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Potassium transport in opossum kidney cells: Effects of Na-selective and K-selective ionizable cryptands, and of valinomycin, FCCP and nystatin

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Abstract

The effects of two ionizable cryptands, the Na-selective (221)C_{10} and the K-selective (222)C_{10}, and of valinomycin, FCCP and nystatin on K^+ fluxes in opossum kidney (OK) cells have been quantified. The Na,K-ATPase (ouabain-sensitive ^{86}Rb influx) was stimulated by nystatin ($\geq$ 20%), and inhibited by the other ionophores (50–80%), by barium (K-channel blocker) (61%) and by amiloride (Na entry blocker) (34%). The V_{max} of the Na,K-ATPase phosphatase activity was unmodified by the ionophores, indicating the absence of direct interaction with the enzyme. The ATPi content was unmodified by the inhibitors and nystatin, but was lowered by (221)C_{10} (47%), (222)C_{10} (75%), valinomycin (72%) and FCCP (88%). Amiloride was found to partially remove the inhibition caused by (222)C_{10} (51%) and valinomycin (49%). Rb efflux was stimulated by nystatin (32%), unmodified by valinomycin, and was inhibited by (221)C_{10} (19%), (222)C_{10} (19%) and FCCP (10%). Barium (39%) and amiloride (32%) inhibited this efflux and, in their presence, the nystatin effect persisted, whereas that of the other ionophores vanished. At pH 6.4, the Rb efflux decreased by 14% of its value at pH 7.4, with no additional inhibition by cryptands. Cryptands are shown to inhibit the pH-sensitive K^+-conductance, probably by inducing a K^+–H^+ exchange at the plasma membrane, and by uncoupling oxidative phosphorylation by inducing the entry of K^+ and H^+ (and possibly Ca^{2+}) ions into the mitochondria. © 1997 Elsevier Science B.V.

Keywords: OK (opossum kidney) cell; Ionizable and neutral ionophore; K^+ flux; Na,K-ATPase activity; Cell ATPi content; Inhibitor

1. Introduction

One third of the energy requirement of animal cells is expended in keeping K^+ inside and Na^+ outside the plasma membrane. Whether cells maintain high Na^+ and K^+ gradients simply to preserve their metabolic integrity or, as in kidney, because transport of these ions is inherent to the function of the organ, they all use essentially the same basic mechanism utilizing the sodium- and potassium-activated adenosine triphosphatase (Na,K-ATPase), an enzyme located in the plasma membrane [1]. Since this enzyme helps maintain the osmotic balance across

Abbreviations: (221)C_{10}-cryptand, diaza-1,10-decyl-5-penta-oxa-4,7,13,16,21-bicyclo[8.8.5]tricosane; (222)C_{10}-cryptand, diaza-1,10-decyl-5-hexaoxa-4,7,13,16,21,24-bicyclo[8.8.8]hexacosane; Na-selective (221)C_{10}-cryptand, Na^+ over K^+ selective (221)C_{10}-cryptand; K-selective (222)C_{10}-cryptand, K^+ over Na^+ selective (222)C_{10}-cryptand; FCCP, carbonylcyanide *p*-trifluoro-methoxyphenyl-hydrazone; ouabain-sensitive Rb influx, ouabain-sensitive ^{86}Rb influx; Rb efflux, ^{86}Rb efflux; V_{max}, maximal rate of the Na,K-ATPase phosphatase activity; ATPi content, cell ATP content; HEPES, *N*-[2-hydroethyl]piperazine-*N*′-[2-ethane-sulfonic acid]

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cell membranes, by keeping the intracellular Na concentration low, balancing the negatively charged organic molecules, confined inside the cell, is performed largely by K^+. This ion is actively pumped into the cell by the Na,K-ATPase and can also move freely in or out through the K^+ leak channels in the plasma membrane [2].

