifuged at 27,000 × *g* for an additional hour at 0°C. The microsomal pellets were resuspended in minimal volumes of the homogenizing solutions either for study on the same day or for freezing in liquid nitrogen. Satisfactorily large transport activity could be measured for at least 5 weeks after storage in the frozen state.

### Assay of $^{86}Rb^+$ Transport

Influx and efflux of $^{86}Rb^+$ were measured analogously to the measurements of $^{22}Na^+$ reported previously (Garty, 1984; Garty & Asher, 1985; Garty, Civan & Civan, 1985). The microsomes were initially eluted through short Dowex (50 WX8) columns (50 to 100 mesh, $Tris^+$ form) with 0.9 to 2.1 ml of 175 mM sucrose solution. This passage established a chemical gradient for $K^+$ across the vesicular walls by the exchange of external $K^+$ for $Tris^+$ and by dilution of the extravesicular medium with isotonic sucrose solution. The chemical gradient produced an electrical gradient across vesicles formed from the basolateral membranes. The electrical gradient amplifies conductive cation uptake and slows the kinetics of such channel-mediated fluxes from milliseconds to minutes (Garty, Rudy & Karlish, 1983). In the absence of added valinomycin the $K^+$ gradient *per se* provides too insubstantial an electrical driving force to cause measurable $^{22}Na^+$ uptake by apical vesicles (Garty, 1984). Aliquots were thereupon immediately distributed among vials containing a final tracer concentration of approximately 13 $\mu Ci \cdot cm^{-3}$ (17 to 31 $\mu M$ $Rb^+$),

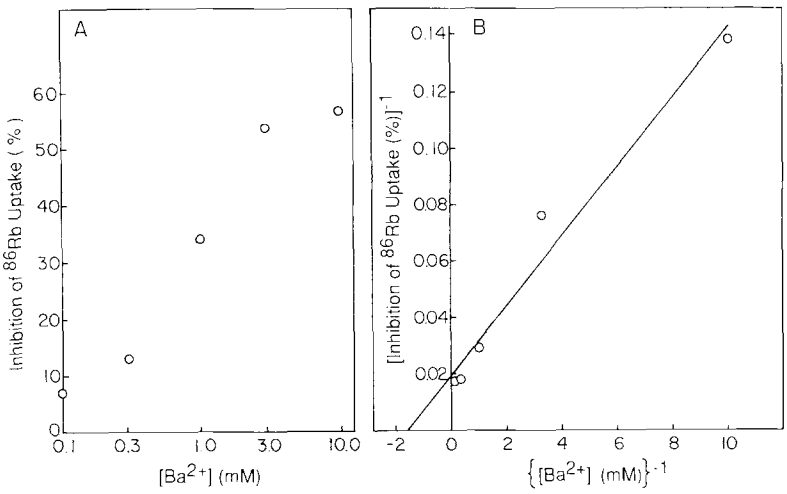


**Fig. 2.** Dose-response relationship for the inhibition of $^{86}Rb^+$ influx as a function of external $[Ba^{2+}]$. The same data are presented in the two panels. In (*B*), the results are displayed in double-reciprocal Lineweaver-Burk form. The solid line has been constructed from the equation obtained by linear regression analysis: $[\%\ \text{Inhibition}]^{-1} = 0.012\ [Ba^{2+}]^{-1} + 0.020$. The regression coefficient is 0.985. The intercept with the *y*-axis is 1/(maximal % block); in this case, 50% of the $^{86}Rb^+$ flux was maximally inhibited

and varying concentrations of Tris buffer and isotonic sucrose, with or without $BaCl_2$, ouabain, and other reagents. Unless otherwise stated, the external pH of the incubation medium was 7.1 to 7.5 U. The total and barium-insensitive uptakes were measured by taking aliquots (150 $\mu$l, 3 to 19 $\mu$g protein) from the radioactive suspensions after 1.5 and 3.5 min; the aliquots were transferred to Dowex columns prewashed in isotonic sucrose solution containing 1 mg · $cm^{-3}$ bovine serum albumin, and eluted into counting vials with 1.5 ml ice-cold isotonic sucrose solution. A permeability ($P_{Rb}$) characterizing the $^{86}Rb^+$ uptake was calculated either as $cm^3 \cdot min^{-1}$ or $cm^3 \cdot min^{-1} \cdot g\ protein^{-1}$. The efflux measurements are described below. The protein contents of most of the samples were measured (Bradford, 1976) with separate aliquots of eluted vesicles. The $^{86}Rb^+$ contents were assayed by measuring the Cerenkov radiation with a $\beta$-counter. Unless otherwise stated, values are presented as means ± SE.

