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Polarized $^{86}Rb^+$ effluxes in primary cultures of rabbit kidney proximal cells: role of calcium and hypotonicity

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Isolated proximal cells from rabbit kidney were seeded on collagen-coated permeable supports. After 8 days, the cultured cells became organized as a confluent monolayer. The proximal origin of the monolayer was confirmed by enzymatic, immunological, electrical and electron microscopical studies. The epithelia exhibited a morphological polarity that allowed for measurements of effluxes across the apical or the basolateral membranes. ^{86}Rb was used as an isotopic tracer to indicate potassium movements. The $^{86}Rb^+$ efflux across the basolateral face was 1.93-times that across the apical face, and both effluxes were pH dependent. Apical and basolateral $^{86}Rb^+$ effluxes increased when the Ca^{2+} ionophore ionomycin (3 μM) was applied and when monolayers were exposed to a hypotonic medium. A pharmacological study revealed that $BaCl_2$ (5 mM), tetraethylammonium (TEA, 20 mM) and *Leiurus quinquestriatus hebraeus* scorpion venom (from which charybdotoxin is extracted) abolished both ionomycin and hypotonically-stimulated effluxes, whereas apamin had no significant effect on the hypotonically-stimulated $^{86}Rb^+$ efflux. This stimulated efflux was also abolished when monolayers were preincubated with pertussis toxin, but did not decrease in a Ca^{2+}-free medium.

Introduction

Like cells from many other tissues [1], proximal tubules are capable of regulatory volume decrease [2,3] If exposed to hypotonic media, the cells swell but subsequently return to their original volume. Although it is known that potassium plays an important role in this regulatory phenomenon, different membrane transport pathways seem to be involved depending on the cell type studied [4] In the proximal nephron, an increase of the basolateral potassium conductance has been described [5–7], but no further investigation was made as to the type of channel involved Neither is the apical membrane response well known, since only cultured renal cell lines such as MDCK or Opossum kidney have as yet been studied [8,9] In order to study the potassium conductances in both apical and basolateral membranes, and their behavior during a hypotonic shock, we undertook $^{86}Rb^+$ efflux measurements on primary cultures of freshly isolated proximal cells The simple geometry and the free access to the apical and basolateral membranes of cultured monolayers are experimental factors suited for efflux measurements In the present paper, we describe two pathways for $^{86}Rb^+$ effluxes present in both apical and basolateral membranes One of them, which appears to be involved during a hypotonic shock, presents the characteristics of the maxi K^+ channel This channel, described in many tissues, has recently been reported in the apical membrane of proximal convoluted tubules in primary culture [10]

Materials and Methods

Animals

Young male New-Zealand white rabbits (body wt, 600–800 g) were used for the experiments. All animals were fed a standard diet and had free access to tap water For cell preparations, kidneys were removed under sterile conditions from animals killed by 2 ml pentobarbital and 1250 U heparin (Roussel, France) injected through the vein of the ear

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Preparation of sterile isolated proximal cells

Isolated cells were prepared as previously described in our laboratory by Poujeol et al [11] For each experiment, four kidneys were used Briefly, kidneys were perfused with a medium composed of an equal mixture of DMEM and HAM F12 (Gibco, Grand Islands, NA, U S A or Eurobio, Paris, France) containing 15 mM $NaHCO_3$, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid (Hepes) (pH 7 5), 100 U/ml penicillin and 100 μg/ml streptomycin Slices from the superficial cortex were then passed through a tissue press and successively filtered through 100-, 40-, and 20-μm nylon meshes Finally, cells were placed in a hormonally-defined culture medium composed of DMEM, HAM F12, 15 mM $NaHCO_3$, 20 mM Hepes, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine, 5 μg/ml insulin, 5 10^{-8} M dexamethasone, 10 ng/ml EGF, 5 μg/ml transferrin, 3 10^{-8} M sodium selenite, and 1 10^{-8} M triiodothyronine

The total number of cells was estimated by microscopic observation of 20 μl of cell suspension diluted in 0 5% eosin solution A final concentration of 20 10^6 cells/ml was generally obtained, corresponding to a total cell count of 100 10^6 Isolated cells were then seeded on collagen-coated culture wells (millicell HA 30 mm diameter, Millipore, U S A) at a concentration of 1 10^6 cells/cm^2 The cells were placed in a 6 wells culture dish (Costar, New York, NY, U S A) containing 2 ml of culture medium This medium was changed 3 days after seeding and then every 2 days, with the same culture medium but without penicillin and streptomycin Cultures were maintained at 37 °C in a 5% CO_2-water-saturated atmosphere Eight days after seeding, cultures became confluent

Enzyme studies

Enzyme analyses were performed on freshly isolated proximal cells and on 8-day-old cultures Leucine aminopeptidase (LAP) was determined by the technique of Kramers and Robinson [12] using leucine *p*-nitroanilide as substrate Gamma-glutamyl *p*-nitroanilide as substrate (Sigma list kit) Alkaline phosphatase (AKP) was assayed with *p*-nitrophenyl phosphate as substrate at pH 8 6 as described by Hubscher and West [13], while the method of Quigley and Gotterer [14] was used to estimate ouabain-sensitive Na/K-ATPase activity The phosphate liberated by the action of the enzyme after ATP hydrolysis was measured using ATP as substrate For these four enzyme assays, the reactions were developed directly in the culture wells, without pretreatment of the cells Hexokinase (HK) was determined by Bergmeyer's method [15] The method used to assay fructose-1,6-bisphosphatase, was based on that of Hintz et al [16] without the enzymatic cycling procedure For this metabolic enzyme the cultured cells were detached from the wells by trypsinization and permeabilized by the freeze-thawing procedure The results were normalized for the DNA content of each sample

Cyclic AMP production in primary cultures

Cyclic adenosine monophosphate (cAMP) produced by the 8-day-old cultures was measured by radioimmunoassay [17] The epithelia were incubated in RPMI medium at 37 °C with 0 1 μg/ml parathyroid hormone (PTH1–34) or 1 10^{-8} M arginine vasopressin (AVP) for 15 min in the presence of 1 mM 3-isobutyl-1-methylxanthine (IBMX) The hormones were added to both apical and basolateral sides of the culture well cAMP was measured in monolayer after cells were lysed in a mixture of formic acid in absolute ethanol (5% v/v)

DNA determination

DNA was measured by a modification of the fluorimetric micromethod of Switzer and Summer [18]

