Biochimica et Biophysica Acta, 1070 (1991) 387–400

BBAMEM 75438

Apical membrane ionic channels in the rabbit cortical thick ascending limb in primary culture

Jean Merot, Valérie Poncet, Michel Bidet, Michel Tauc and Philippe Poujeol

Département de Biologie Cellulaire et Moléculaire, Service de Biologie Cellulaire, Centre d'Etudes de Saclay, Gif sur Yvette (France)

(Received 22 February 1991)
(Revised manuscript received 15 July 1991)

Key words: Potassium ion conductance; Patch clamp; (Rabbit)

Cortical thick ascending limbs of Henle's loop (cTAL) were microdissected from rabbit kidneys and cultured in a hormonally-defined medium. The cultured cells grew as a monolayer and retained the morphological and biochemical characteristics of the original tubule. Cyclic AMP production of the cultured cells was increased by human calcitonin (×13) and parathyroid hormone (×2). The cultured epithelial developed a transepithelial potential of 4.1 ± 1.3 mV that was orientated positively towards the apical compartment. The basolateral membrane of the cells exhibited a chloride conductance sensitive to diphenylamine 2-carboxylate (DPC) and the apical membrane a barium-sensitive K^+ permeability. Patch clamp analysis conducted on the apical membrane of the cells revealed the presence of three types of ionic channel. The first is a large conductance Ca^{2+}-activated K^+ channel (95 pS). The second K^+ channel has a much smaller conductance (18.3 pS) and is insensitive to Ca^{2+}. It may represent the conductive pathway for K^+ recycling into the lumen in the original tubule. The last channel is cation selective, does not discriminate between Na^+ and K^+ and was found to have a conductance of 20.5 pS. Channel activity required a high cytoplasmic calcium concentration (1 mM), and was blocked by ATP (10 μM) applied on its cytoplasmic face.

Introduction

The mechanism of NaCl reabsorption along the cortical thick ascending limb (cTAL) has been extensively studied using the in vitro microperfusion technique. In this field, Greger carried out very important work on the cortical portion of the rabbit TAL and demonstrated that the mode of transport is a $Na^+/K^+/2\ Cl^-$ cotransporter in the apical membrane [1]. He also concluded that, the apical membranes are essentially K^+ conductive and the basolateral membranes are Cl^- conductive [2,3]. The nature of this Cl^- conductivity has now been analysed by patch clamp experiments on intact microperfused cTAL (4). Small conductance Cl^- channels were observed which can explain the passive Cl^- exit step suggested by classical electrophysiological data [2]. Culturing functional epithelial cells is useful for investigating apical channels by the patch clamp technique. Detailed studies have been made with this method in cultured rabbit mTAL and large-conductance K^+ channels have been described [5,6]. In the present paper we established primary cultures of microdissected rabbit cTAL segments. Once the main morphological and biochemical characteristics of these cultures had been recorded, we analysed some of the electrical properties of the cultured monolayers. The data indicate the presence of a chloride conductance in the basolateral membrane, inhibited by DPC. Furthermore we found a large conductance K^+ channel in the apical membrane sensitive to Ca^{2+} which shares many of the properties of the maxi K^+ channel described in other tissues [7] including the renal epithelium [8,9]. A second channel of smaller conductance was also characterized. These two channels could account for the K^+ conductance of the membrane in vivo. In addition, we report results concerning a cation selective channel which did not discriminate between Na^+ and K^+ and was sensitive to high Ca^{2+} concentrations and blocked by ATP.

Correspondence: P. Poujeol, DBCM/SBCe, Centre d'Etudes Nucléaires Saclay, 91191 Gif sur Yvette Cedex, France.

Materials and Methods

Primary cultures

The primary cell culture technique has been described in detail in previous papers [10,11]. The cortical

fragments of the thick ascending limb were carefully microdissected under sterile conditions from 4–5 weeks-old New Zealand rabbit kidneys. The kidneys were cut into small pyramids which were incubated in a dissection medium containing 0.1 mg/ml collagenase (Cooper, U.S.A.). The tubules were seeded in collagen-coated culture wells filled with primary culture medium composed of equal mixtures of Dulbecco's modified Eagle medium (DMEM) and Ham F12 (Gibco) containing 15 mM $NaHCO_3$, 20 mM Hepes (pH 7.5), 2 mM glutamine, 5 μg/ml insulin, $5 \cdot 10^{-8}$ M dexamethasone, 10 ng/ml EGF, 5 μg/ml transferrin, $3 \cdot 10^{-8}$ M sodium selenite, 10^{-8} M triiodothyronine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained at 37 °C in 5% CO_2/95% air water-saturated atmosphere. The medium was changed 4 days after seeding and then every 2 days. The antibiotics were definitively removed from the medium after the first rinsing. The culture chamber was custom made. A polycarbonate filter formed the bottom of the cylinder and was coated with rat tail collagen. The surface available for tubular growth was 0.2 cm^2.

Enzyme studies and cAMP production in primary cultures

To verify the cultured cTAL cells retained the enzymatic characteristics of the original segment, enzyme activities were measured and compared with those of cultured proximal tubules. The enzyme assays were made at 37 °C after 15–20 days seeding on Petri dishes. Alkaline phosphatase (AKP) was assayed with *p*-nitrophenyl phosphate as substrate at pH 8.6. Leucine aminopeptidase (LAP) was determined by using leucine para nitroanilide as substrate. γ-Glutamyltransferase (γGT) was measured using γ-glutamyl *p*-nitroanilide as substrate (Sigma list kit). For these three enzyme assays, the reactions were developed directly in the culture wells without pretreatment of the cells.

Hexokinase (HK) was determined as previously reported [11]. For studying this metabolic enzyme, the cells were detached from the plates by trypsinization and permeabilized by the freeze-thawing procedure. The results were normalized for the DNA content of each sample.

Intracellular cyclic adenosine monophosphate (cAMP) produced by 15–20-day-old cells grown on a permeable support was measured by radioimmunoassay. The radioimmunoassay is based on the competitive reaction between cAMP succinyl tyrosine methyl ester labelled with iodine 125 and the sample with a specific cAMP antibody (Institut Pasteur Production, France). The epithelia were incubated at 37 °C with 0.1 μg/ml bovine parathyroid hormone (PTH 1–34) (Sigma), or 30 mU/ml human calcitonin (HCT) (Ciba Geigy) for 15 min in the presence of 1 mM 3-isobutyl-1-methylxanthine (IBMX) and the cAMP extracted in formic acid and 5% ethanol. The hormones were added to both apical and basolateral sides of the culture well.

DNA determination

DNA was measured by a modification of the fluorimetric micromethod of Switzer and Summer [12].

Electrophysiological study

The transepithelial potential (V_t) was measured on primary cultures grown on permeable chambers by recording the electrical potential difference between two calomel electrodes connected to the bathing solutions by KCl-saturated Agar bridges (Keitley 601 electrometer). The basal side was grounded. The transepithelial resistance (R_t) was estimated by applying square-current pulses (1.5 μA, 2 s duration) through Ag-AgCl electrodes and measuring V_t changes with electrodes placed as near as possible to the epithelium surface. V_t was measured with RPMI medium (Gibco) bathing apical and basolateral compartments. R_t measurements were corrected for series resistances measured after removing the cells from the support at the end of the experiment. All the experiments were performed at room temperature.

Patch clamp experiments

The single-channel current recordings were made on the apical membrane of 14–15-day-old cultured cells grown on a permeable support according to the method described by Hammil et al. [13]. Patch pipettes were made in two steps from hematocrit capillaries using a vertical puller (Kopf, Tujunga, CA, U.S.A.), coated with Sylgard 184 (Dow Corning) and fire polished immediately before using. Pipettes filled with a Na rich solution had resistances of 5- 10 MΩ. Seals between 10 to 50 gigaΩ were achieved by applying a slight suction when the pipette was lowered onto the cell membrane.

Channel currents were recorded with a RK 300 patch clamp amplifier (Biologic, France), stored on digital audio tapes using a DTR 1200 recorder (Biologic, France) and visualized on a digital oscilloscope (Nicolet Instruments, Madison, WI, U.S.A.). For consistency throughout the paper the potential is defined as the potential on the cytoplasmic face of the membrane relative to the pipette. In cell-attached experiments, the intracellular potential that contributes to the actual membrane potential was assumed to be −60 mV. On the recordings given in the figures, upward deflections represent currents of positive charge moving from the cell interior to the pipette.

In some experiments, single channel currents were recorded on 'rightside-out' excised patches. This configuration was obtained after the seal formation by disrupting the patch membrane by additional suction. The pipette was then pulled away from the cell to obtain an excised patch with the cytoplasmic side fac-

ing towards the interior of the pipette and the external side facing the bath. All experiments were performed at room temperature.

Data analysis

Channel current amplitudes were measured by replaying the tape onto a digital oscilloscope (Nicolet Instruments, Madison, WI, U.S.A.). The current-voltage (I/V) relations of the channels were constructed from the average amplitude of 10 to 20 well defined transitions between closed and open current levels at each applied potential. The Goldman-Hodgkin-Katz (GHK) equation or a straight line was fitted to the data by the least square method. The best fit obtained is shown as a solid line in the I/V plots.

The K^+ to Na^+ permeability ratio (P_K/P_{Na}) was calculated from current reversal potentials (E_{rev}) in excised patches according to the GHK equation:

$$P_K/P_{Na} = \frac{[Na]_{pip} - [Na]_{bath} \cdot \exp[-E_{rev}/(RT/F)]}{[K]_{bath} \cdot \exp[-E_{rev}/(RT/F)] - [K]_{pip}}$$

where R, T, F have their usual meanings. pip and bath refer to the ionic concentration in the pipette and the bath, respectively.