### Chemicals

EGTA, bovine serum albumin (Fraction V), quinine, quinidine and lidocaine were obtained from the Sigma Chemical Company (St. Louis, Mo.), Dowex beads from Fluka AG (Buchs, Switzerland) and $^{86}RbCl$ from NEN Products (Boston, Mass.). Amiloride was a gift from Merck, Sharp and Dohme (GmbH, Munich, FRG).

## Results

### Effects of External Ouabain and $Ba^{2+}$

Figure 1 presents the intravesicular $^{86}Rb^+$ content as a function of time in the absence and presence of 5.7 mM $Ba^{2+}$. With or without external $Ba^{2+}$, the vesicles continued to accumulate $^{86}Rb^+$ throughout the 60 min of measurement, consistent with the known ability of the intact cells to exchange (at least partially) internal $K^+$ for external $Rb^+$ (Robinson & Macknight, 1976*b*). $Rb^+$ has also been found to be a satisfactory replacement for $K^+$ in sustaining the transepithelial electrical properties of toad bladder (Leb, Hoshiko & Lindley, 1965; Robinson & Macknight, 1976*a*). The $^{86}Rb^+$ uptake of Fig. 1 could have reflected at least two distinct transport processes: electrodiffusive entry through aqueous channels, and antiport Rb/K exchange through the Na,K-activated ATPase (which does not require ATP hydrolysis) (Karlish & Stein, 1982; Kenney & Kaplan, 1985). Both processes were likely active in the present preparation of vesicles.

As displayed by Fig. 1, the external $Ba^{2+}$ reduced the magnitude of the $Rb^+$ uptake throughout the entire period of measurement, suggesting that part of the influx proceeded through aqueous channels. Barium is widely used as a blocker of $K^+$ channels, in excitable (Sperelakis, Schneider & Harris, 1967; Standen & Stanfield 1978; Armstrong & Taylor, 1980; Eaton & Brodwick, 1980) and epithelial cells (Nagel, 1979; Nielsen, 1979; Van Driessche & Zeiske, 1980; DeLong & Civan, 1983; Kirk & Dawson, 1983; Hunter et al.,1984). This point was further investigated in the experiment of Fig. 2, which demonstrates that the inhibition by $Ba^{2+}$ was dose dependent. Linear regression analysis of the Lineweaver-Burk presentation of the data in Fig. 2*B* indicates that the apparent $K_m$ for the action of external $Ba^{2+}$ is approximately 0.6 mM. Although this value is relatively large, the pharmacological properties displayed by $K^+$ channels are variable (Hille, 1984), and little information is available concerning the dose-response relationship for $K^+$ channels in epithelia. Van Driessche and Zeiske (1980) have reported a value of 40 to 80 $\mu$M for the apical $K^+$ channel of the skin of *Rana temporaria*. The observation of a dose-dependent inhibition of $Rb^+$ flux by $Ba^{2+}$ is consistent with the concept that part of the observed $^{86}Rb$ uptake proceeds through aqueous channels.