Indirect immunofluorescence on primary cultures

Primary cultures from isolated proximal cells grown on 30 mm collagen-coated teflon wells were fixed for 15 min at room temperature with 4% paraformaldehyde (PAF) in 50 mM phosphate buffer (pH 7 1) (PBS) After rinsing, wells were incubated in PBS for 30 min at room temperature with a 1/100 dilution (10–20 μg/ml) of the monoclonal antibody (Mab 5A4), raised against LAP [19] After several washings in PBS, wells were incubated in FITC-coupled rabbit anti-mouse IgG (Miles, Elkhardt, IN, U S A) diluted 1/100 in PBS containing 0 3% bovine serum albumin (BSA) After 30 min incubation at room temperature, cultures were rinsed three times, covered with glass coverslips and observed with an epifluorescent microscope (Zeiss, Oberkochen, F R G)

Electron microscopy

8-Day-old cultures were fixed with 2 5% glutaraldehyde in 0 1 M cacodylate buffer (pH 7 4) for 30 min and postfixed in 1% reduced osmium tetroxide, after which they were dehydrated in ethanol and embedded in Epon Thin sections were cut and examined at 80 kV with a Philips 400 electron microscope The sections were counterstained for 2 min with lead citrate prior to examination with the electron microscope

Electrophysiological measurements

Transepithelial voltage (V_{TE}) and resistance (R_T) were measured on 8-day-old primary cultures grown on Millipore supports coated with collagen V_{TE} was measured as the difference between two calomel electrodes connected to the bathing solutions by KCl saturated agar bridges (Keithley 601 electrometer) The basal side was earthed The transepithelial resistance was estimated by applying square current pulses (1 5 μA, 2 s duration) through Ag-AgCl electrodes and measuring

transepithelial voltage changes with electrodes positioned as near as possible to the epithelial surface Measurements were made at room temperature with a mixture of DMEM/F12 bathing the apical and basolateral compartments

$^{86}Rb^+$ efflux from confluent monolayers

8-Day-old cultures were loaded with $^{86}Rb^+$ (1 5 μCi/ml) for 4 h at room temperature in RPMI 1640 medium (Gibco, U S A) devoid of sodium bicarbonate and buffered with 25 mM Hepes at pH 7 4, under continuous air stream After rinsing the wells in unlabelled RPMI to remove isotope from the extracellular space, apical and basolateral $^{86}Rb^+$ effluxes were measured simultaneously Every 2 min, the totality of the medium (2 apical ml and 2 basolateral ml of RPMI) was collected directly from the apical and basolateral sides of the culture well and replaced by fresh medium The remaining radioactivity in the epithelium at the end of the experiment was determined by counting filters in vials The $^{86}Rb^+$ content of samples was determined in a Packard liquid scintillation counter (Minaxi 400) by the Cerenkov effect

Calculation

From the backaddition of the radioactivity in the efflux samples to the radioactivity remaining in the cells, we calculated the apical and basolateral efflux rate constants (according to Eqns 1 and 2), i e , the fraction of the total radioactivity lost per unit time

$$(k_a)_t = \frac{(C_a)_t}{C_{ep} + \sum_{t=t_f}^{t+1} [(C_a)_t + (C_b)_t] + 1/2[(C_a)_t + (C_b)_t]} (1/T) \quad (1)$$

$$(k_b)_t = \frac{(C_b)_t}{C_{ep} + \sum_{t=t_f}^{t+1} [(C_a)_t + (C_b)_t] + 1/2[(C_a)_t + (C_b)_t]} (1/T) \quad (2)$$

where $(k_a)_t$ and $(k_b)_t$ are the apical and basolateral efflux rate constants at time t, $(C_a)_t$ and $(C_b)_t$ are the radioactivity lost from the apical and basolateral sides at time t and during the period T, respectively C_{ep} is the radioactivity remaining in the epithelium at the end of the measurements, and t_f corresponds to the final time of the experiment

For comparing the different experimental conditions, the results were expressed in percent of the initial value ($t = 4$ min) of the basolateral efflux rate constant

Determination of intracellular pH

Intracellular pH was determined by fluorescent video microscopy Cultures were loaded with 4 μM BCECF/AM in RPMI medium during 15 min at 37 °C After rinsing the cultures were observed on an inverted microscope coupled to a low light level video camera (Lhesa LH 4036). Fluorescence excitation was provided by a mercury vapor lamp and was controlled by an automatic shutter (Uniblitz) The excitation beam was filtered through a filter selector fitted with 10 nm band pass filters centered at 450 and 490 nm The separation between incident and emitted fluorescence radiations was done through a Zeiss chromatic beam splitter (FT 510) and a longwave pass filter (LP 520) Fluorescence images were digitized by a PIP image card and stored on a hard disk controlled by a PC-AT computer Experiments were carried out as follows the cells were excited at 490 nm and a first image was digitized Immediately after, the cells were excited at 450 nm and a second image was stored After the collection of a sequence of images, arithmetic operations were performed with an image processing system (Biocom) The grey level intensity values at 490 and 450 nm were corrected for the photobleaching, the background was subtracted and the ratio value between the two wavelengths was calculated The mean ratio value of ten zones was used to estimate the intracellular pH values of the monolayers by reference to calibration curves [20]

Experimental solutions

Experiments were carried out in a RPMI medium continuously aerated, except when barium was present, a Krebs solution was used (137 mM NaCl, 5 4 mM KCl, 1 2 mM $MgCl_2$, 0 3 mM NaH_2PO_4, 0 3 mM KH_2PO_4, 5 mM glucose, 1 mM $CaCl_2$, 20 mM Hepes (pH 7 4)) When pH dependency was studied, the control medium contained 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0 4 mM $MgSO_4$, 2 mM glutamine, 5 mM alanine, 5 mM glucose and 20 mM Hepes (pH 7 4) In experimental solutions, 140 mM NaCl was replaced by 100 mM NaCl and 40 mM NH_4Cl or 40 mM $NaCH_3COO$

Chemicals

Barium and TEA were purchased from Merck, Darmstadt, F R G Ionomycin was obtained from Calbiochem, San Diego, CA, U S A , apamin and pertussis toxin from Sigma, U S A , and crude *Leiurus quinquestriatus hebraeus* venom from Latoxan, France $^{86}Rb^+$ as the Cl^- salt was purchased from Amersham International Ltd

Statistical analysis

Values reported in the text are means ± S E Unpaired Student's t-test was used for statistical analysis

Results

Microscopic studies

Purified proximal cells were seeded at relatively high density on collagen coated permeable filters Within