For the purpose of understanding the molecular basis of cellular processes controlled by ions, ionophores have been widely used by cell biologists over the past thirty years as tools to increase the ion permeability of cell membranes. Among these ionophores, the K^+-selective carrier, valinomycin, the H^+-carrier, FCCP, and the channel-forming compound, nystatin, deserve special attention. Valinomycin, a neutral antibiotic, has been used to investigate the involvement of K^+ gradients, membrane potentials and K^+ permeability within cell membranes [3,4]. While inducing K^+ entry into the mitochondrial matrix, valinomycin also promotes the uncoupling of oxidative phosphorylation from respiration [5,6]. The protonophore, FCCP, a lipid-soluble weak acid, has been used mainly as a potent uncoupler of mitochondrial phosphorylation [6,7], and also has frequently been used to allow H^+ exchange across membranes against alkali cations carried by ionophores like valinomycin [8]. The polyene antibiotic, nystatin, which displays a channel-forming activity, increases the permeability of sterol-containing membranes to anions, and to Li^+, Na^+, K^+ and H^+ ions [9,10]. This compound has been used essentially to stimulate the Na,K-ATPase activity by increasing intracellular Na [11], and it does not exert an uncoupling effect on the mitochondria [12]. A detailed study of the effects of these three ionophores on K^+ transport in the proximal tubule has shown that their actions were localized at the plasma membrane (nystatin), the mitochondrial membrane (FCCP), or at both (valinomycin) [12].

More recently, another type of ionophore, i.e., the synthetic agents called cryptands, have attracted increasing interest [13]. These compounds form very stable complexes with various substrates, especially with monovalent and divalent cations [14] (Table 1), and it has been demonstrated that the $(221)C_{10}$ and $(222)C_{10}$ lipophilic cryptands induced the transport of competing K^+ and Na^+ ions through thin lipid membranes [15,16]. Due to the stated difference in specificity, these two cryptands may be expected to produce different effects. However, the affinity of either cryptand is sufficient to bind cellular concentrations of either Na^+ or K^+ [15,16]. Outside the cell, where K^+ is low, either cryptand should bind and transport Na^+; whereas inside, where Na^+ is low, the same may be true for K^+. Also (since they are ionizable), these cryptands have been found to elicit a cation-H^+ exchange across membranes without the need for a proton carrier [17,18].

Table 1
Ionic radii of cations and association constants (K_a) of cations to cryptands, in water at 25°C [14]

Ionic radius (Å)		K_a (M^{-1})	
		(221)-cryptand Cavity radius 1.1 Å	(222)-cryptand Cavity radius 1.4 Å
Na^+	0.98	$2.5 \cdot 10^5$	$7.9 \cdot 10^3$
K^+	1.33	$8.9 \cdot 10^3$	$2.5 \cdot 10^5$
Rb^+	1.49	$3.5 \cdot 10^2$	$2.2 \cdot 10^4$
Ca^{2+}	1.06	$8.9 \cdot 10^6$	$2.5 \cdot 10^4$
Ba^{2+}	1.43	$2.0 \cdot 10^6$	$3.2 \cdot 10^9$

The Na^+ over K^+ selectivity of the hydrophilic (221)-cryptand and the K^+ over Na^+ selectivity of the hydrophilic (222)-cryptand, calculated using the K_a values, are equal to ~ 30.
Note: When competing Na^+ and K^+ ions are transported through thin lipid membranes at identical concentrations, the competitive transport selectivities have lower values, i.e., the Na^+ over K^+ transport selectivity of the $(221)C_{10}$-cryptand and the K^+ over Na^+ transport selectivity of the $(222)C_{10}$-cryptand at 25°C are equal to 1.5 and 2.5, respectively [15,16].
Our interpretations of the present data are based upon this observation.

Due to the selectivity of $(221)C_{10}$ for Na^+ over K^+ and its potential ability to carry Ca^{2+} ions under physiological conditions, whereas $(222)C_{10}$ exhibits selectivity for K^+ over Na^+ and might be blocked by barium (K^+-channel blocker), it seemed of interest to investigate the action of such compounds at the cellular level. Until now, it has only been shown that $(221)C_{10}$ was as efficient as nystatin at reversing the cellular Na^+ and K^+ gradients [19], and at stimulating the oxidative metabolism of isolated tubules of rat nephron [20]. However, the molecular mechanisms underlying these effects have not yet been elucidated.