It is likely that part of the total uptake also reflects passive Rb/K exchange through the Na,K-exchange pump (Karlish & Stein, 1982; Kenney &

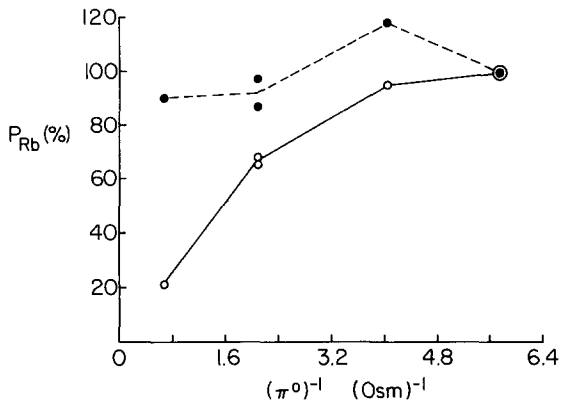


**Fig. 3.** Dependence of barium-sensitive (open symbols) and barium-insensitive (filled circles) $Rb^+$ permeability on external osmolality. The osmolality was varied by adding different concentrations of sucrose to the assay media. The lines are intended to facilitate the identification of the two sets of data points and have no theoretical significance. As in almost all of the experiments conducted in the present study, the data of Fig. 3 were obtained with 1 mM ouabain in the external medium

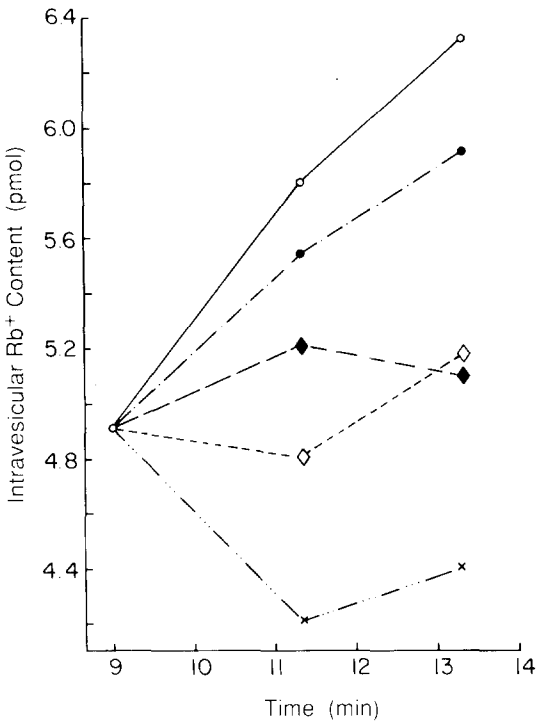


**Fig. 4.** Intravesicular $^{86}Rb^+$ content as a function of external cationic composition. After preincubation for 9 min in the presence of tracer, aliquots of vesicles were added to solutions containing: no added cation (open circles), 6.1 mM $BaCl_2$ (filled circles), 10.0 mM LiCl (filled rhomboids), 10 mM NaCl (open rhomboids), or 10 mM KCl (crosses). The addition of the KCl produced a net efflux of $^{86}Rb^+$ from the vesicles

Kaplan, 1985). In a separate experiment, addition of 1 mM ouabain reduced the barium-insensitive uptake from 6.87 to 3.82 $cm^3 \cdot min^{-1} \cdot g^{-1}$ without substantially affecting the barium-sensitive uptake; the $P_{Rb}$ for the latter flux was reduced only slightly from 4.69 to 4.26 $cm^3 \cdot min^{-1} \cdot g^{-1}$. In the present work, we have been concerned specifically with transport through aqueous channels. Therefore, in order to reduce the level of the background barium-insensitive $^{86}Rb^+$ flux, 1 mM ouabain has been included in the external assay medium in all of the experiments described below.

## Effects of External Osmolality and Temperature

The effects observed with external ouabain and $Ba^{2+}$ suggested that the measured $^{86}Rb^+$ uptake reflected entry into a membrane-bound space, rather than simple membrane adsorption. This point was further examined by the experiment of Fig. 3. The $Rb^+$ permeability ($P_{Rb}$) was measured as a function of increasing external osmolality ($\pi_o$), produced by the addition of sucrose to the assay medium. The direct dependence of $P_{Rb}$ on $(1/\pi_o)$ documents that the barium-sensitive $Rb^+$ influx enters an intravesicular space. However, the absence of such an effect on the barium-insensitive flux does not necessarily preclude the possibility that this fraction also enters a membrane-bound, albeit different, space. The $Rb^+$ influx is expected to be linearly dependent on $(1/\pi_o)$ (Beck & Sacktor, 1975) only if the measurements are conducted after equilibration has been attained and if the equilibrium ratio of the intra- to extravesicular $Rb^+$ activities is unchanged. In the present circumstance, the osmotically induced shrinkage increases the electrical driving force for $Rb^+$ uptake by increasing the internal $K^+$ activity, complicating the form of the dependence of $P_{Rb}$ on external osmolality. It was not feasible to conduct the current experiments at equilibrium because of the very small barium-blockable $Rb^+$ influx under those conditions.