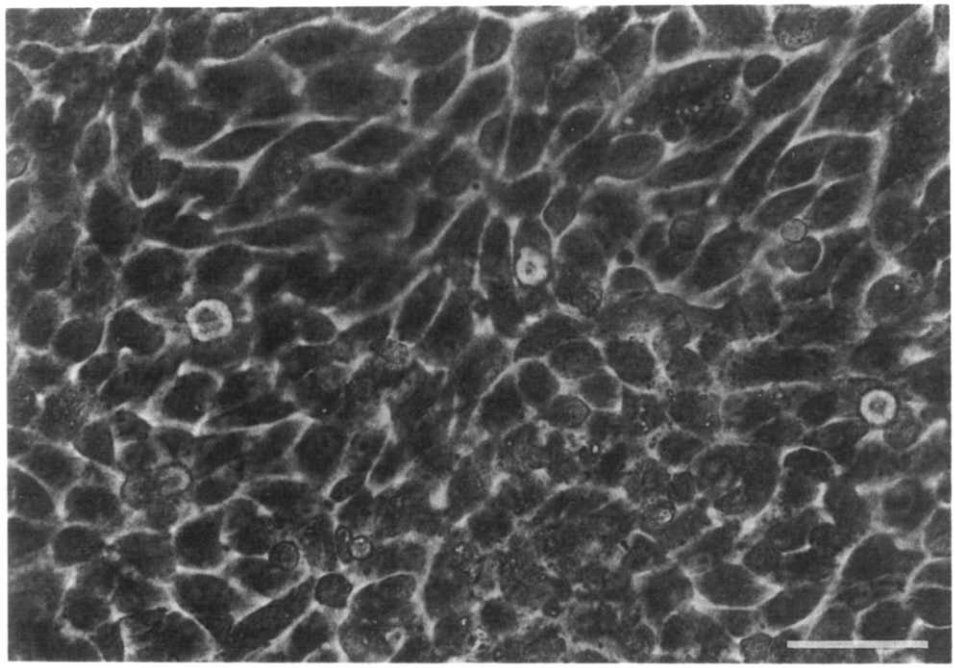

Fig 1 Micrograph of 8-day-old cultured proximal cells Bar is 50 μm

four hours isolated cells were observed to attach to the collagen bed When cultures were seeded at 1 10^6 cells/cm^2, the plating efficiency after 24 h was 5 to 8% and the unattached cells were subsequently removed with the first medium change at 3 days Completely confluent cultures were obtained between 7 and 8 days after seeding at 10^6 cells/cm^2 Using higher seeding densities (up to 2 10^6 cells/cm^2) did not modify either the plating efficiency after 24 h or the time elapsing before total confluency (data not given) Fig 1 shows a phase contrast micrograph representing a confluent monolayer 8 days after seeding The cells exhibited a homogenous shape and size and were obviously contiguous Fig 2 shows an electron micrograph of a 8-day-old monolayer attached to a permeable filter Cultured cells had a morphological polarity shown by the presence of microvilli on the apical membrane exclusively and of delimiting interdigitating cytoplasmic processes restricted to the basal part of the cell

Immunological studies

The proximal tubules have various biochemical properties that can be used to characterize the cells To obtain control values, we determined the enzyme activities of the initial freshly isolated proximal cells The data are reported in Table I Cultured proximal cells showed LAP, γ-GT, AKP, and ouabain-sensitive Na/K-ATPase activities after 8 days seeding The activities of the first two hydrolases (LAP, γ-GT) were lower in primary cultures than in isolated cells, whereas activi-

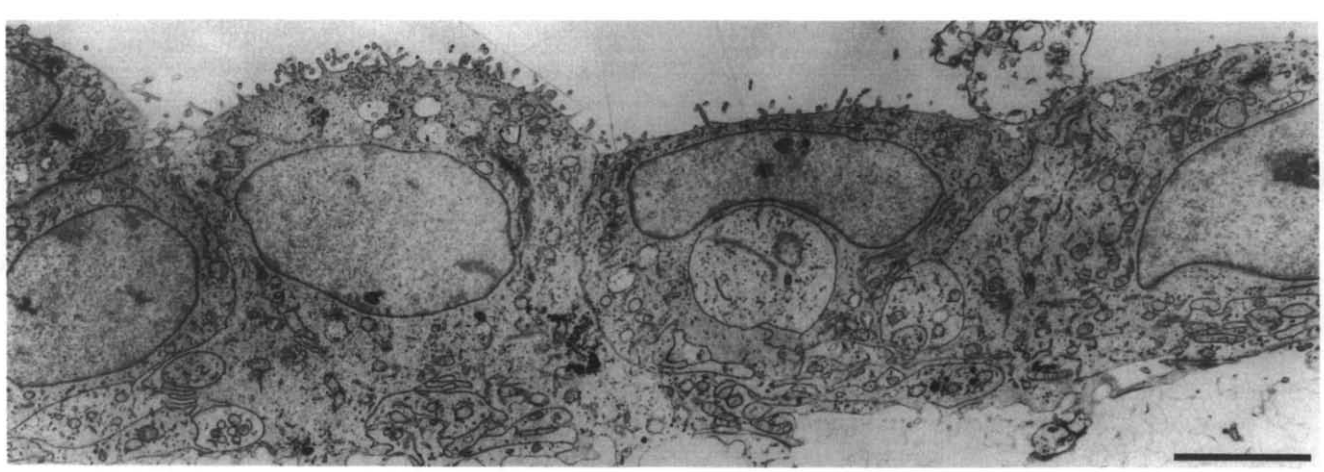

Fig 2 Electron micrograph of 8-day-old cultured proximal cells Bar is 5 μm

TABLE I

Enzymatic characteristics of cultured and freshly isolated proximal cells from rabbit kidney

Leucine aminopeptidase (LAP), gamma-glutamyl transferase (γGT), alkaline phosphatase (AKP), hexokinase (HK) and Na/K-ATPase activities measured in freshly isolated proximal cells and 8-day-old cultures grown on collagen-coated permeable supports Activities are expressed in nmol/min per μg of DNA Values are means ± S E of *n* experiments

	Isolated cells	Cultured cells
LAP	4 5 ±0 7 **	2 04 ±0 19
	(*n* = 6)	(*n* = 7)
γGT	20 0 ±2 3 ***	4 05 ±0 68
	(*n* = 7)	(*n* = 8)
AKP	0 61 ± 0 05 ***	0 97 ±0 04
	(*n* = 3)	(*n* = 7)
HK	0 66 ± 0 10 **	0 277 ± 0 009
	(*n* = 12)	(*n* = 8)
Na/K-ATPase	1 78 ± 0 21 *	3 54 ±0 56
	(*n* = 6)	(*n* = 12)