The present study is the first to focus on the effects of ionizable cryptands on K^+ transport by an established cell line, the opossum kidney (OK) cell, de-

rived from the cortex of the American opossum. These cells are known to express several K^+ channels [21–24], and K^+-conductance, via these pathways, is intracellular pH-sensitive and can be blocked by barium [21,23]. Besides, K^+ exit from these cells also occurs via a K/H antiporter that, under normal conditions, would participate in intracellular pH regulation [25], together with an amiloride-sensitive Na/H exchanger [26]. As in other animal cells, a major part of K^+ entry into OK cells is ensured by the Na,K-ATPase and, to a limited extent, by a Na,K/2Cl cotransporter. Because the complex interplay between the intracellular pH and Na^+, and the K^+ fluxes across their apical and basolateral membranes, is well known, the OK cells present a unique opportunity to study the effects of two ionophores transporting Na^+, K^+ and H^+ ions, i.e., the Na-selective $(221)C_{10}$ and the K-selective $(222)C_{10}$. This study quantifies the effects of the $(221)C_{10}$- and $(222)C_{10}$-cryptands on: The ouabain-sensitive ^{86}Rb influx, which is attributable to the functioning of the Na,K-ATPase; the maximal rate of the Na,K-ATPase phosphatase activity (V_{max}); the cells' ATP content and ^{86}Rb efflux, in the presence or absence of barium (K^+-channel blocker) and amiloride (Na^+-channels and Na/H antiporter blocker). The effects of valinomycin, FCCP and nystatin on these parameters have also been quantified and compared to those of the two cryptands. The results are discussed in terms of the ionic selectivities of the ionophores and of their structural characteristics. They are interpreted in terms of the ionophore's action at the plasma membrane (nystatin), or at the mitochondrial membrane (valinomycin) or both [$(221)C_{10}$, $(222)C_{10}$ and FCCP].

2. Materials and methods

2.1. Materials

$^{86}RbCl$ (0.5–10 mCi/mg Rb) was obtained from Amersham (Buckinghamshire, UK) and the Quicksafe A scintillator was from Zinsser Analytic (Frankfurt, Germany). The ATP assay kit was purchased from Calbiochem (San Diego, CA, USA) and FCCP was from Boëhringer (Mannheim, Germany). $(221)C_{10}$-cryptand, $(222)C_{10}$-cryptand, absolute ethanol for spectroscopy, NaCl, KCl, $MgSO_4$, $CaCl_2$, 2 H_2O, D-glucose, NaOH, KOH, $MgCl_2$, 6 H_2O, perchloric acid (70–72%), $BaCl_2$, 2 H_2O, NaH_2PO_4, H_2O, hydrochloric acid (37%) and ammonium heptamolybdate tetrahydrate were obtained from Merck (Darmstadt, Germany). Ouabain, amiloride, valinomycin, nystatin, dimethyl sulfoxide, trypan blue, ATP(2Na), BRIJ (polyoxyethylene-20-cetyl ether), triton X-100 and HEPES were purchased from Sigma (St. Louis, MO, USA). D-Mannitol and sulfuric acid (98% min.) were from Prolabo (Paris, France) and HCl was from Prosciences (Paris, France). Culture media and reagents were from Gibco-BRL (Paisley, UK), except for sodium selenite, insulin, transferrin and hydrocortisone, which were purchased from Sigma. Plastic-ware was from Costar (Cambridge, MA, USA).

The liquid scintillation counter was an LKB-Wallac 1209 RackBeta from EGG Wallac (Turku, Finland) and was used for β-emission counting. The production of inorganic phosphate by the cell homogenates was measured at 390 nm using a Uvikon 930 spectrophotometer from Kontron Instruments (Milan, Italy). The light emitted by the luciferin/luciferase system, which was used to quantify the ATP content of the supernatants, was measured using a luminometer (LUMAT LK 9501/16) from EGG Berthold (Bad Wilbad, Germany).