The influence of temperature on the barium-sensitive and barium-insensitive $^{86}Rb^+$ uptakes was also examined. The activation energy ($A$) for diffusion in simple solutions is 3 to 6 kcal $\cdot$ $mol^{-1}$ (Helfferich, 1962). Taking $A$ to be the mid-value within this range (4.5 kcal $\cdot$ $mol^{-1}$), the $Q_{10}$ for the coefficient of diffusion at 288 to 298 K is calculated to be 1.3. In contrast, the $Q_{10}$ for a chemically mediated process is commonly taken to be $\simeq 2$. Therefore, raising the temperature from 273 to 298 K should increase $P_{Rb}$ by factors of about 1.9 and 5.7 for diffusion through an aqueous pore and for chemically mediated transfer, respectively. In two experi-

**Table 1.** $^{86}Rb^+$ uptake as a function of external cations[a]

| Experiment | Experimental composition | $P_{Rb}$ ($cm^3 \cdot min^{-1} \cdot g\ protein^{-1}$) | |
|---|---|---|---|
| | | $Ba^{2+}$-sensitive | $Ba^{2+}$-insensitive |
| Control: | Sucrose | 6.58 ± 1.55 (2) | 2.86 ± 0.13 (2) |
| | Sucrose + Tris | 3.73 ± 0.30 (2) | 2.70 ± 0.06 (2) |
| | Averaged data | 5.15 ± 1.04 (4) | 2.78 ± 0.08 (4) |
| $Li^+/Na^+$: | $Li^+$ | 1.06 ± 0.17 (3) | 2.21 ± 0.14 (3) |
| | $Na^+$ | 1.26 ± 0.08 (3) | 1.49 ± 0.13 (3) |
| | Averaged data | 1.16 ± 0.09 (6) | 1.85 ± 0.18 (6) |
| $K^+/Rb^+/Cs^+$: | $K^+$ | 0.43 ± 0.14 (3) | 0.60 ± 0.14 (3) |
| | $Rb^+$ | 0.51 ± 0.02 (3) | 0.56 ± 0.09 (3) |
| | $Cs^+$ | 0.39 ± 0.16 (2) | 0.82 ± 0.10 (2) |
| | Averaged data | 0.45 ± 0.06 (8) | 0.64 ± 0.07 (8) |

[a] The $^{86}Rb^+$ influxes were measured with three sets of vesicles, and have been expressed as $Rb^+$ permeabilities (in $cm^3 \cdot min^{-1} \cdot g\ protein^{-1}$). In each experiment, the total volume of each experimental assay suspension was 360 μl, and included 7.2 μl of a 0.5 M solution of LiCl, NaCl, KCl, RbCl or CsCl, producing a final alkali cation concentration of 10.0 mM. The control assay suspensions contained 7.2 μl of either deionized water or 6% sucrose instead of the 0.5 M solutions of the alkali cations. In two of the studies, each of the experimental and control assay suspensions contained 23.0 mM sucrose; in the third study, the suspensions all contained 42.1 mM Tris at constant pH, instead of the sucrose. The results were qualitatively and quantitatively similar for all three vesicle preparations. The numbers in parentheses indicate the number ($n$) of experimental determinations. The uncertainties entered for $n \geqq 3$ are the standard errors. For $n = 2$, each uncertainty has been estimated as half the difference between the two experimental measurements. For the data of this and the succeeding Tables, the $Ba^{2+}$-insensitive fluxes were measured in the presence of 6.1 mM $Ba^{2+}$.

ments, the barium-sensitive fraction of $P_{Rb}$ was increased by factors of 2.3 and 1.5, while the barium-insensitive flux was increased by factors of 3.0 and 3.6. Thus, the temperature dependence of the barium-sensitive uptake was precisely that expected for diffusion through an aqueous channel, while the temperature dependence of the barium-insensitive fraction was consistent with either diffusion or chemically mediated transport.