* $P < 0\,02$, ** $P < 0\,01$, *** $P < 0\,001$ represent the statistical significances of the differences of enzymatic activities between freshly isolated and cultured cells

ties of AKP and Na/K-ATPase were higher in primary cultures

Effect of peptide hormones on cAMP production

After 15 min exposure to PTH, cAMP significantly increased in the proximal cell monolayers (Table II) However, AVP did not modify the cAMP production

TABLE II

Adenylate cyclase activity in cultured proximal cells

Cyclic AMP production in fmol/15 min per μg DNA measured on 8-day-old cultures Arginine vasopressin (AVP) and parathyroid hormone (PTH) concentrations were 10^{-8} M and 0 1 μg/ml, respectively Values are means ± S E of *n* experiments

Control	AVP	PTH
473 ± 82	644 ± 139	6661 ± 689 *
(*n* = 11)	(*n* = 7)	(*n* = 11)

* $P < 10^{-6}$ statistical significance between stimulated and basal production of cAMP.

Immunofluorescence localization of leucine aminopeptidase

Leucine aminopeptidase (LAP) is a hydrolase expressed on the apical membrane of the proximal tubule To assess the polarity of the monolayer, proximal cultures grown on transparent teflon supports were labelled with a monoclonal antibody (Mab 5A4), raised against LAP [19] Cultures were then stained with FITC-conjugated anti-mouse IgG Fig 3 shows the apical face of the cultured monolayer on which membrane fluorescence was clearly visible with the antibody The specific apical labelling was confirmed by incubating the Mab 5A4 on the basal side of the culture, in which case no labelling was observed (data not given).

Transepithelial voltages and resistances

The 8-day-old proximal cultures exhibited a small apical negative transepithelial voltage ($V_{TE} = 0\,48 \pm 0\,05$

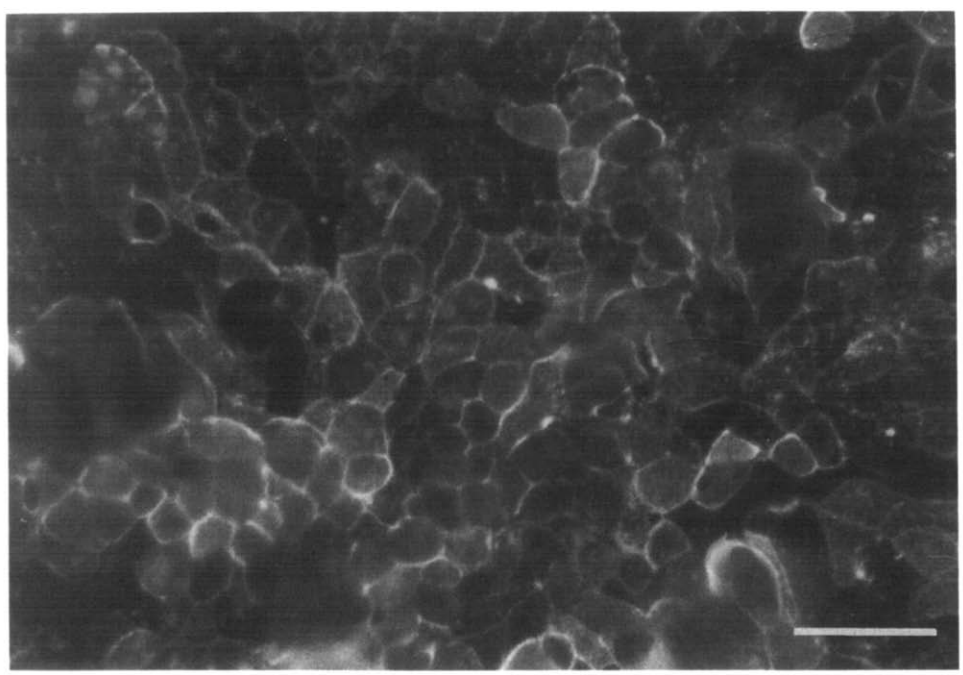

Fig 3 Fluorescence pattern obtained in 8-day-old cultures with a monoclonal antibody raised against leucine aminopeptidase Bar is 50 μm

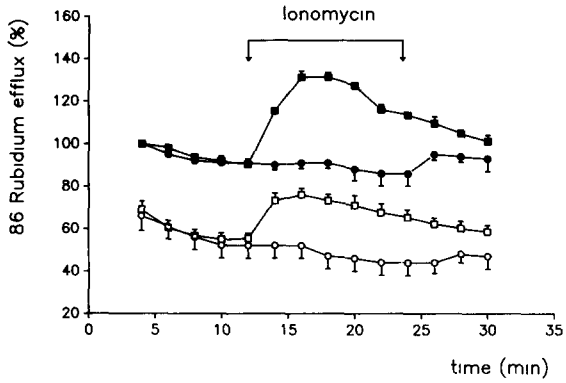

Fig 4 Effect of ionomycin on $^{86}Rb^+$ effluxes Apical (○) and basolateral (●) control effluxes ($n = 3$) measured in a RPMI medium containing 1 mM $caCl_2$ During the period between the two arrows, apical (□) and basolateral (■) effluxes were measured in a medium containing 3 μM ionomycin ($n = 5$) The data are expressed in percent of the basolateral efflux rate constant at the beginning of measurements ($t = 4$ min) The points are the mean values ± S E of n experiments

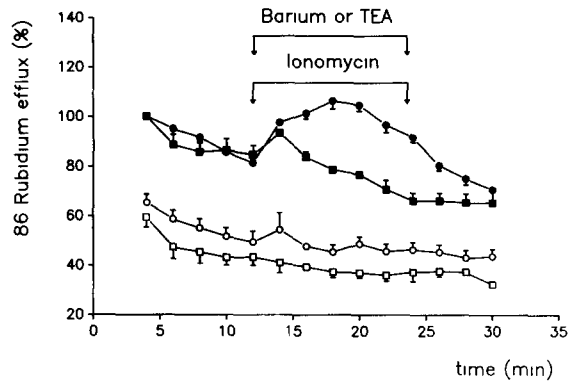

Fig 5 Effects of $BaCl_2$ and TEA on the ionomycin-stimulated $^{86}Rb^+$ efflux After an initial period in a control medium, apical (□, ○) and basolateral (■, ●) effluxes were measured in medium containing either 3 μM ionomycin and 5 mM $BaCl_2$ (□, ■, $n = 3$) or ionomycin and 20 mM TEA (○, ●, $n = 3$) Experimental solutions were applied during the period between the two arrows The points are the mean values ± S E of n experiments

mV, $n = 8$) and a low transepithelial resistance (R_T = 36 7 ± 3 2 Ω cm^2, $n = 23$)