To estimate the channel-open probability, the current recordings were filtered at 300 Hz with an 8 pole Bessel filter (902 LPF, Frequency Device, U.S.A.) and transferred at 1 kHz sampling frequency to a Hewlett Packard computer. Current amplitude histograms were constructed and analysed with Bio-Patch software (Biologic, France). The ratio of the areas of the Gaussian like distributed current amplitude histograms corresponding to the close and open state of the channel was taken as an estimate of the channel-open probability and denoted P_o.

Drugs and solutions

Diphenylamine 2-carboxylate (DPC) was purchased from Aldrich Chimie (F.R.G.). To dissolve it in aqueous solution, it was first dissolved in DMSO (Sigma) and then the DPC-DMSO mixture was slowly added to a rapidly stirred RPMI medium (0.1% final (DMSO)).

In patch clamp experiments the Na-rich solution contained (in mM): 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 5 glucose, 10 Hepes (pH 7.4, adjusted with NaOH). The K-rich solution contained (in mM): 140 KCl, 5 NaCl, 1 $MgCl_2$, 10 Hepes (pH 7.2, adjusted with KOH). In K-rich solutions, the calcium concentration (10^{-5} or 10^{-3} M) was adjusted by addition of $CaCl_2$. The K-rich solutions with free-calcium concentrations of 10^{-7} and 10^{-9} M contained 1 mM $CaCl_2$, 2.7 mM EGTA and 0 mM $CaCl_2$, 5 mM EGTA, respectively [14]. In Cl^--free solution 140 mM KCl was replaced by 140 mM Na-gluconate.

Calculations

The values are presented as means ± S.E. Student's t-test was employed to determine statistical significance of differences.

Results

Morphology of the primary cultures

Collagenase treatment of rabbit kidney cortex enables well-defined segments to be isolated. After dissection the cTAL segment quickly sticks to the collagen

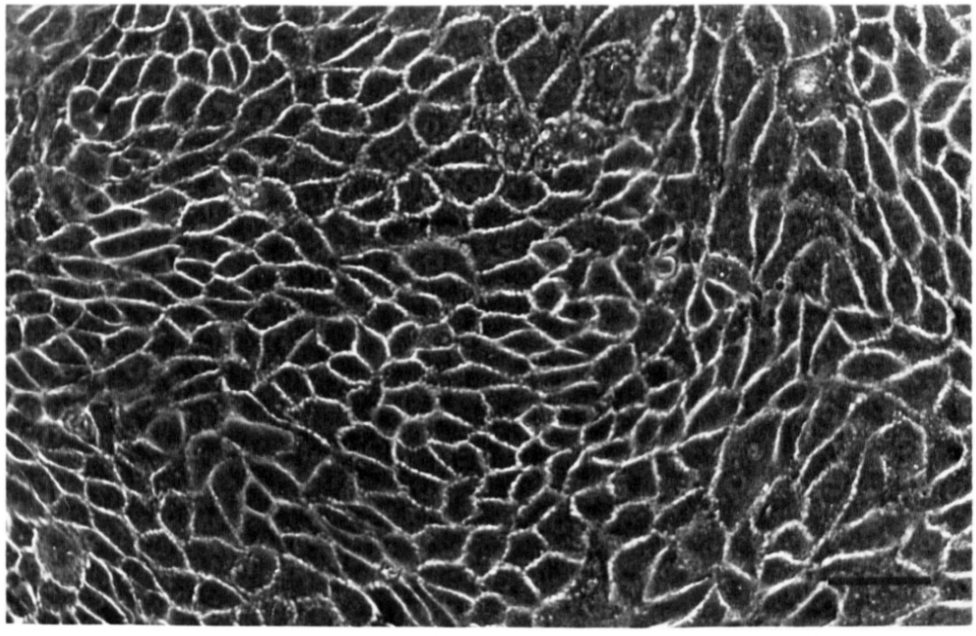


Fig. 1. Migrograph of a 10-day-old culture of cortical thick ascending limb. Bar is 40 μm.

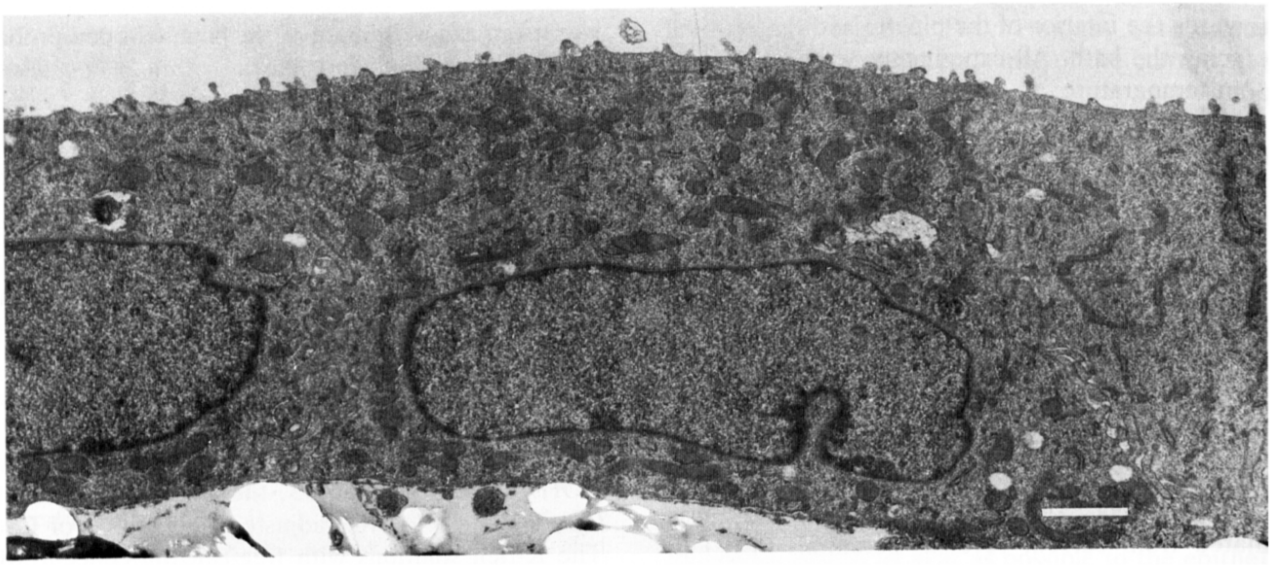


Fig. 2. Electron micrograph of a 20-day-old culture of cTAL growing on a permeable support. Bar is 1.2 μm.

bed and growth occurs after a lag period of 2 to 3 days. Fig. 1 shows that the cultured epithelium is composed of contiguous cells similar in size and shape. At the ultrastructural level (Fig. 2), the cells show a polarized morphology with the basement membrane close to the permeable support and the apical membrane fringed with few short microvilli.

Enzyme activities of primary cultures

Enzyme activities of leucine aminopeptidase (LAP), γ-glutamyltransferase (γGT), alkaline phosphatase (AKP) and hexokinase (HK) were measured in 15-20-day-old cultures. The results were compared with those obtained on primary cultures of proximal tubules. As shown on Fig. 3A the levels of the three hydrolases are lower in the cTAL cultures than in PCT cultures, whereas the glycolytic enzyme hexokinase is higher in the cTAL cultures.

cAMP production of cultured cells

The results are summarized in Fig. 3B and show that the basal cAMP production of the cultured cells was stimulated 15-fold and 2-fold by exposure to human calcitonin (HCT) and parathyroid hormone (PTH), respectively.

Electrophysiological study

The cultures growing on permeable chambers reached the edges of the culture wells 9 to 12 days after seeding. They had a transepithelial potential that was oriented positively towards the apical surface of the monolayer (V_t: $+4.1 \pm 1.3$ mV ($n = 6$)) and a transepithelial resistance of 303 ± 58 Ω cm^2 ($n = 6$). The apical and basolateral ionic membrane conductances of the cells were assessed by examining the effects of different ionic channel blockers on V_t.

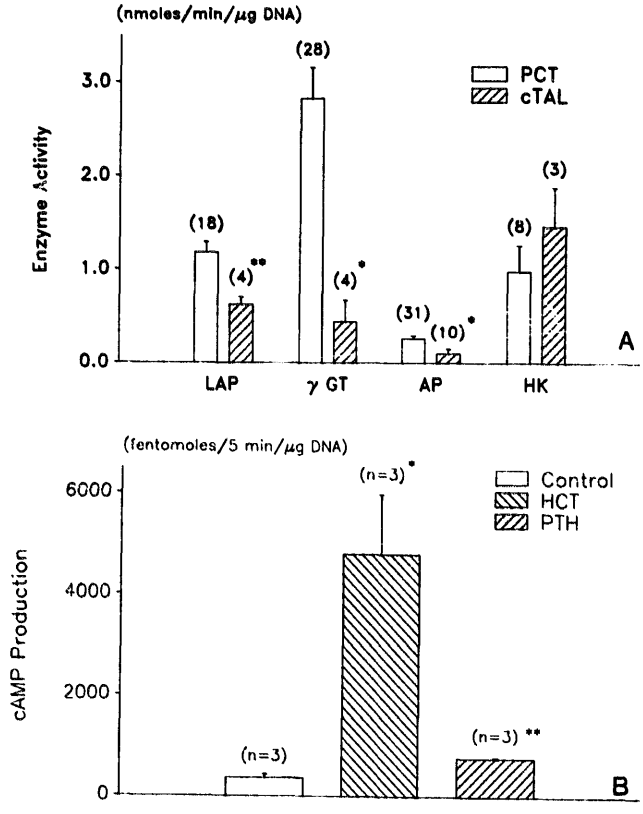


Fig. 3. (A) Leucine aminopeptidase (LAP), γ-glutamyltransferase (μGT), alkaline phosphatase (AP) and hexokinase (HK) activities measured in 15–20-day-old primary cultures of proximal tubule (PCT) and cortical thick ascending limb (cTAL). Values are means ± S.E. of n experiments. * ($P < 0.02$) and ** ($P < 0.05$) indicate the statistical significance of the differences between enzymatic activities in PCT and cTAL cultures. (B) cAMP production measured in 15–20-day-old primary cultures of cortical ascending limbs. Human calcitonin (HCT) and parathyroid hormone (PTH) concentrations were 30 mU/ml and 0.1 μg/ml, respectively. Values are means ± S.E. of three experiments in each condition. * $P < 0.05$ and ** $P < 0.02$ represent the statistical significances of the differences between basal and stimulated productions of cAMP.