## Effects of External Alkali Cations

Whether or not the $^{86}Rb^+$ transport was conductive was explored by examining the effects of externally added alkali cations on both efflux and influx. In the experiment of Fig. 4, vesicles were first preloaded with $^{86}Rb$ over a period of 9 min, and then transferred either to control solutions containing simply sucrose (CON), or to solutions also containing 6.1 mM $Ba^{2+}$ or 10 mM $Li^+$, $Na^+$ or $K^+$. In the control assay solution, the vesicles continued to accumulate $^{86}Rb$. In the presence of 6.1 mM $Ba^{2+}$, $^{86}Rb$ uptake persisted, but at a slower rate than for the control. The uptake was further slowed both by $Li^+$ and $Na^+$; in these cases, the $^{86}Rb$ content was relatively constant over the subsequent 4 min. The addition of external $K^+$ was more dramatic, producing a substantial loss of intravesicular $Rb^+$. The most direct interpretation of these data is that addition of permeable cations to the outside medium depolarized the electrical gradient established across aqueous conductive channels, reducing the driving force for intracellular accumulation of $^{86}Rb^+$.

The alkali cations exerted similar effects on $^{86}Rb^+$ influx. Table 1 presents the results of three sets of influx experiments. $Li^+$ and $Na^+$ reduced $^{86}Rb$ to comparable extents. In comparison to the control measurements, these ions reduced the barium-sensitive influx by 77% and the barium-insensitive flux by 33%. $K^+$, $Rb^+$ and $Cs^+$ were about equally effective in lowering $^{86}Rb^+$ uptake to a greater extent; the barium-sensitive and barium-insensitive influxes were reduced by 91 and 77%, respectively.

Thus, measurements of both $^{86}Rb^+$ efflux and influx following addition of external cations indicate that the tracer movement in the pesence of ouabain proceeds largely through a conductive pathway. This pathway appears more permeable to $K^+$, $Rb^+$ and $Cs^+$ than to $Li^+$ and $Na^+$.

## Effects of External pH

Figure 5 presents the results of two sets of experiments in which $^{86}Rb^+$ uptake was measured as a function of external pH ($pH_o$). The mean values for

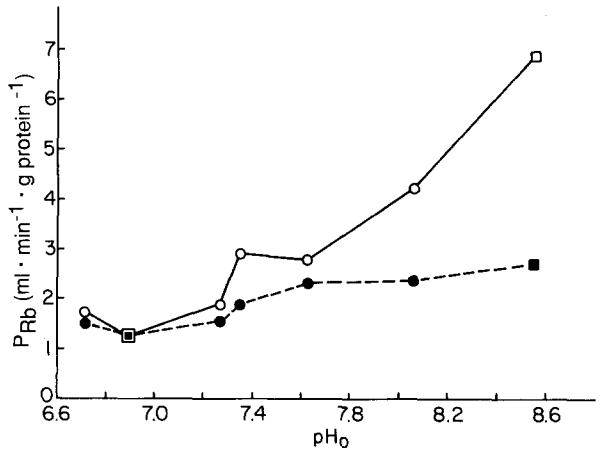


**Fig. 5.** $^{86}Rb^+$ influx as a function of external pH ($pH_o$). The data have been expressed in terms of $Rb^+$ permeabilities ($P_{Rb}$). The closed symbols refer to the $Ba^{2+}$-insensitive uptake, measured in the presence of 6.1 mM external $Ba^{2+}$. The data presented as open symbols are the $Ba^{2+}$-sensitive fluxes. The results are the means calculated from two sets of experiments. Most of the data points were taken from two measurements at the same external pH (circles); the points reflecting single measurements are symbolized by squares

the barium-sensitive and barium-insensitive permeabilities are displayed as open and closed symbols, respectively. A modest fall in barium-insensitive uptake was noted when external pH was reduced from 8.6 to 6.7. A much more striking effect was noted with the barium-sensitive influx. Reducing $pH_o$ from 8.6 to 7.6 reduced the uptake by two- to threefold; the subsequent decline in $^{86}Rb^+$ flux with further reductions in $pH_o$ was comparable to that observed for the barium-insensitive component.