Characterization of K^+ permeability in cultured cells

Effect of Ca^{2+} ionophore

Isolated cultured cells grown on permeable Millipore supports allowed for the separate measurements of K^+ effluxes across the apical and the basolateral membranes. Fig 4 presents the $^{86}Rb^+$ efflux rate constant (in percent of the initial basolateral value at time $t = 4$ min) as a function of time in the absence and presence of 3 μM ionomycin In control conditions, the efflux of $^{86}Rb^+$ from the monolayer into the apical and basolateral bathing solutions was independent of time The basolateral efflux rate constant ((1 99 ± 0 02) 10^{-2} min^{-1}, mean ± S E, $n = 270$) exceeded by a factor of 1 93 the apical rate constant ((1 03 ± 0 01) 10^{-2} min^{-1}, $n = 313$) Addition of 3 μM of the calcium ionophore to the control solution containing 1 mM $CaCl_2$ caused a simultaneous increase in $^{86}Rb^+$ efflux of 38 8 ± 1 4% ($n = 5$) and 45 7 ± 5 7% ($n = 5$) from apical and basolateral borders, respectively After rinsing away the ionophore, both apical and basolateral effluxes returned to their control levels

Pharmacological properties of the ionomycin-stimulated efflux

To characterize this Ca^{2+}-sensitive $^{86}Rb^+$ efflux further, we examined the effects of known blockers of K^+ conductance Fig 5 shows that when 5 mM barium was added to a medium containing 3 μM ionomycin, no efflux stimulation was observed TEA partly inhibited the ionomycin-stimulated efflux Identical experiments were made to determine the effect of crude scorpion venom and apamin The data are shown in Fig 6 the ionomycin-stimulated efflux was reduced by 75% in the presence of 20 μg/ml scorpion venom and by 50% in the presence of 10^{-7} M apamin

Effect of pH

To examine the pH dependency of the K^+ conductance, the intracytoplasmic pH of the cells was varied by addition of either NH_4Cl or $NaCH_3COO$ to the bathing control medium (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0 4 mM $MgSO_4$, 2 mM glutamine, 5 mM alanine, 5 mM glucose and 20 mM Hepes (pH 7 4)) The initial pH in the control medium was 7 25 ± 0 02 ($n = 23$) The replacement of 140 mM NaCl with 100 mM NaCl and 40 mM NH_4Cl caused the pH_i to increase by 0 55 ± 0 05 ($n = 7$), whereas the replace-

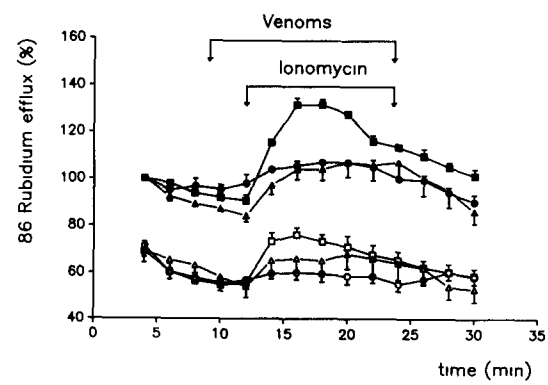

Fig 6 Effects of apamin and crude scorpion venom on the ionomycin-stimulated $^{86}Rb^+$ efflux After an initial control period, apical (□, ○, △) and basolateral (■, ●, ▲) effluxes were measured in a medium containing either 3 μM ionomycin (□, ■, $n = 5$) or ionomycin with 20 μg/ml scorpion venom (○, ●, $n = 6$) or ionomycin with 10^{-7} M apamin (△, ▲, $n = 6$) Scorpion venom or apamin was added 4 min before ionomycin At $t = 24$ min, the control medium was applied again The points are the mean values ± S E of n experiments

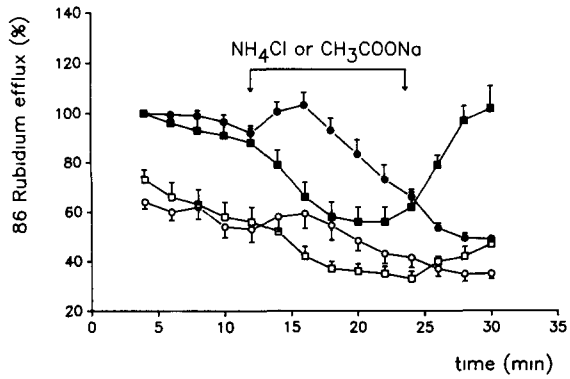

Fig 7 Effect of pH_i on the $^{86}Rb^+$ efflux rate constant After an initial period in the control medium (140 mM NaCl, 5 mM KCl, 1 mM $CACl_2$, 0 4 mM $MgSO_4$, 2 mM glutamine, 5 mM alanine, 5 mM glucose, 20 mM Hepes (pH 7 4)), apical (○, □) and basolateral (●, ■) $^{86}Rb^+$ effluxes were measured in a medium in which 140 mM NaCl was replaced by 100 mM NaCl and 40 mM NH_4Cl (○, ●, $n = 6$) or by 100 mM NaCl and 40 mM CH_3COONa (□, ■, $n = 5$) Experimental solutions were applied during the period between the two arrows The points are the mean values ± S E of n experiments

ment with 40 mM $NaCH_3COO$ induced a decrease of 1 00 ± 0 16 ($n = 7$) The pH dependence of the $^{86}Rb^+$ effluxes is demonstrated in Fig 7 Increasing the pH_i induced a basolateral increase of 12 8 ± 2.4% and an apical increase of 16 0 ± 2 0% ($n = 6$) of the $^{86}Rb^+$ efflux On the other hand, decreasing the pH_i decreased the basolateral $^{86}Rb^+$ efflux by 39 0 ± 7 5% and the apical efflux by 40 2 ± 9 7% ($n = 5$)

Effect of hypotonic media

We examined the $^{86}Rb^+$ efflux variations when cultured monolayers were exposed to a hypotonic medium Initial effluxes were measured in the isotonic control medium The hypotonic medium was obtained by diluting the control solution The osmolality of the isotonic and hypotonic solutions were 289 ± 2 ($n = 7$) and 196