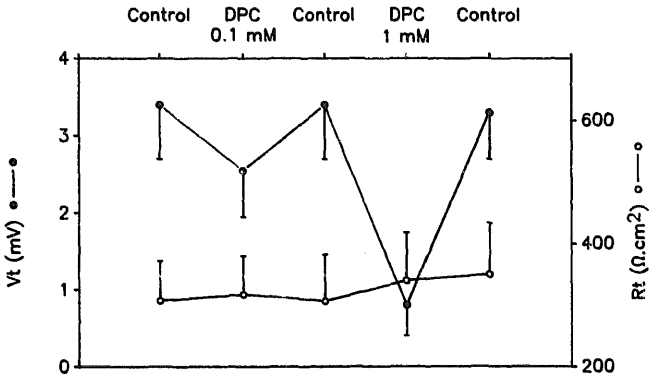


Fig. 4. Effect of diphenylamine 2-carboxylate (DPC) applied apically on transepithelial voltage (V_t) and resistance (R_t) of cultured cortical ascending limbs. Values are means ± S.E. of four experiments. The transepithelial potential is positive and refers to the apical side of the culture with respect to the basolateral side.

The effect of the chloride channel blocker diphenylamine 2-carboxylate (DPC) was tested on the basolateral membrane. We first verified that the DMSO (0.1%) used to solubilize DPC did not modify V_t significantly (Control: $+2.8 \pm 0.6$ mV ($n = 4$); DMSO: $+3.0 \pm 1.5$ mV ($n = 4$)). The effects of DPC are summarized in Fig. 4. DPC (0.1 mM) decreased V_t by $30.0 \pm 7.0\%$ ($n = 4$) which returned to its control value when DPC was rinsed out. Addition of 1 mM DPC further decreased V_t by $79.7 \pm 7.4\%$ ($n = 4$). At neither concentration did R_t vary significantly.

On the apical membrane, the application of the potassium channel blocker barium (5 mM) reduced V_t from 3.2 ± 1.2 to 1.1 ± 0.9 mV ($n = 4$). On the other hand, the sodium channel blocker amiloride (1 mM) did not modify V_t when applied on the apical side of the epithelia (Control: $+3.1 \pm 1.0$ mV; Amiloride: $+3.2 \pm 1.1$ mV ($n = 3$)).

Patch clamp experiments

In order to characterize the ionic channels present in the apical membrane of the cultured cells, single-channel currents were recorded using the patch clamp technique. Stable gigaseals were obtained in 55% of the trials and single channel activity was recorded in 34 patches out of 163. Large and small conductance K channels and cation-selective channel were observed in 6, 18 and 10 patches respectively.

Large conductance calcium activated K^+ channels (BK)

Cell attached configuration. An example of this channel recorded in a cell attached patch when pipette and bath both contained the Na-rich solution is given in Fig. 5A. At resting membrane potential ($V_m = -60$ mV, $V_p = 0$ mV) channel currents were directed from the cell interior to the pipette. Channel openings appeared in bursts of activity alternating with closed periods lasting from a few milliseconds to seconds. Depolarizing the membrane increased the current amplitude without affecting the channel activity. The corresponding I/V relationship of the channel is given in Fig. 5B. The maximal channel conductance, calculated as the slope of the curve between 0 and +20 mV, was

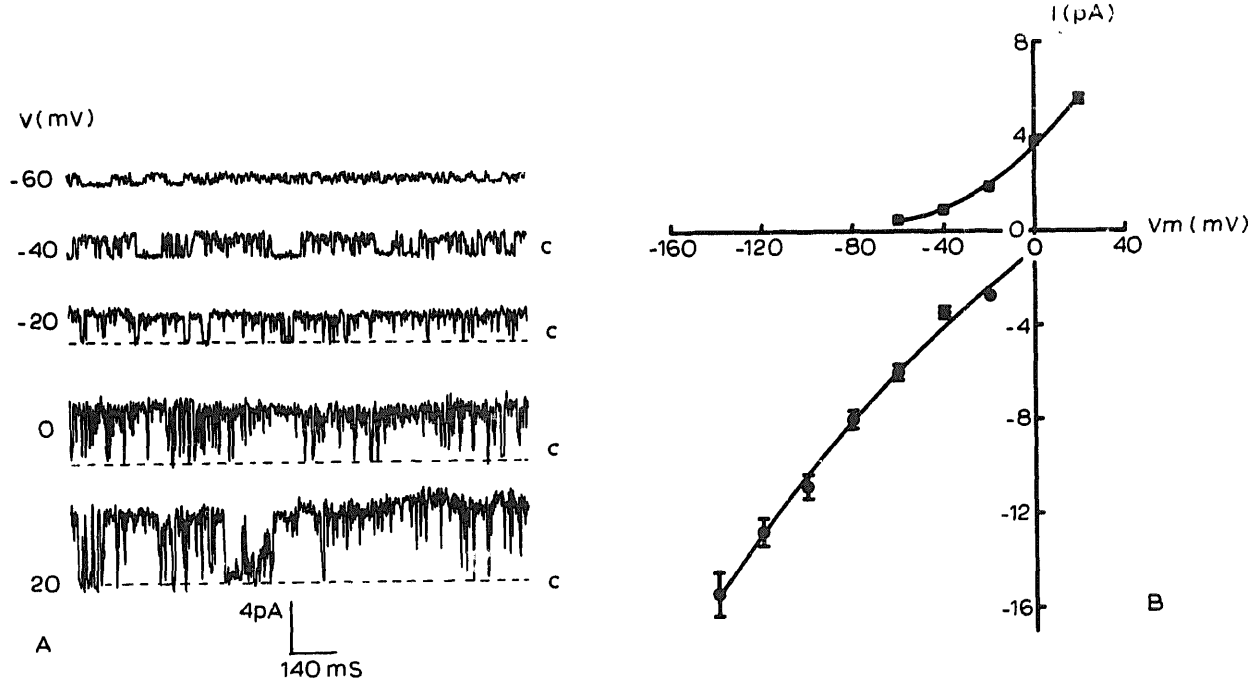


Fig. 5. (A) Single-channel current recordings of the large K^+ channel in a cell-attached patch. Channel openings are upward. The pipette and the bath contained the Na-rich solution. The membrane holding potentials are indicated on the left of each recording. 'C' on the right of the recordings indicates the closed state of the channel. (B) I/V relationship of the channel in the cell-attached condition. ■, The pipette and the bath contained the same solution as in (A). ●, The pipette contained the K-rich solution (1 mM Ca^{2+}).