$(221)C_{10}$-cryptand, $(222)C_{10}$-cryptand, valinomycin and FCCP were dissolved in absolute ethanol. Nystatin, ouabain and amiloride were dissolved in dimethyl sulfoxide, and triton X-100 and $BaCl_2$, 2 H_2O in water. Final overall ethanol plus dimethyl sulfoxide concentrations never exceeded 0.6%; control experiments demonstrated that these solvent concentrations had no detectable effect on the parameters measured. The percentage of viable cells was over 99% under all experimental conditions under study (trypan blue exclusion procedure).

2.2. Cell culture

OK cells (passages 19–27) were grown to confluence in 6- or 24-well trays in a medium consisting of a 1:1 (v/v) mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium, which, in addition, contained 21.5 mM $NaHCO_3$, 15 mM HEPES, 4 mM L-glutamine, 0.5 mM sodium pyru-

vate, 100 nM sodium selenite, 50 IU/ml penicillin, 50 μg/ml streptomycin, 10 μg/ml insulin, 5 μg/ml transferrin, 50 nM hydrocortisone and 2.5% fetal calf serum. Cultures were maintained at 37°C in an atmosphere of 5% CO_2/95% air, and the medium was changed on alternate days. Cells were fed with hormone- and serum-free medium 24 h before experiments were started, and they were studied when confluent, after six to seven days. They were subcultured weekly using 0.05% trypsin and 0.02% EDTA in saline solution (Puck-modified) and seeded (approx. $1 \cdot 10^6$ cells/disk) onto 100-mm plastic petri dishes. Since trypsin might modify K^+ transport systems, it should be stressed that any tryptic activity present in the initial passaging would be inhibited by the added serum and reduced to negligible levels by dilution, as each culture was fed three times before experimental study, with all old media being aspirated and replaced by fresh medium.

2.3. Unidirectional K^+ flux

Unidirectional K^+ flux was measured using ^{86}RbCl following procedures adapted from the methods by Cheval and Doucet [27] for K^+ influx measurements, and by Venglarik et al. [28] for K^+ efflux measurements. Cells were preincubated in a buffered solution adjusted to 340 mOsm with D-mannitol and containing (in mM): 137 NaCl, 5.4 KCl, 1.2 $MgSO_4$, 1 D-glucose, 1 $CaCl_2$, 14 HEPES (pH 6.4 or 7.4). Then, transport was induced by incubation in the same buffer without D-glucose and supplemented with the components to be investigated (inhibitors, ionophores, tracer).

The ^{86}Rb influx was determined as follows: after removal of the culture medium, the monolayers were washed three times with 500 μl/well of the buffer solution and were preincubated for variable times at 37°C in 250 μl of buffer, with or without ionophores and inhibitors. After removing this solution, ^{86}Rb uptake was induced by adding 250 μl of the D-glucose-free buffer, supplemented with 0.2 μCi/ml ^{86}RbCl (0.5–10 mCi/mg Rb) in the presence or absence of inhibitors. At 3 min, the uptake was stopped by washing the monolayers three times with 500 μl/well of ice-cold rinsing solution, adjusted to 340 mOsm with D-mannitol and containing (in mM): 137 *N*-methyl glucamine, 3 $BaCl_2$, 1 ouabain, 1 amiloride, 14 HEPES (pH 6.4 or 7.4). The cells were then solubilized in 500 μl/well of 0.5% triton X-100 and the tracer activity was measured by liquid scintillation counting using 400 μl aliquots. The protein concentration was measured according to the method of Bradford [29]. Ouabain-sensitive ^{86}Rb influx, calculated as the difference between the values of the ^{86}Rb influxes measured in the presence or absence of 1 mM ouabain, was used as an indicator of Na,K-ATPase activity. It was expressed in nmoles K^+/mg protein/3 min.