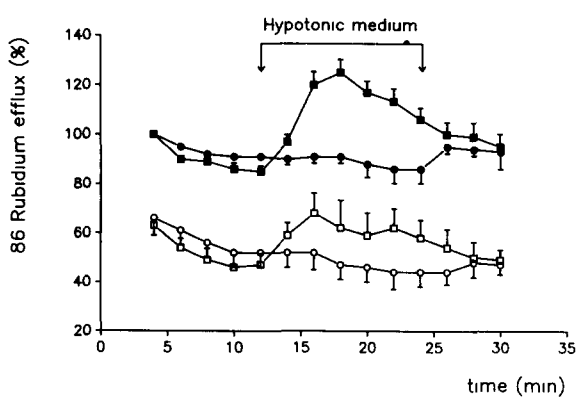

Fig 8 Effect of a hypotonic medium on $^{86}Rb^+$ effluxes Apical (○, □) and basolateral (●, ■) $^{86}Rb^+$ effluxes were measured in a control medium (○, ●, $n = 3$) and when the monolayers were exposed to a hypotonic medium (□, ■, $n = 9$) Hypotonic medium was applied during the period between the two arrows The points are the mean values ± S E of n experiments

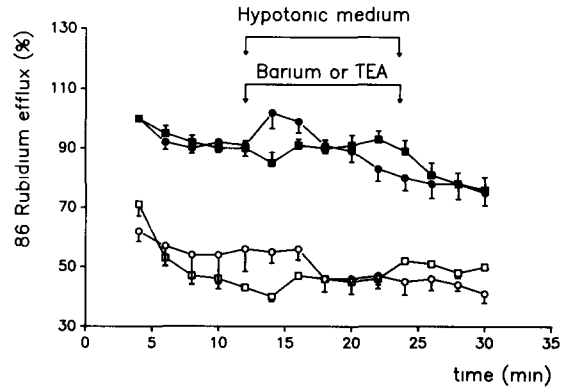

Fig 9 Effects of $BaCl_2$ and TEA on the $^{86}Rb^+$ efflux stimulated by hypotonicity After an initial period in the control medium, apical (□, ○) and basolateral (■, ●) effluxes were measured in a hypotonic medium containing either 5 mM $BaCl_2$ (□, ■, $n = 5$) or 20 mM TEA (○, ●, $n = 6$) The points are the mean values ± S E of n experiments

± 1 ($n = 7$) mosmol/kg, respectively, measured with an osmometer (Roebling, F R G) Fig 8 shows that exposure of the monolayers to a hypotonic medium led to an increased $^{86}Rb^+$ loss through both basolateral and apical membranes This efflux stimulation reached a maximum level after 6 min exposure the mean increase of the $^{86}Rb^+$ efflux across the basolateral and apical sides was 50 0 ± 5 7% and 61 5 ± 10 0% ($n = 8$), respectively

Pharmacological properties of hypotonically-stimulated efflux

To characterize the stimulated $^{86}Rb^+$ efflux in hypotonic conditions, we studied the effects of K^+ channel inhibitors The action of 5 mM $BaCl_2$ and 20 mM TEA is shown in Fig 9 Both channel blockers almost com-

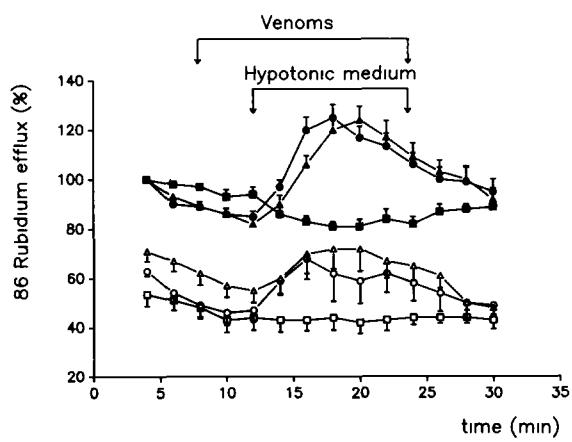

Fig 10 Effects of apamin and crude scorpion venom on the $^{86}Rb^+$ efflux stimulated by hypotonicity After an initial control period, apical (○, □, △) and basolateral (●, ■, ▲) effluxes were measured in a hypotonic medium (○, ●, $n = 9$) containing either 20 μg/ml scorpion venom (□, ■, $n = 4$) or 10^{-7} M apamin (△, ▲, $n = 5$) Scorpion venom and apamin were added 4 min before the hypotonic medium At $t = 24$ min, cells were again exposed to the isotonic control medium The points are the mean values ± S E of n experiments

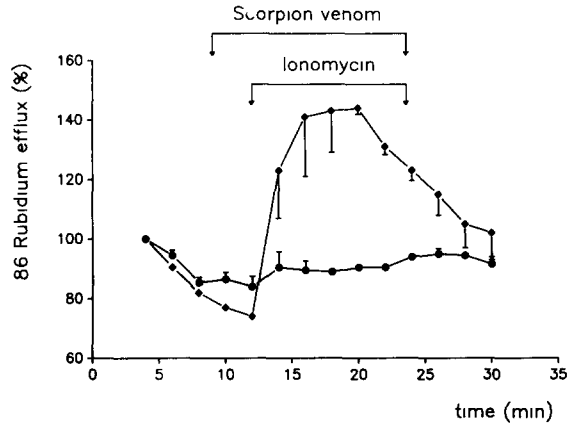

Fig 11 Effects of ionomycin and scorpion venom on apical effluxes after blocking the basolateral sides of the cells with water-saturated oil After an initial period in a control medium, apical effluxes were measured after the addition of ionomycin (3 μM) (◆, $n = 3$) or ionomycin plus venom scorpion (●, $n = 3$), the venom being added 4 min before the ionomycin At $t = 24$ min, the control medium was applied again Apical effluxes are expressed in percent of the initial value of the apical efflux rate constant The points are the mean values ± S E of n experiments