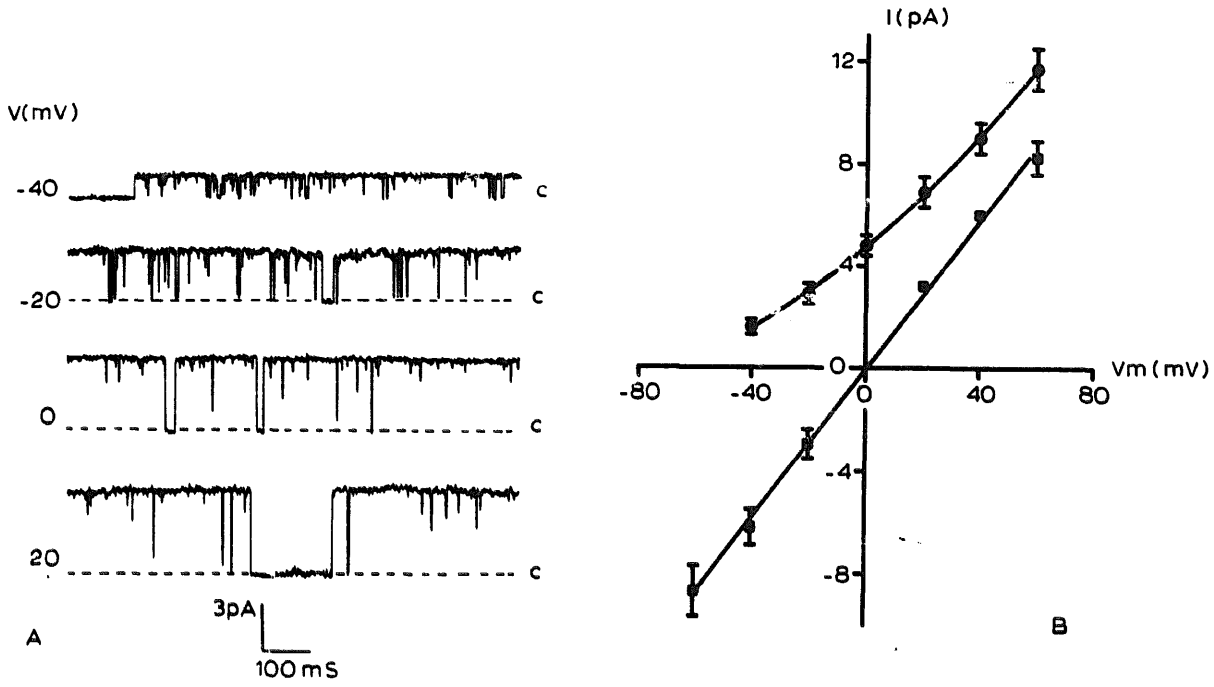
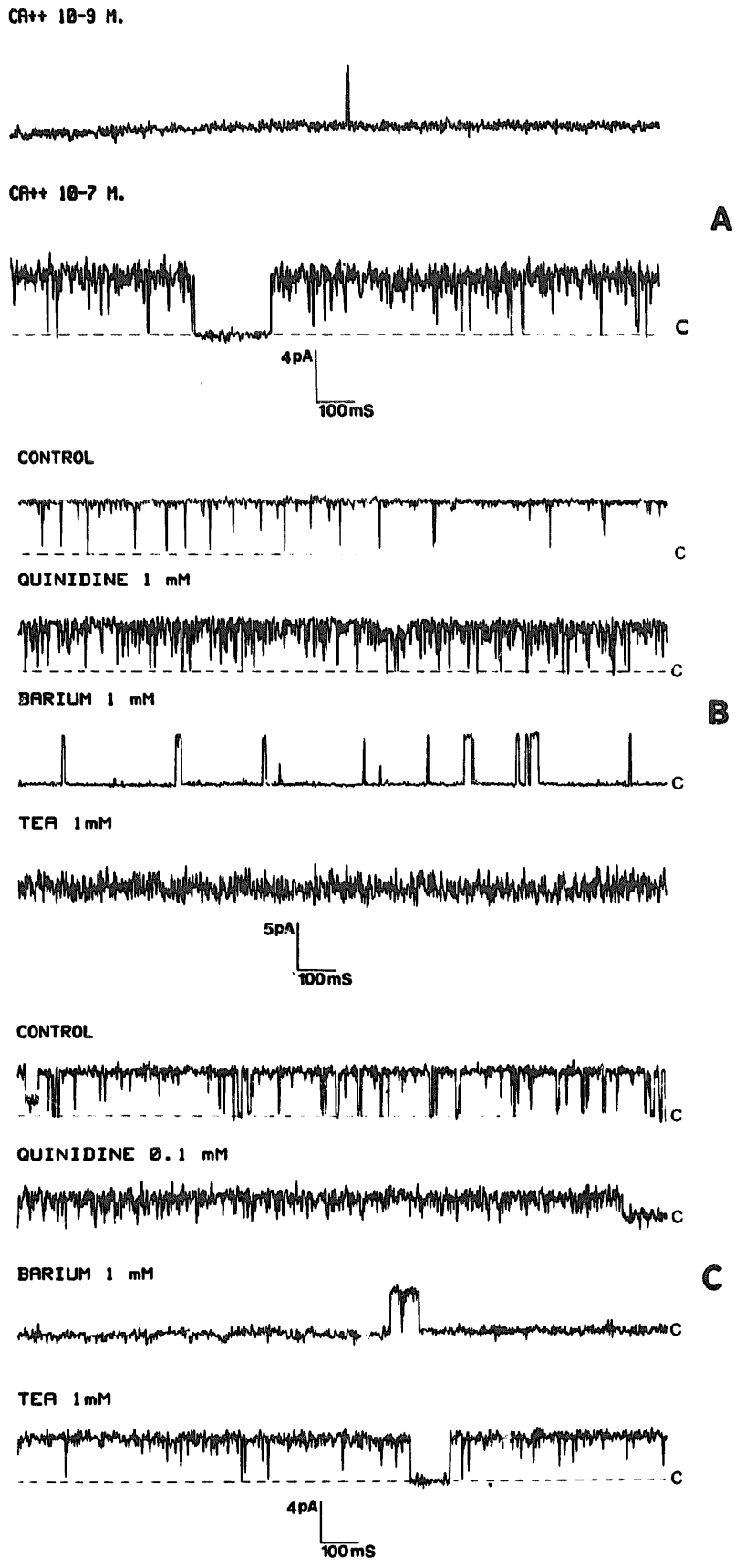


Fig. 6. (A) Single-channel currents recording of the large K^+ channel in an inside-out patch. The pipette contained the Na-rich solution and the bath the K-rich solution (1 mM Ca^{2+}). The membrane holding potentials are given on the left of the recordings. 'C' and dotted lines indicate the closed state of the channel. (B) I/V relationships of the channel in excised patches. The pipette and bath contained the same solutions as in A (●) or both contained the K-rich solution, 1 mM Ca^{2+} (■).

94.7 ± 2.57 pS ($n = 3$). The GHK equation was applied to the data assuming that the channel was K^+ selective. This revealed a mean permeability of (2.49 ± 0.08 · 10^{-13} cm^3/s ($n = 3$). We also examined the channel with K-rich solution in the pipette and Na-rich solution in the bath (Fig. 5B). Under these conditions the maximal channel conductance was 117.0 ± 68 pS ($n = 3$) and the mean K^+ permeability was (1.72 ± 0.091) · 10^{-13} cm^3/s ($n = 3$).

Cell excised configuration. The ionic channel selectivity was studied in excised patch experiments. Fig. 6A illustrates the single-channel currents recorded in an excised inside-out patch when the pipette contained the Na-rich solution and the bath the K-rich solution. At 0 mV holding potential, the channel openings are upward deflections and must correspond to K^+ currents since Na^+ currents would produce downward deflections and Cl^- is at electrochemical equilibrium. Negative membrane potentials decreased the current amplitude whereas positive potentials increased it. The mean I/V relationship of five experiments performed in these conditions is shown in Fig. 6B. From the current reversal potential estimated by extrapolation (−67 mV) a K^+ to Na^+ permeability ratio $P_K/P_{Na} \approx 29$ was calculated. The mean channel conductance calculated between +40 and +60 mV was 122 ± 7 pS ($n = 5$). The I/V curve could be fitted by the GHK equation giving a mean permeability of (3.46 ± 0.34) · 10^{-13} cm^3/s ($n = 5$). Fig. 6B also gives the I/V relationship of the channel when pipette and bath both contained 140 mM KCl. In this condition the I/V relation was linear, the current reversal potential was 0 mV and the channel conductance 144.7 ± 5.7 pS ($n = 3$). The calcium sensitivity of the channel was studied on excised patches at 0 mV holding potential (Fig. 7A). In the upper tracing the pipette contained the Na-rich solution and the bath the K-rich solution. At 10^{-9} M Ca^{2+} in the bath, few channel openings were recorded ($P_o = 0$). Raising the calcium concentration (10^{-7} M) increased the channel activity ($P_o = 0.88$).

Fig. 7. (A) Effect of cytoplasmic calcium concentration on the large K^+ channel activity. The pipette contained the Na-rich solution and the membrane potential was 0 mV. In the upper recording the bath contained the K-rich solution. Free Ca^{2+} concentration was adjusted to 10^{-9} M with EGTA. In the lower recording the bath calcium concentration was increased to 10^{-7} M. Channel openings are upward and the 'C' indicates the closed state of the channel. (B) Effects of quinidine, barium and tetraethylammonium (TEA) on K^+ channel currents recorded in an excised rightside-out patch. In the control recording the pipette contained the K-rich solution (1 mM Ca^{2+}) and the bath the Na-rich solution. In the lower recordings, quinidine (1 mM), barium (1 mM) or TEA (1 mM) were added to the bath. The membrane holding potential was 0 mV. (C) Effects of quinidine, barium and tetraethylammonium (TEA) on K^+ channel currents recorded in an inside-out patch. In the control recording the pipette contained the Na-rich solution and the bath the K-rich solution. In the lower recordings, quinidine (0.1 mM), barium (1 mM) and TEA (1 mM) were added to the bath. The membrane holding potential was 0 mV.


CA++ 10-9 M.
CA++ 10-7 M.
A
C
4pA
100mS
CONTROL
C
QUINIDINE 1 mM
C
BARIUM 1 mM
B
C
TEA 1mM
5pA
100mS
CONTROL
C
QUINIDINE 0.1 mM
C
BARIUM 1 mM
C
C
TEA 1mM
C
4pA
100mS

The effects of three agents which block large-conductance K^+ channels were studied on excised patches. In order to gain access to both external and cytoplasmic sides of the membrane, channel currents were recorded in both rightside-out and inside-out patches. As shown in Fig. 7B, quinidine (1 mM) barium (1 mM) and tetraethylammonium (TEA 1 mM) applied on the external side of the channel considerably modified the current recordings. Quinidine ($n = 3$) and TEA ($n = 3$) reduced the channel amplitude and the channel recording had a flickering appearance, whereas barium blocked the channel for long periods without affecting current amplitude when the channel opened ($n = 3$).

Quinidine (0.1 mM) and barium (1 mM) had similar effects when applied on the cytoplasmic side of the channel whereas TEA (1 mM) was without effect (Fig. 7C, $n = 3$). The effects of the blockers were found to be reversible when they were rinsed away irrespective of the side of application.

Small conductance K^+ channels

Cell attached configuration. In cell attached experiments we also recorded a second type of ionic channel. Fig. 8A is an example of the channel currents obtained with the Na-rich solution both in the pipette and the bath. From +20 to −40 mV membrane potential, the currents were outwardly directed. They reversed around −60 mV. Single-channel conductance ranged from 18.3 ± 2.7 pS near the resting membrane potential ($V_m = -60$ mV, $V_p = 0$ mV) to 58.1 ± 3.0 mV ($n = 3$) when the membrane was depolarized between 0 and +20 mV (Fig. 8B).