In principle, the effects produced by external acidification could have been mediated by secondary changes induced in the internal pH. This possibility is unlikely with the present preparation. Fluorescence measurements of intravesicular pyranine have indicated that the inner pH is little changed by step changes in $pH_o$ over the time frames of the current measurements (Garty, Civan & Civan, 1985).

## Effects of External Amiloride

The heterogeneous preparation of vesicles used in the present study contains $Na^+$ channels which are highly sensitive to amiloride [$K_I = 2 \times 10^{-8}$ M (Garty & Asher, 1985)]. Although $K^+$ movement through these apical $Na^+$ channels has been reported to be barely detectable (Palmer, 1982), the vesicles do display a similar dependence on external pH for both $^{22}Na^+$ and $^{86}Rb^+$ uptake. To exclude the possibility that both uptakes proceed through the same channel, the $^{86}Rb^+$ uptake was studied as a function of external amiloride concentration. In contrast to the high amiloride sensitivity characterizing the apical $Na^+$ channels, little effect on $^{86}Rb^+$ uptake was noted at amiloride concentrations as large as 45 $\mu$M. Thus, the conductive channels mediating the $^{22}Na^+$ and $^{86}Rb^+$ fluxes are pharmacologically distinct.

**Table 2.** Effects of $Ca^{2+}$ on $^{86}Rb^+$ uptake[a]

| Presence of external: | | $P_{Rb}$ (cm$^3$ · min$^{-1}$ · g protein$^{-1}$) | |
|---|---|---|---|
| $Ca^{2+}$ ($\mu$M) | A23187 ($\mu$M) | $Ba^{2+}$-sensitive | $Ba^{2+}$-insensitive |
| 0 | 0 | 2.36 ± 0.30 | 1.55 ± 0.02 |
| 0 | 4 | 2.33 ± 0.09 | 2.14 ± 0.02 |
| 10 | 0 | 1.89 ± 0.17 | 1.47 ± 0.08 |
| 10 | 4 | 1.83 ± 0.15 | 1.81 ± 0.20 |

[a] The tabulated values are the means of two sets of experimental measurements performed in the presence or absence of $10^{-5}$ M external free $Ca^{2+}$ and of 4 $\mu$M A23187. Each uncertainty has been estimated as half the difference between the two measurements performed under the same conditions. Each of the suspensions contained 1 mM EGTA inside and outside the vesicles. It should be appreciated that during the flux assays, only the internal $Ca^{2+}$ activity was varied. The external free $Ca^{2+}$ concentration was negligible ($\simeq 10^{-9}$ M) in each of the four assays, following passage through the Dowex column and chelation by the external EGTA.

## Effects of Calcium

Intracellular $Ca^{2+}$ activity has been reported to activate some potassium channels and not others (Petersen & Maruyama, 1984), and has even been reported to inhibit potassium channels of human T cells (Bregestovski, Redkozubov & Alexeev, 1986). Activation may result from either a direct effect (i.e, by binding to the channel protein or to a gating site nearby) or indirectly by triggering an intermediate enzymatic process. Both types of mechanisms have been found to play a role in the down regulation of $Na^+$ channels in toad bladder (Garty & Asher, 1985, 1986; Garty et al., 1987). We have examined effects of $Ca^{2+}$ on $^{86}Rb^+$ fluxes using two approaches. In the first, vesicles prepared containing no internal $Ca^{2+}$ were incubated for 1 hr at 25°C in a medium containing 1 mM EGTA, with or without 1 mM $CaCl_2$ ($10^{-5}$ M free $Ca^{2+}$ at pH 7.8), and with or without 4 $\mu$M A23187 (Table 2). Incubation with A23187 equalizes the intra- and extravesicular $Ca^{2+}$ activities of these membrane preparations (Garty et al., 1987). Incorporation of $10^{-5}$ M free

$Ca^{2+}$ in the isolated vesicles failed to stimulate the barium-blockable $^{86}Rb$ flux.