^{86}Rb efflux was determined as follows: after removal of the culture medium, the monolayers were washed three times with 500 μl/well of the buffer solution and were preincubated for 60 min at 37°C in 250 μl of buffer solution containing 0.4 μCi/ml ^{86}RbCl, in the presence or absence of inhibitors. After removing the loading solution by suction, the monolayers in one of the 24-well plates experimented on were washed three times with 500 μl/well of ice-cold rinsing solution. Then, the cells were solubilized in 500 μl/well of 0.5% triton X-100, and the tracer activity of the cell layer was determined by liquid scintillation counting, in order to determine the total ^{86}Rb load (qRb_0) under each experimental condition. Following removal of the loading solution, the monolayers in the wells of the other plates being used for experiments were washed four times with 500 μl/well of warmed D-glucose-free buffer solution (1.5 min total time). Then, Rb efflux was induced by adding 250 μl of the glucose-free buffer, with or without the ionophores and the inhibitors. At 5 min, the efflux was stopped by rinsing the monolayers three times, as indicated above. Cells were then solubilized in 0.5% triton X-100 and the tracer activity remaining in the cell layer (qRb_t) was counted. Results are expressed as the percentage of the total ^{86}Rb load that was released in 5 min, i.e., $100(qRb_0 - qRb_t)/qRb_0$.

2.4. Maximal rate of the Na,K-ATPase phosphatase activity (V_{max})

Ouabain-sensitive phosphatase activity was measured according to the method of Post and Sen [30] on cell homogenates with 1 mM ouabain and 3 mM ATP. The release of inorganic phosphate from ATP was measured by spectrophotometric absorption at

390 nm using a procedure adapted from the method of Atkinson et al. [31]. The results are expressed in μmoles Pi/mg protein/h.

2.5. Cell adenosine triphosphate content (ATPi)

For these experiments, cells were grown to confluence in six-well plates. After removal of the culture medium, the monolayers were washed three times with 2 ml/well of the buffer solution and were then preincubated for 40 min at 37°C in 1 ml of buffer, with or without ionophores and inhibitors. After removing this solution, 1 ml/well of the glucose-free buffer, with or without inhibitors, was added for 3 min, i.e., ^{86}Rb influx procedure without tracer. The monolayers were then washed three times with 2 ml/well of ice-cold rinsing solution and twice with 2 ml/well of ice-cold 0.9% NaCl, and were extracted with 1 ml/well of ice-cold 0.3 N perchloric acid. After scraping off the monolayers from the bottom of the wells with a plastic cell scraper, the extracts were centrifuged for 30 min at 4°C (14 000 rpm $\sim 88\ g$) and the supernatant was collected. The remaining protein pellet in the tube was dissolved in 1 ml of NaOH and the protein concentration was determined according to Bradford [29]. The supernatant was neutralized to pH 7 with 3 M and 0.3 M KOH at 4°C, and was maintained for 30 min at this temperature and centrifuged for 30 min at 4°C and 14000 rpm. ATP was measured in the supernatant using Calbiochem's ATP assay kit, which uses the luciferin/luciferase system. The emitted light produced by the enzymatic reaction, which is proportional to the ATP content of the sample, was measured using a luminometer. The results are expressed in nmoles ATP/mg protein.

2.6. Statistical treatment

Data are expressed as means ± SEM. Means were compared by variance analysis with repeated measurements for the ionophore factor followed either by a Dunnet's test for comparisons to the control (''ionophore effect'') or the standard conditions (''inhibitor effect''), or by a Newman-Keul's test for comparisons between the ionophores (''inter-ionophores differences'') or the pH (''inter-pH differences''). Differences were considered as significant for $P < 0.05$.