Cell excised configuration. Experiments were then performed in the inside out excised configuration. Fig. 9A shows the single-channel currents when the pipette contained the K-rich solution and the bath the Na-rich solution. At 0 mV, negative currents were observed which could correspond to an exit of K + ions from the pipette interior into the bath. The current-voltage relation (Fig. 9B) was fitted by the GHK equation (dotted line) giving a mean permeability of $(7.29 \pm 0.28) \cdot 10^{-14}$ cm^3/s ($n = 5$). The extrapolated reversal potential was +74.4 ± 6.7 mV and the slope conductance calculated at −60 mV was 43.3 ± 4.7 pS $n = 5$. The time constants of the channel were analysed at −60 mV by tracing the dwell time histograms. The mean open time was 43.7 ± 8.9 ms whereas the mean closed time was 137.5 ± 16.3 ms $n = 5$ (Fig. 9 lower). The open probability of this channel was voltage-dependent. The P_o decreased when the membrane potential was hyperpolarized (Fig. 10A). Fig. 10B shows the effect of barium of the channel-open probability. When added on the cytosolic side Ba^{2+} blockade was clearly dose-dependent ($n = 3$). On the other hand, changes in the cytoplasmic Ca^{2+} concentration did not modify the channel activity. At −60 mV holding potential, P_o was

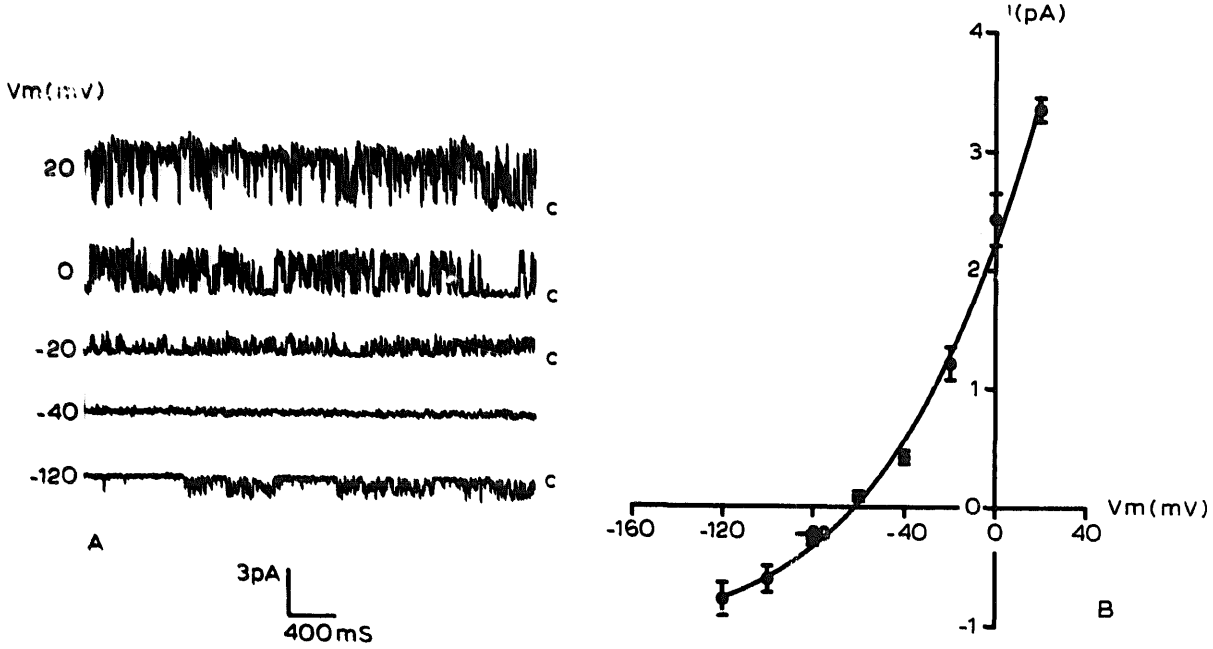


Fig. 8. (A) Single-channel current recordings of the small K^+ channel in a cell-attached patch. Channel openings are upward. The pipette and the bath contained the Na-rich solution. The membrane holding potentials are indicated on the left of each recording. 'C' on the right of the recordings indicates the closed state of the channel. (B) I/V relationship of the channel in the cell-attached condition. The pipette and bath contained the same solutions as in (A).

0.16 ± 0.02, 0.11 ± 0.02 and 0.16 ± 0.05 ($n = 3$) with 10^{-3}, 10^{-6} and 10^{-8} M calcium, respectively.

Non-selective cationic channel

Cell attached configuration. Besides the two kinds of K^+ channels, a non selective channel was found in the cultured cTAL. The typical channel activity in a cell attached patch can be seen in Fig. 11A when the pipette contained the K-rich solution and the bath the Na-rich solution. The current voltage relation of the channel under these conditions was linear over the range of potential examined and it indicated a conductance of 27.0 ± 5.9 pS ($n = 4$) (Fig. 11B). The kinetic analysis of recordings done at -60 mV, revealed that the channel showed two open states with time constants of 362.3 ms and 31.9 ms and two closed states with time constants of 1107.8 ms and 19.7 ms ($n = 4$) (Fig. 11, lower).

Cell excised configuration. In order to investigate the ionic selectivity of the channel, experiments were done on inside out excised patches. Typical channel current recordings are presented in Fig. 12A where the pipette contained the Na-rich solution and the bath the K-rich solution. The channel openings are downward at negative holding membrane potentials (-40, -60 mV), and upward at positive potentials. The I/V relationship of the channel in these experiments was linear (Fig. 12B) and a channel conductance of 21.3 ± 1.5 pS ($n = 9$) was calculated. The current reversal potential was -0.4 ± 0.7 mV ($n = 9$). Since the equilibrium potential for Na^+, K^+ and Cl^- were $+84$, -84 and 0 mV, respectively, the channel must either not have discriminated

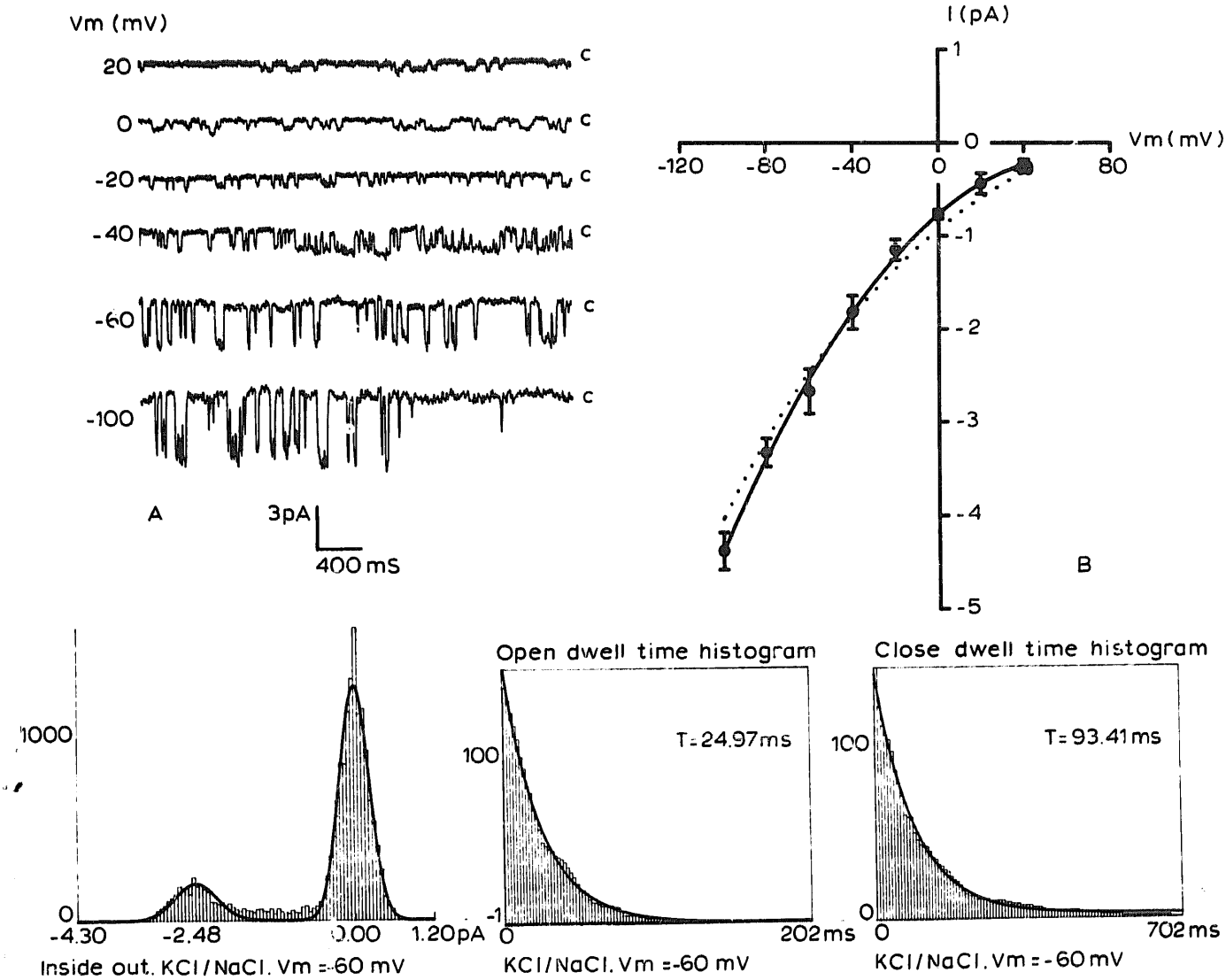


Fig. 9. (A) Single-channel currents recording of the small K^+ channel in an inside-out patch. The pipette contained the K-rich solution (1 mM Ca^{2+}) and the bath the Na-rich solution. The membrane holding potentials are given on the left of the recordings. 'C' indicates the closed state of the channel. (B) I/V relationships of the channel in excised patches. The pipette and bath contained the same solutions as in (A). (Bottom) Amplitude and dwell time histograms at membrane voltage = -60 mV.