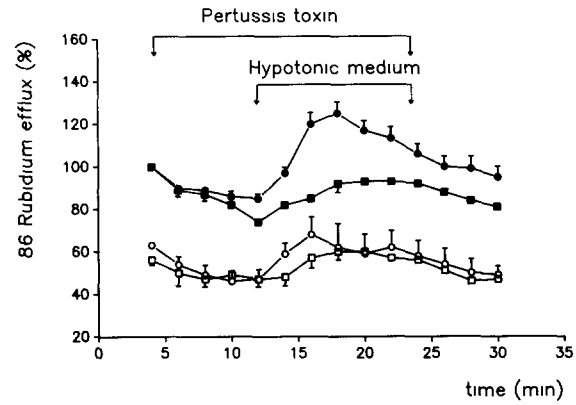

Fig 13 Effect of pertussis toxin on the effluxes stimulated by hypotonicity Apical (○, □) and basolateral (●, ■) $^{86}Rb^+$ effluxes were measured after a hypotonic shock in the presence (□, ■, $n = 3$) or in the absence (○, ●, $n = 9$) of 500 ng/ml pertussis toxin The toxin was added during the $^{86}Rb^+$ load period and during the efflux measurements The hypotonic medium was used during the period defined by the two arrows The points are the mean values ± S E of n experiments

pletely abolished the action of the hypotonic medium on the basolateral and apical $^{86}Rb^+$ effluxes Fig 10 shows the effect of scorpion venom and apamin The $^{86}Rb^+$ efflux stimulation elicited by the hypotonic medium was completely inhibited by 20 μg/ml scorpion venom on both sides of the culture On the other hand, the addition of 5 10^{-7} M apamin remained without significant effect on the stimulated effluxes

Apical $^{86}Rb^+$ effluxes

Similar efflux measurements were made after blocking the basolateral side of the culture with water-saturated oil In these conditions only the apical efflux rate constant was measured Fig. 11 presents the apical $^{86}Rb^+$ efflux (in percent of the initial rate constant) and shows that addition of 3 μM ionomycin to the control medium caused the apical efflux to increase rapidly, the increase spontaneously declining after 8 min When 20 μg/ml scorpion venom was added 4 min before the ionomycin, there was no stimulation. In the same way, as shown in Fig 12, apical effluxes were enhanced when the cells were exposed to a hypotonic medium, and stimulation of the efflux was blocked in the presence of 20 μg/ml scorpion venom

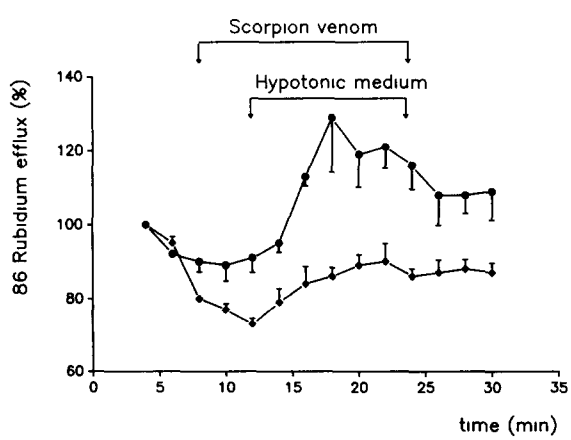

Fig 12 Effects of hypotonicity and venom scorpion on apical effluxes after blocking the basolateral side After an initial period in a control medium, apical effluxes were measured in a hypotonic medium (●, $n = 3$) or in a hypotonic medium with scorpion venom (◆, $n = 3$), the venom being added 4 min before the hypotonic medium At $t = 24$ min, the cells were again exposed to the isotonic control medium The points are the mean values ± S E of n experiments

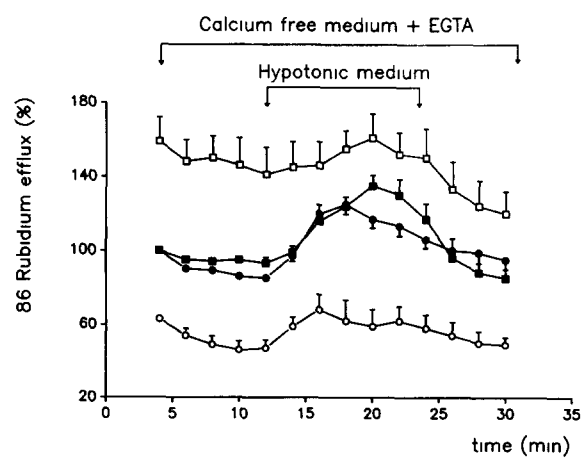

Fig 14 Effect of a Ca^{2+}-free medium on the effluxes stimulated by hypotonicity Apical (○, □) and basolateral (●, ■) $^{86}Rb^+$ effluxes were measured during a control period in an isotonic medium followed by a hypotonic shock defined by the two arrows Measurements were realized in a RPMI medium containing 1 M $CaCl_2$ (○, ●, $n = 9$) and in a Ca^{2+}-free medium containing 10 mM EGTA (□, ■, $n = 6$) The points are the mean values ± S E of n experiments

Effect of pertussis toxin and Ca^{2+}-free medium on the effluxes stimulated by hypotonicity

To elucidate the mechanism by which hypotonicity activates $^{86}Rb^+$ efflux, we tested the effect of pertussis toxin Fig 13 shows that preincubation of the monolayers with 500 ng/ml pertussis toxin altered the basolateral response to hypotonicity by more than 70% and the apical response by more than 55% These results indicate that the action of hypotonicity is mediated by a mechanism involving G-protein stimulation Fig 14 shows the $^{86}Rb^+$ efflux when monolayers were exposed to a Ca^{2+}-free hypotonic medium The effluxes were first measured in an isotonic Ca^{2+}-free medium containing 10 mM EGTA Encouraging a paracellular pathway, the absence of calcium led to a dramatic increase of the apical efflux, without modifying the basolateral efflux When the Ca^{2+}-free hypotonic medium was applied, both apical and basolateral effluxes increased The variation induced by the hypotonic medium was not significantly different in the presence and in the absence of calcium