In a second set of experiments, the scraped epithelial cells were first incubated for 45 min at 25°C in the presence of EGTA with or without $10^{-5}$ M free $Ca^{2+}$, and then homogenized in the same media. Exposing whole, permeabilized, toad bladder cells to $10^{-5}$ M $Ca^{2+}$ has been shown to irreversibly inhibit the amiloride-sensitive $^{22}Na$ fluxes subsequently measured in the vesicles (Garty & Asher, 1985, 1986). Here, however, pretreating the cells with $Ca^{2+}$ and trapping $Ca^{2+}$ within the vesicles had little effect on the barium-sensitive $^{86}Rb$ flux. In four sets of paired measurements, internal $Ca^{2+}$ increased the ratio of the barium-sensitive to barium-insensitive $^{86}Rb$ uptakes by only 27 ± 12%. Thus, $Ca^{2+}$ did not appear to exert a substantial indirect effect on $Rb^+$ transport, either. However, one possibility which we could not assess is that the calcium-induced activation is reversed (even in the sustained presence of $Ca^{2+}$), once the cell is disrupted and its cytoplasmic contents infinitely diluted. This would be the case if, e.g., channels are activated by an ATP-requiring, calcium-dependent kinase, and are inhibited following dephosphorylation by a membrane-bound phosphatase.

### Effects of Quinine, Quinidine and Lidocaine

As noted in the Introduction, quinidine is known to inhibit $Na^+$ transport across toad bladder ($J_{Na}^{ms}$) (Taylor et al., 1979), an effect initially ascribed to a quinidine-induced elevation of intracellular calcium activity. However, quinine, quinidine and lidocaine have also been reported to block $K^+$ channels directly in other tissues; a quinidine-induced depolarization could also inhibit $J_{Na}^{ms}$ by reducing the electrical driving force favoring apical $Na^+$ entry. It was therefore of interest to examine whether quinine, quinidine and lidocaine exerted any effects on the $P_{Rb}$ of the channels characterized in the current study.

Table 3 presents the means ± SE for the results of three experiments. No significant effects were noted on either the barium-sensitive or -insensitive fluxes by 0.5 mM quinine, 0.5 mM quinidine or 0.2 mM lidocaine.

**Table 3.** Effects of quinine, quinidine and lidocaine on $^{86}Rb^+$ uptake[a]

| Experimental condition | $P_{Rb}$ ($cm^3 \cdot min^{-1} \cdot g\ protein^{-1}$) | |
|---|---|---|
| | $Ba^{2+}$-sensitive | $Ba^{2+}$-insensitive |
| Control | 1.8 ± 0.7 | 1.7 ± 0.3 |
| Quinine (500 μM) | 1.3 ± 0.4 | 2.1 ± 0.8 |
| Quinidine (500 μM) | 1.6 ± 0.6 | 2.5 ± 1.0 |
| Lidocaine (200 μM) | 1.7 ± 0.6 | 1.7 ± 0.5 |

[a] The tabulated values are means ± SE from three sets of experiments.

## Discussion

The results of the present study document that a $Rb^+$ channel from the urinary bladder of the toad can be studied by measurements of $^{86}Rb$ flux across the walls of vesicle preparations. The great advantage of using vesicles is the capability it affords of studying the direct effects of perturbations, in isolation from the complexities of whole cell preparations. The vesicles currently used are particularly satisfactory because of the technical ease of their preparation and the stability of their transport properties. Readily measurable, barium-inhibitable $^{86}Rb$ fluxes can be studied after storage of the frozen vesicles for weeks.

The $Rb^+$ channel characterized in the current work is barium-inhibitable; the concentration for half-maximal inhibition is 0.6 mM. As demonstrated by the effects of external cations on influx and efflux, the channel is conductive with a considerable selectivity for $K^+$, $Rb^+$ and $Cs^+$ over $Li^+$ and $Na^+$.