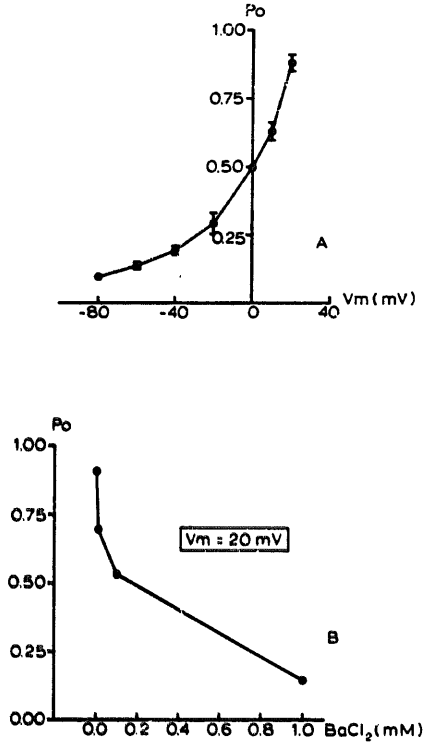


Fig. 10. (A) Open probability of the small K^+ channel as a function of membrane voltage. Values are means ± S.E. of five experiments. (B) Dose response curve for Ba^{2+} at $V_m = 20$ mV. The curve is representative of three different experiments.

between Na^+ and K^+ or have been Cl^- selective. To investigate these possibilities the KCl in the bath was replaced by 140 mM Na gluconate. Because this maneuver did not significantly modify the reversal potential (Fig. 12B), we concluded that the channel is cation permeable and dose not discriminate between Na^+ and K^+. To compare with cell attached conditions, kinetic analysis was done on inside out patches at −60 mV holding potential. We found two open states (T_1 = 29.3 ± 6.3 and T_2 = 7.0 ± 1.3 , $n = 4$) and two closed states (T_1 = 145.7 ± 22.4 and T_2 + 11.9 ± 3.0, $n = 4$) (Fig. 12, lower part).

The open-state probability of the channel was also found dependent upon the membrane potential. As illustrated on Fig. 13A, depolarization produced an increase in the open probability of the channel ($n = 4$).

Another characteristic of the channel is the high calcium concentration needed on the cytoplasmic side for its activation. Fig. 13B illustrates an experiment in which a patch was excised in a K-rich solution containing 1 μM calcium. Only one channel of low activity was observed. Increasing the calcium concentration to 1 mM enhanced the channel activity and there was a stimultaneous opening of two channels.

We examined the effects of ATP on channel activity when applied on the cytoplasmic side of the membrane (Fig. 13C). Addition of 10^{-5} M ATP decreased channel-open probability by 82% ($n = 3$). This effect was immediate and reversed when the nucleotide was removed.

Discussion

To study the nature of the ionic channels present in the apical membrane of the cortical ascending limb, we established primary cultures of microdissected cTAL. Of the techniques for preparing monolayers we chose the culture of defined segments obtained by microdissection [15]. The principal avantage of this technique is the exact identification of the nephron segment inoculated on the collagen-coated permeable support. To ensure that the primary culture resembles the original epithelium, biochemical, morphological and physiological studies were first performed. The ultrastructural examination indicated that the monolayers are composed of a single type of cell with an obvious morphological polarity. Parallel enzyme assays showed that these primary cultures contained a high level of hexokinase. Furthermore, the activities of γ-glutamyltransferase, leucine aminopeptidase and alkaline phosphatase were significantly lower than those measured in isolated PCT cultured under the same conditions. This confirms the distal origin of the cultured cells. The cAMP production was stimulated by calcitonin (HCT) and parathyroid (PTH) treatments. The stimulation factor (× 13) determined in the presence of HCT was of the same order of magnitude as that reported for microdissected segments [16]. Although PTH also induced a significant increase in cAMP level in cultured cTAL, the amplitude of the stimulation was lower than that obtained with the initial segment [16]. Reduced responsiveness to PTH has already been reported in cultures originating from PCT [10]. Moreover, Wilson et al. [17] found a lack of stimulation in primary cultures of human cTAL. It is therefore possible that the process of making primary cultures alters the sensitivity of receptors to PTH and/or the intracellular coupling to adenylate cyclase.

Starting from a single microdissected segment of cTAL, the cultures reached the edges of the permeable culture well (0.20 cm^2) after 12–15 days. By this time the cultures had developed an apical positive potential averaging 4.1 mV which is characteristic of the original segment [1,18]. Similar values have been reported in cultured mTAL [15,19,20]. On the other hand, the resistance (303 Ω cm^2) we measured in our experimental conditions is higher than the mean values reported for rabbit microperfused mTAL or cTAL segments (35 Ω cm^2) [1]. This was also reported for cultured PCT [10,21], mTAL [18] and CCT [22]. As previously suggested [23] culture conditions could determine the resistance of monolayers by shifting the distributions of the sealing and conductive elements of the occluding junction. In addition, a reduction of the cell size in

culture could increase the number of cell junctions per unit surface area and result in an increased resistance. The V_t was inhibited by DPC (1 mM) applied on the basolateral face of the monolayer, indicating the presence of a conductive Cl^- pathway. This feature of the cTAL basolateral membrane was well established from microperfusion experiments [2] and patch clamp data [4].

Our results indicate that the apical membrane of cTAL in culture has no measurable conductance to Na^+. The depolarizing effect of Ba^{2+}, however, is consistant with the presence of a conductive path for K^+ across the apical membrane.

From the results discussed above, it is reasonable to conclude that monolayers obtained from microdissected cTAL retain, at least qualitatively, characteristics similar to those of in vivo cTAL.

Single-channel analysis performed on the apical membrane revealed the existence of three kinds of channel. One is highly selective to potassium as against sodium ions and its conductance is about 120 pS. This high-conductance K^+ channel shows certain properties of the maxi K^+ channels found in other tissues [7]. In renal epithelia, a homologous channel has been described in the apical membrane of collecting tubules [8,9], primary cultures of PCT [10], cloned cultures of mTAL [5] and MDCK cell lines [24]. In these studies, however, the channel activity increased with membrane depolarization. This is at variance with the present data which show the opening probability of the channel to be little modified by the potential. The large increase of channel activity between 10^{-9} and 10^{-7} M Ca^{2+} lies within the range previously recorded [5,7]. As expected, the K^+ channel was blocked by Ba^{2+}, TEA

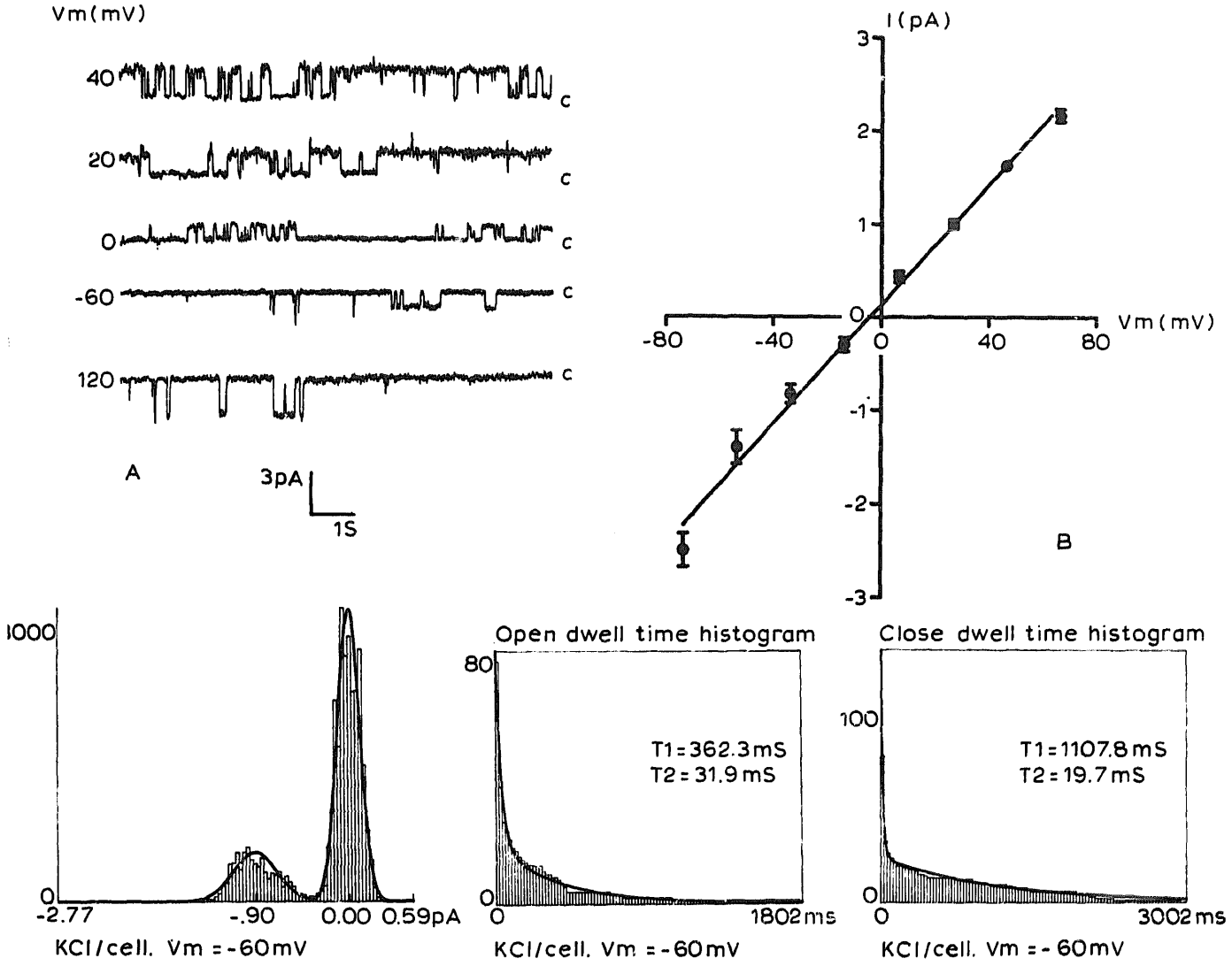


Fig. 11. (A) Single-channel current recordings of the non-selective cationic channel in a cell-attached patch. The pipette contained the K-rich solution (1 mM Ca^{2+}) and the bath the Na-rich solution. The membrane holding potentials are indicated on the left of each recording and the 'C' on the right indicates the closed state of the channels. (B) I/V relationship of the channel in cell attached configuration. The pipette and bath contained the same solutions as in (A). (Bottom) Amplitude and dwell time histograms at membrane voltage = −60 mV.