3. Results

3.1. Ouabain-sensitive ^{86}Rb influx

At 40 min preincubation, about 90% of the overall Rb influx observed at pH 7.4 was ouabain-sensitive and, therefore, was attributable to the functioning of the Na,K-ATPase. In many cell types, K^+ enters cells via a Na,K/2Cl cotransport system. The presence of such a cotransport system in OK cells was therefore checked, using bumetanide as an inhibitor (not shown). At 500 μM bumetanide and a preincubation time of 40 min, only 7% of the overall Rb influx was ouabain-resistant and bumetanide-sensitive and, therefore, attributable to the Na,K/2Cl cotransporter. Thus, bumetanide was not used in future experiments.

In preliminary experiments, the effect of cryptands on ouabain-sensitive Rb influx in OK cells was studied after preincubating the monolayers in HEPES (pH 7.4) for various times and carrier concentrations. It was found that (221)C_{10} and (222)C_{10} inhibited ouabain-sensitive Rb influx in a time- and concentration-dependent manner (not shown). Regardless of the cryptand concentration, in the 2 to 20 μM range (or 5.4 to 54.0 nmoles/mg protein), the inhibition reached a steady-state within 40 min of preincubation.

The effects of the Na-selective (221)C_{10} and K-selective (222)C_{10} (10 μM or 27 nmoles/mg protein) on the ouabain-sensitive Rb influx were then compared to those of valinomycin, a K^+-selective antibiotic (1 μM or 3 nmoles/mg protein), nystatin, a channel-forming compound that increases the permeability of membranes to Na^+, K^+ and H^+ ions (50 μM or 125 nmoles/mg protein), and of the protonophore FCCP, an uncoupler of oxidative phosphorylation (10 μM or 26 nmoles/mg protein). The experiments were performed with or without inhibitors (3 mM barium, a K^+-channel blocker, or 1 mM amiloride, a Na^+-channel and Na/H antiporter blocker), and 40 min was chosen for the preincubation period so that the effects of all the ionophores and inhibitors were nearly at steady-state levels (Fig. 1).

Under standard conditions, ouabain-sensitive Rb influx was equal to 247.8 ± 19.0 nmoles/mg pro-

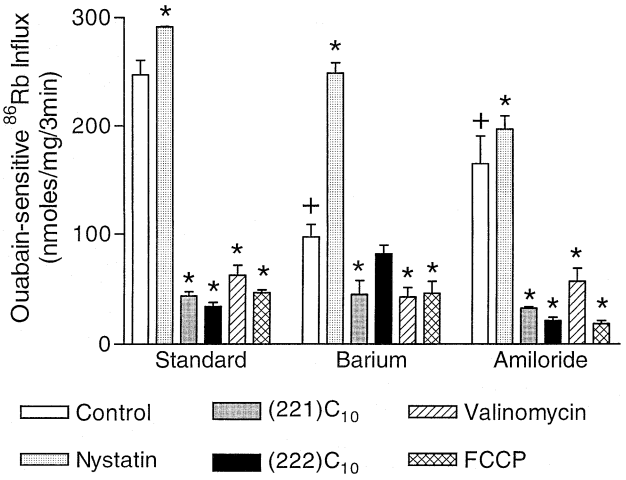

Fig. 1. Effect of ionophores and inhibitors on the ouabain-sensitive ^{86}Rb influx in OK cells. OK cells were preincubated for 40 min in HEPES buffer (pH 7.4), with or without nystatin (50 μM or 125 nmoles/mg protein), $(221)C_{10}$ (10 μM or 27 nmoles/mg protein), $(222)C_{10}$ (10 μM or 27 nmoles/mg protein), valinomycin (1 μM or 3 nmoles/mg protein) and FCCP (10 μM or 26 nmoles/mg protein), in the presence or absence of 3 mM barium or 1 mM amiloride. Ouabain-sensitive Rb influx was measured after 3 min incubation in glucose-free buffer (pH 7.4) containing 0.2 μCi/ml ^{86}RbCl, with or without 3 mM barium or 1 mM amiloride. Each value (±SEM) is the mean of five–seven experiments (in duplicate) for the control, $(221)C_{10}$, $(222)C_{10}$ and valinomycin, two–four experiments (in duplicate) for FCCP and two experiments (in duplicate) for nystatin. The statistical significance (at least $P < 0.05$) of the ionophore effect (*), i.e., ionophore vs. appropriate control, and of the inhibitor effect (+), i.e., control plus inhibitor vs. control standard, are indicated on the figure.

tein/3 min. This value was significantly decreased by 3 mM barium (61%) and 1 mM amiloride (34%).