Discussion

In order to study $^{86}Rb^+$ movements in the rabbit proximal tubules, we chose primary cultures of freshly isolated proximal cells This choice of material has the advantage of giving a high yield of cells, and thus makes it possible to obtain sufficiently large surfaces of epithelial cell layers for effective measuring of effluxes The method used for cell preparation, which avoids any chelating or proteolytic treatment has already been described The enzymatic and morphological characteristics of these cells clearly indicate that they are mainly proximal cells [11] The present work shows that isolated cells in primary culture preserved their proximal characteristics and recovered their membrane polarity when growing on collagen-coated permeable supports Electron microscopic study revealed the presence on the apical membrane of microvilli characteristic of the original epithelium [21]. The characterization of several enzymatic activities indicated that the primary culture maintained high levels of LAP, γ-GT, AKP and ouabain-sensitive Na/K-ATPase The activities of the two brush border enzymes LAP and γ-GT were, however, lower than in freshly isolated proximal cells Similarly reduced activities have also been recorded by Chung et al [22] and Merot et al [23] and may well be due to reduced development of the brush border system during growth On the other hand, the increased activity of the basolateral enzyme Na/K-ATPase may reflect recovery of the membrane polarity lost during cell isolation [11] After 8 days, the cultures presented a low hexokinase activity, even lower than in isolated cells This is consistent with the fact that glycolytic pathways do not occur in the proximal tubule A further typical feature of the proximal tubule is its marked response to PTH, whereas AVP has no significant effect Such results had already been observed by Poujeol et al [11] in isolated proximal cells and our data confirm the presence of this effect in the cultured cells In our study, the order of magnitude for the cAMP stimulation by PTH was similar to that previously described for cultured proximal tubule cells from man [24] and rabbit [25,26] Monoclonal antibodies, the localization and identification of which have been precisely defined on the original tubules, represent the ideal markers for identification. In our experiments, the Mab 5A4 demonstrated to the specific for LAP was completely reactive only with the apical membrane of cultured cells [19] The labelling obtained with the Mab indicated that the genetic expression of the cells is not greatly disturbed by the culture process and that no appreciable dedifferentiation occurred, at least as far as hydrolase functions are concerned The cultured monolayers reached the edge of the 4 52 cm^2 well after 8 days, at which time measurements of the transepithelial voltage and resistance were possible The values that we obtained are compatible with the expected values for a leaky epithelium In proximal cultures obtained from microdissected tubules [23], similar values were obtained, whereas Bello-Reuss and Weber [25] found a lower transepithelial resistance Thus, the data presented here demonstrate that isolated cells from rabbit proximal tubule reestablish a structurally polarized epithelial cell layer in primary culture

In the proximal tubule [26], as well as in MDCK cells [27], $^{86}Rb^+$ was believed to be a good indicator for K^+ movements In luminal-membrane vesicles, Jacobsen et al [26] have shown a K^+ channel which is somewhat selective for potassium comparing to rubidium Hence, $^{86}Rb^+$ would seem to be an appropriate substitute for K^+ The present work shows that stimulated $^{86}Rb^+$ effluxes were inhibited by known K^+ channel blockers, indicating that the effluxes measured under these conditions were therefore due to the transport of Rb^+ through K^+ channels

The present study shows that ionomycin increased the $^{86}Rb^+$ efflux through both apical and basolateral membranes of proximal cells in culture. This effect could be assimilable to a macroscopic potassium permeability which appears to be calcium sensitive The pharmacology of the ionomycin-stimulated efflux was investigated by adding known blockers of potassium conductance in epithelial cells Of these, Ba^{2+} was very effective Its action, reported in a wide variety of tissues, is not very specific to a given type of K^+ channel Crude scorpion venom and apamin only partly inhibited the ionomycin-stimulated efflux. Charybdotoxin (CTX), contained in scorpion venom, strongly inhibits high conductance Ca^{2+}-activated K^+ channels (BK) [28,29], while apamin, extracted from bee venom, is a selective blocker of small conductance Ca^{2+}-activated

K^+ (SK) channels [30], but does not act on maxi channels [29] Thus our results suggest the presence of at least two types of K^+ channels which could contribute to the macroscopic conductance of the cultured proximal cells One interesting observation was the pH sensitivity of the $^{86}Rb^+$ efflux Along with the Ca^{2+} sensitivity, pH sensitivity is a typical feature of the maxi K^+ channel irrespective of the tissue in which it has been recorded [31] Using a patch clamp technique, Merot et al [10] have recently characterized two types of K^+ channels in the apical membrane of the proximal convoluted tubule (PCT) in primary culture The variation of K^+ permeability that we observed after ionomycin application is possibly due to the activation of the same kind of channel, although we made no direct conductance measurements to confirm this Under these conditions, the basolateral membrane could also contain a high conductance K^+ channel similar to the 'BK' Ca^{2+}-sensitive channel However, the only K^+ channels that have been demonstrated in the basolateral membrane of the proximal tubule were of smaller conductance and Ca^{2+} dependency or CTX sensitivity were not reported [32,33]

Our results clearly demonstrate that a hypotonic shock gives an increase in apical and basolateral $^{86}Rb^+$ effluxes within a few minutes The efflux measurements after blocking the basolateral border suggest that the increase of the apical efflux is not due to a leak of K^+ via paracellular shunt pathways from the basal to the apical solution Moreover, similar results were obtained when cell monolayers were grown upon plastic petri dishes (data not given) The involvement of the K^+ conductance variation after a hypotonic shock has already been reported [1] However, in the proximal tubule, only the basolateral membrane has as yet been investigated [34] Experiments on the apical membrane of cultured renal cell lines have shown an involvement of Ca^{2+}-activated K^+ channels in hypotonic shock [35] Our data clearly demonstrate that only the TEA and CTX-sensitive K^+ channel could be responsible for the increased K^+ conductance Thus the response to the hypotonic shock could be selective and would not involve all populations of K^+ channels present in cultured proximal cells A survey of the literature revealed that the activation of the K^+ channels seemed to be mediated by a rise in $[Ca^{2+}]_i$, but the detailed mechanism is not yet known and several hypotheses are possible In the choroid plexus epithelium, Christensen suggested that stretch-activated Ca^{2+} channels are the transducing element for volume regulation through Ca^{2+}-activated K^+ channels [36] Our results showed that pretreatment of cultured cells with pertussis toxin for 4 h abolished the rise in $^{86}Rb^+$ efflux, suggesting an involvement of G-protein The same effect was reported by Suzuki et al [37] in cultured single proximal tubule cells from rabbit kidney Furthermore, although a Ca^{2+}-free medium leads to a distortion of the cell, increasing the paracellular pathway, and thus equilibrating the apical and basolateral effluxes, an increased $^{86}Rb^+$ efflux was observed after a hypotonic shock in a Ca^{2+}-free medium This result suggests that external Ca^{2+} is not essential for an increased K^+ channel activity In the same way, Ubl et al [35] demonstrated that exposure of Opossum kidney cells to hypotonic shock evokes an elevation of cytoplasmic Ca^{2+}, released from internal stores

In conclusion, the present study shows that isolated proximal cells in primary culture present at least two distinct K^+ conductances One of them, CTX-sensitive, shares many characteristics with the maxi K^+ channel, and seems to be involved in regulation phenomena after hypotonic shock Further experiments are necessary however to identify the accompanying anion At present, published studies [38,39] give conflicting results, both chlorine and bicarbonate being suggested as possible candidates

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