and quinidine. The modification of the channel gating behaviour by these various blocking agents was similar to that obtained in cultured clones from mTAL.

We also observed a second K^+ channel the conductance of which ranged from 18 to 58 pS in cell attached and was 43 pS in excised configuration. The channel was very selective for K^+ over Na^+ ions, was blocked by Ba^{2+} and its opening probability was strongly voltage-dependent. Potassium channels of conductance ranging from 20 to 80 pS were described in a large variety of epithelia, i.e. the proximal tubule [25–27], the distal convoluted tubule [28] and the rectal gland [29]. In a very detailed study performed in the rat cTAL perfused in vitro, Bleich et al. [30] have reported the existence of a luminal K^+ channel of conductance around 60–80 pS. This channel shares similarities to that described by Wang et al. [31] on rabbit cTAL. In the present study the small K^+ channel is not Ca^{2+} activated. This corroborates the results of Wang et al. [31] and Bleich et al. [30]. The insensitivity to cytosolic Ca^{2+} was also demonstrated for the basolateral K^+ channel found in the rabbit proximal tubule [26]. In rat CCT, Frindt and Palmer [32] have observed a low-conductance K^+ channel insensitive to Ca^{2+} and blocked by Ba^{2+} ions. Taken together these data indicated that in spite of variations of conductance values, all these channels recording could correspond to one kind of K^+ channel only.

In addition to these channel species, the apical membrane contains a third channel which does not discriminate between Na^+ and K^+. Its gating behaviour, conductance and pronounced Ca^{2+} sensitivity are very similar to those reported for the Ca^{2+} sensitive cation channel in various tissues [33,34]. In the

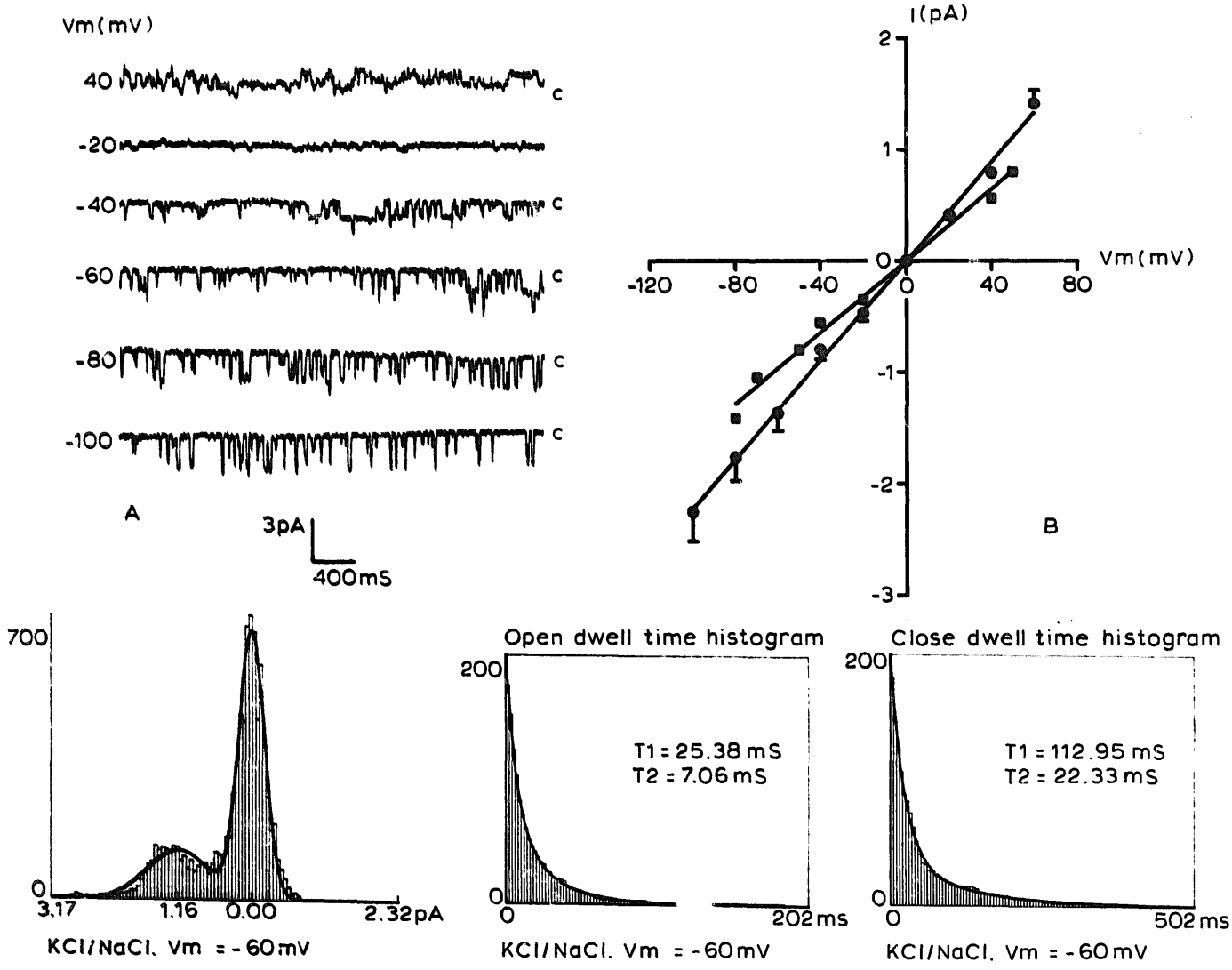


Fig. 12. (A) Single-channel current recordings of the non-selective cationic channel in an excised inside-out patch. The pipette contained the K-rich solution (1 mM Ca^{2+}) and the bath the Na-rich solution. The membrane holding potentials are indicated on the left of each recording and the 'C' on the right indicates the closed state of the channels. (B) I/V relationships of the channel in different conditions. ●, Corresponds to experiments in which pipette and bath contained the same solutions as in (A). ■, Represents experiments in which the bath contained 140 Na gluconate, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Hepes (pH 7.4). (Bottom) Amplitude and dwell time histograms at membrane voltage = −60 mV.

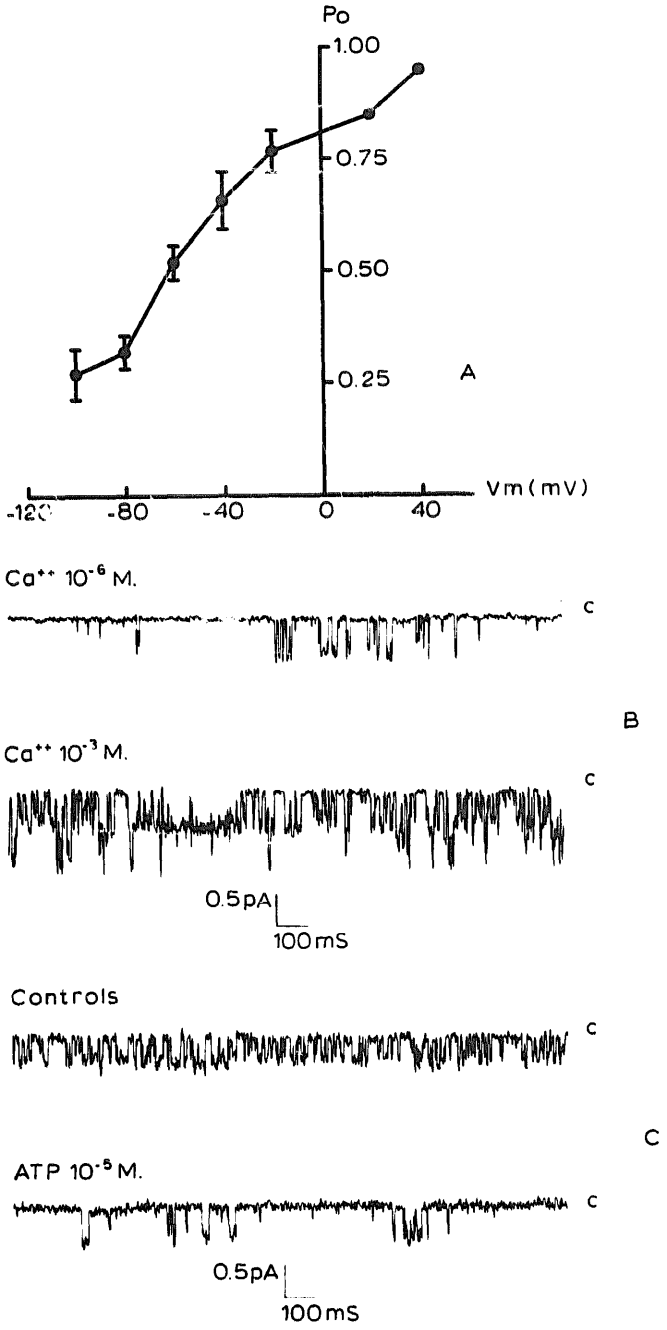


Fig. 13. (A) Open probability of the non-selective cationic channel as a function of membrane voltage. Values are means ± S.E. of four experiments. (B) Effect of the cytoplasmic calcium concentration on non-selective cationic channel activity recorded in an inside-out patch. The pipette contained the Na-rich solution and the bath the K-rich solution with two calcium concentrations: 1 μM (upper recording) and 1 mM (lower recording). The membrane holding potential was kept constant at -40 mV. The traces are representative of three different experiments. (C) Effect of adenosine triphosphate (ATP) on non-selective cationic channel activity. In the control recording the pipette contained the Na-rich solution and the bath the K-rich solution (1 mM Ca^{2+}). In the lower recording ATP (10^{-5} M) was added to the bath. The membrane holding potential was -40 mV. The traces are representative of three different experiments.