As in the case of any heterogeneous preparation, the anatomic identity of the uptake sites cannot be precisely identified. The osmostic dependence of the barium-sensitive $Rb^+$ uptake indicates that this flux enters a membrane-bound vesicular space. The pathway for the barium-sensitive uptake is pharmacologically distinct from the apical $Na^+$ channels already characterized in this preparation. The selectivity for $K^+$ over $Na^+$ strongly suggests that the channels studied are not located in the apical membranes, altogether. It is also implausible that the channels could originate from mitochondria; conductive $K^+$ channels would dissipate the electrical gradient critically necessary for mitochondrial function. Therefore the $K^+$ channels defined in the present study likely arise from the basolateral membranes, although the possibility of other, as yet unidentified intracellular origins cannot be excluded.

The apical $Na^+$ channels of the current preparation are oriented right-side-out (Garty, 1984). In the present study, addition of 1 mM external ouabain reduced $Rb^+$ accumulation. This phenomenon likely reflected inhibition of Rb/K exchange through the Na,K-exchange pump (Karlish & Stein, 1982; Kenney & Kaplan, 1985). Therefore, a significant fraction of the vesicles incorporating the barium-

blockable $Rb^+$ channel studied are also likely oriented right-side-out. The current data do not exclude the possibility that some of the basolateral vesicles display the opposite orientation.

The physical basis for the barium- and ouabain-insensitive $Rb^+$ uptake is unclear. This flux was distinctly different from the barium-sensitive component in its responses to changes in the external osmolality, temperature, and external pH. In part, the barium-insensitive uptake may reflect adsorption of $Rb^+$ onto microsomal membranes.

The barium-sensitive $Rb^+$ channel characterized in this study is not substantially activated by internal $Ca^{2+}$. Whether this pathway is identical with the principle $K^+$ channel in the whole cells, and whether its properties have been unaltered during the preparative procedures is as yet unclear. Subject to these caveats, we have used this preparation to address a problem of current physiologic interest. The mechanism by which quinidine inhibits $Na^+$ transport across toad bladder has been uncertain. This agent has been documented to raise intracellular $Ca^{2+}$ activity ($a^c_{Ca}$) in *Necturus* proximal tubule (Lorenzen et al., 1984) and in muscle (Isaacson & Sandow, 1967; Carvalho, 1968; Batra, 1974); the increased ($a^c_{Ca}$) could inhibit apical $Na^+$ directly (Chase & Al-Awqati, 1983). The alternative possibility, that quinidine inhibits $J^{ms}_{Na}$ by depolarizing the cell through a direct block of the $K^+$ channels seems less likely in view of the present results. Quinine, quinidine and lidocaine all had no significant effects on the barium-sensitive and barium-insensitive $^{86}Rb$ fluxes of these vesicles.

In the present work, we have used measurements of $^{86}Rb^+$ flux to characterize one $K^+$ channel in toad bladder. Additional $Rb^+$-permeable $K^+$ channels with different properties may exist in this tissue. However, we should emphasize that we have monitored all the $Rb^+$-permeable channels in the preparation, since the membranes are not fractionated. Thus, additional putative, $Rb^+$-permeable channels must be: (i) present in the preparation used, and (ii) inactivated during the formation and isolation of the vesicles.

It is also possible that the experimental approach used selects for one specific type of $K^+$ channel which happens to be permeable to $Rb^+$, as well. Toad bladder may indeed contain additional $K^+$ channels, which are impermeable to $Rb^+$ and sensitive to internal $Ca^{2+}$ and external quinidine. However, if the use of $Rb^+$ selects for one type of $K^+$ channel, that pathway is likely to correspond to the physiological $K^+$ channel observed under baseline conditions in the baseline membrane of turtle colon (Germann et al., 1986*a*,*b*). The latter channel was found to be quinidine-insensitive and unselective for $K^+$ over $Rb^+$, in contrast to the $K^+$ channel which appeared under conditions of extreme osmotic stress. The physiological relevance of the present data is also supported by the observations that: (i) $Rb^+$ can satisfactorily replace $K^+$ in sustaining the transepithelial electrical properties of toad bladder (Leb et al., 1965; Robinson & Macknight, 1976*a*), and (ii) external $Rb^+$ at least partially exchanges for the intracellular $K^+$ of the intact preparation (Robinson & Macknight, 1976*b*).

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