Whether or not inhibitors were present, nystatin induced a significant increase (≥ 20%) of the ouabain-sensitive Rb influx, whereas all of the other ionophores decreased this influx significantly (50–80%), with the exception of $(222)C_{10}$ in the presence of barium. In the latter case, the stability of the complexes formed with Ba^{2+} being 10^4 times greater than with K^+ [14], the binding and transport of K^+ ions by $(222)C_{10}$ were prevented (Table 1).

3.2. Maximal rate of the Na,K-ATPase phosphatase activity (V_{max})

In order to check if the ouabain-sensitive Rb influx decrement induced by cryptands, valinomycin and FCCP was due to a direct interaction between the ionophores and the Na,K-ATPase or its lipidic environment, the maximal rate of the Na,K-ATPase phosphatase activity (V_{max}) of OK cell homogenates was quantified at 37°C and pH 7.4, in the presence and absence of the ionophores. The V_{max} of the phosphatase activity of the enzyme was determined at final substrate concentrations of 120 mM Na^+, 20 mM K^+ and 3 mM ATP (Mg/ATP = 1.2), in the presence or absence of 1 mM ouabain. The ionophore concentrations used here were the same as in Rb influx experiments, when expressed in nmoles/mg protein (Fig. 2). Statistical treatment of the data indicated that none of the ionophores had a significant effect on the V_{max} value.

3.3. Adenosine triphosphate cell content (ATPi)

In an attempt to explain, at the molecular level, the ouabain-sensitive Rb influx inhibition by an uncoupling effect of cryptands, valinomycin and FCCP, the ATPi content of OK cells was quantified following a 40-min preincubation of the cell monolayers in

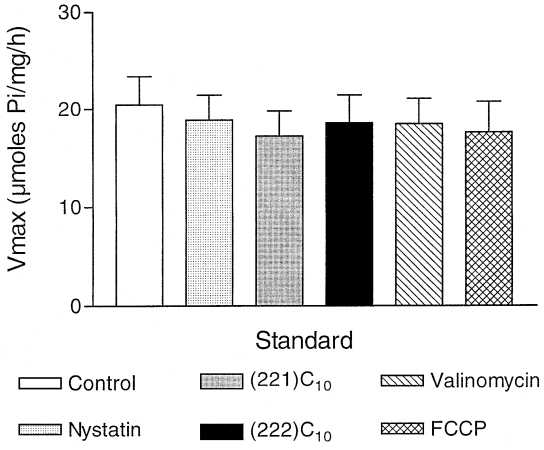

Fig. 2. Effect of ionophores on the maximal rate of Na,K-ATPase phosphatase activity (V_{max}) in OK cells. The maximal rate of Na,K-ATPase phosphatase activity of OK cell homogenates was measured according to the method of Post and Sen [30], with or without nystatin (6.23 μM or 125 nmoles/mg protein), $(221)C_{10}$ (1.35 μM or 27 nmoles/mg protein), $(222)C_{10}$ (1.35 μM or 27 nmoles/mg protein), valinomycin (0.12 μM or 3 nmoles/mg protein) and FCCP (1.30 μM or 26 nmoles/mg protein). Each value (±SEM) is the mean of five experiments (in duplicate). No statistically significant differences were found between the ionophores and the control.

HEPES (pH 7.4), with or without inhibitors and ionophores, and a 3-min incubation in glucose-free HEPES (pH 7.4), with or without inhibitors, i.e., under the same conditions as used for Rb influx experiments performed without tracer.