cultured cTAL the channel was recorded in the cell attached configuration and was found to be active at the resting cell potential. However, the analysis of the open and closed time constants indicated that the kinetic of the channel was very slow. Excising the patch did not modify the conductance properties but caused the time constant to be strongly shortened. In fact the open probability was 0.18 ± 0.02 ($n = 3$) in cell attached at resting membrane voltage and increased up to 0.52 ± 0.04 ($n = 4$) when the patch was excised. In this condition the application of ATP reduced this value to 0.15 ± 0.03 ($n = 4$). Thus the low activity of the channel in the on cell mode could be due to the action of intracellular ATP. In addition we have demonstrated that the non-specific channel in these cells was voltage-dependent. This confirms the data of Gögelein et al. on rabbit proximal tubule [35]. We have already observed a non-selective cation channel in the apical membrane of all the nephron segments that we have cultured, i.e. proximal convoluted tubule (PCT) [10] bright distal convoluted tubule (DCTb) [36] and cortical collecting tubule (CCT) [37]. Moreover, Paulais et al. [38] have also shown that a Ca^{2+}-activated non-specific cation channel is present in the basolateral membrane of the mouse cTAL. One common finding of these studies is the requirement for such a high calcium concentration to activate the channel in cell excised experiments. Nevertheless, in cell attached configuration, the channel was active at the resting intracellular Ca^{2+} concentrations. It is therefore possible that when the patch is excised the Ca^{2+} sensitivity is drastically reduced. Finally as stated by Sturgess et al. [34], the open probability of the channel could depend upon inter-relations between the intracellular Ca^{2+}, the membrane voltage and the intracellular ATP (or nucleotides) concentration.

In the present study, this channel was never sensitive to amiloride or phenamil. On the other hand, Light et al. [39] observed an amiloride sensitive non-selective cation channel in the apical membrane of IMCT in primary culture. Thus the question arises as to whether there are several kinds of non-specific cation epithelial channel or whether the controversial findings indicate a fragility of the channel integrity. In support of the latter hypothesis, certain properties of the Na^+ epithelial channel are relevant, notably its loss of selectivity due to membrane degradation processes [40], culture conditions or isolation procedures [41].

In conclusion, potassium channels with different properties have been found in the apical membrane of cultured cTAL. If one considers that barium applied on the apical side of the culture induced complete depolarization of V_t, it is clear that the apical membrane conductance corresponds essentially to a K^+ conductance. The two K^+ channels could account for this conductance. According to several authors [31,32]

the small-conductance K^+ channel is probably involved in net K^+ secretion. Therefore, in the cTAL this channel could be the major pathway for K^+ recycling that have been transported by the $Na^+/2Cl^-/K^+$ carrier [1,30]. This is further suggested by the higher number of observations of this channel ($n = 18$) compared to those of the maxi K^+ channel ($n = 6$).

The role of the non-selective cation channel is not known at present. Moreover, when active it will permit the entry of Na^+ into the cell. This could reduce the Na^+ gradient across the apical membrane and impair the efficiency of the $Na^+/2Cl^-/K^+$ cotransporter. Therefore the physiological significance of this channel remains questionable. Finally it is also difficult to ascertain whether the ion selectivity of the channel is similar in cultured cells and in vivo.

Acknowledgment

The technical assistance of M. Gastineau is gratefully acknowledged.

References

1 Greger, R., Schlatter, E. and Lang, F. (1983) Pflügers Arch. 396, 308–314.
2 Greger, R. and Schlatter, E. (1983) Pflügers Arch. 396, 325–334.
3 Greger, R. and Schlatter, E. (1983) Pflügers Arch. 396, 315–324.
4 Gogelein, H. (1988) Biochim. Biophys. Acta 947, 521–547.
5 Guggino, S.E., Guggino, W.B., Green, N. and Sacktor, B. (1987) Am. J. Physiol. 252, C121–C127.
6 Guggino, S.E., Guggino, W.B., Green, N. and Sackor, B. (1987) Am. J. Physiol. 252, C128–C137.
7 Marty, A. (1981) Nature (London) 291, 497–500.
8 Hunter, M., Lopes, A.G., Boulpaep, E.L. and Giebisch, G.H. (1984) Proc. Natl. Acad. Sci. USA 81, 4237–4239.
9 Palmer, L.G. (1986) Am. J. Physiol. 250, F379–F385.
10 Merot, J., Bidet, M., Gachot, B., Le Maout, S. Tauc, M. and Poujeol, P. (1988) Pflügers Arch. 413, 51–61.
11 Tauc, M., Merot, J., Bidet, M., Koechlin, N., Gastineau, M., Othmani, L. and Poujeol, P. (1989) Histochemistry, 91, 17–30.
12 Switzer, B.R. and Summer G.K. (1971) Clin. Chim. Acta 32, 203–206.
13 Hammil, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J. (1981) Pflügers Arch. 391, 85–100.
14 Fabiato, A. and Fabiato, F. (1979) J. Physiol (Paris) 75, 463–505.
15 Burg, M., Green, N., Sohraby, S., Steele, R. and Handler, J. (1982) Am. J. Physiol. 242, C229–C233.
16 Morel, F. and Doucet, A. (1986) Physiol. Rev. 66, 377–468.
17 Wilson, P.D., Dillingham, M.A., Breckon, R. and Anderson, R.J. (1985) Am. J. Physiol. 248, F436–F443.
18 Hebert, S.C., Culpepper, R.M. and Andreoli, T.E. (1981) Am. J. Physiol. 241, F412–F431.
19 Valentich, J.P. and Stokols, M.F. (1986) Am. J. Physiol. 251, C312–C322.
20 Drugge, E.D., Maieread, A.C. and McGiff, J. (1989) Am. J. Physiol. 256, C1070–C1081.
21 Blackburn, J.G., Hazen-Martini, D. J., Detrisac, C.J. and Sens, D.A. (1988) Kidney Int. 33, 508–516.
22 Gross, P., Minuth, W.W., Kriz, W. and Fromter, E. (1986) Pflügers Arch. 406, 380–386.
23 Simmons, N.L. (1981) J. Membr. Biol. 59, 105–114.
24 Bolivar, J.J. and Cereijido, M. (1987) J. Membr. Biol. 97, 43–51.
25 Gogelein, H. and Greger, R. (1987) Pflügers Arch. 410, 288–295.
26 Parent, L., Cardinal, J. and Sauve, R. (1988) Am. J. Physiol. 254, F105–F113.
27 Merot, J., Bidet, M., Le Maout, S., Tauc, M. and Poujeol, P. (1989) Biochim. Biophys. Acta 978, 134–144.
28 Taniguchi, J., Yoshitomi, K. and Imai, M. (1989) Am. J. Physiol. 256, F246–F254.
29 Greger, R., Gogelein, H. and Schlatter, E. (1987) Pflügers Arch. 409, 100–106.
30 Bleich, M., Schlatter, E. and Greger, R. (1990) Pflügers Arch. 415, 449–460.
31 Wang, W., White S., Geibel J. and Giebisch, G. (1990) Am. J. Physiol. 258, F244–F253.
32 Frindt, G. and Palmer, L.G. (1989) Am. J. Physiol 256, F143–F151.
33 Fichtner, H., Frobe, U., Brusse, R. and Kohlhardt, M. (1987) J. Membr. Biol. 98, 125–33.
34 Sturgess, N.C., Hales, C.N. and Ashford, M.L. (1987) Pflügers Arch. 409, 607–615.
35 Gogelein, H. and Greger, R. (1986) Pflügers Arch. 407, S142–S148.
36 Merot, J., Tauc, M., Bidet, M. and Poujeol, P. (1991) Nephrology, 1–15.
37 Merot, J., Bidet, M., Tauc, M. and Poujeol, P. (1987) J. Physiol. (Paris) 82, 63A (Abstr.).
38 Paulais, M. and Teulon, J. (1989) J. Physiol. (London) 413, 315–327.
39 Light, D.B., McCann, F.V., Keller, T.M. and Stanton, B.A. (1988) Am. J. Physiol. 255, F278–F286.
40 Lewis, S.A. and Hanrahan, J.W. (1985) Pflügers Arch. 405, S83–S88.
41 Hamilton, K.L. and Eaton, D.C. (1986) Fed. Proc. 45, 2713–2717.