Fig. 3 shows the effects of the various ionophores and inhibitors on the cells' ATP content. Under standard conditions (absence of inhibitor), the ATPi content of the control was equal to 40.7 ± 7.4 nmoles/mg protein. This value was not significantly modified by 3 mM barium (37.6 ± 6.4 nmoles/mg protein), 1 mM amiloride (41.0 ± 7.5 nmoles/mg protein) or 1 mM ouabain (41.6 ± 7.5 nmoles/mg protein). Also, under standard conditions, nystatin did not significantly modify the ATPi content, whereas $(221)C_{10}$, $(222)C_{10}$, valinomycin and FCCP decreased this content significantly by 47, 75, 72 and 88% of the control value, respectively. In addition,

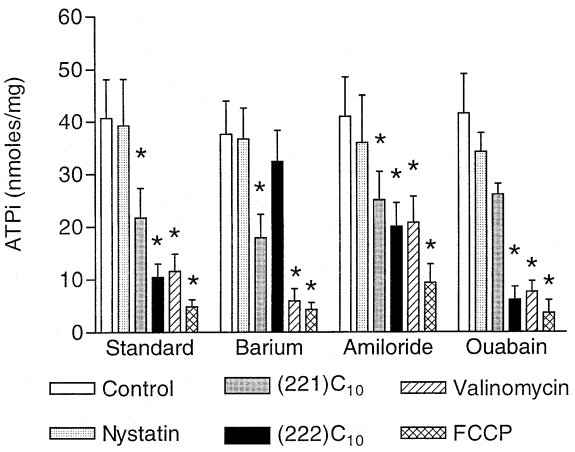

Fig. 3. Effect of ionophores and inhibitors on the ATP content (ATPi) of OK cells. OK cells were preincubated for 40 min in HEPES buffer (pH 7.4), with or without nystatin (50 μM or 125 nmoles/mg protein), $(221)C_{10}$ (10 μM or 27 nmoles/mg protein), $(222)C_{10}$ (10 μM or 27 nmoles/mg protein), valinomycin (1 μM or 3 nmoles/mg protein) and FCCP (10 μM or 26 nmoles/mg protein), in the presence or absence of 3 mM barium, 1 mM amiloride or 1 mM ouabain. They were next incubated for 3 min in glucose-free buffer (pH 7.4) in the presence of the corresponding inhibitor, i.e., the Rb influx procedure performed without tracer, and then checked for the cells' ATP content. Each value (±SEM) is the mean of five–six experiments, except for the ionophores in the presence of ouabain ($n = 2$). The statistical significance (at least $P < 0.05$) of the ionophore effect (*), i.e., ionophore vs. appropriate control, and of the inhibitor effect (+), i.e., control plus inhibitor vs. control standard, are indicated on the figure.

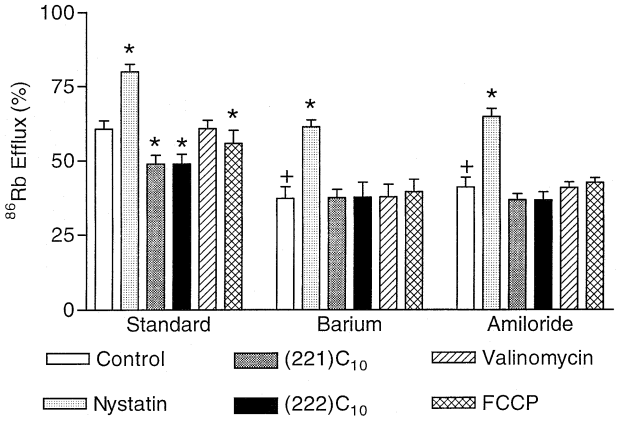

Fig. 4. Effect of ionophores and inhibitors on ^{86}Rb efflux from OK cells. OK cells were preloaded for 1 h in HEPES buffer (pH 7.4) containing 0.4 μCi/ml $^{86}RbCl$, in the presence or absence of 3 mM barium or 1 mM amiloride. Rb efflux was measured after an incubation period of 5 min in the glucose-free buffer (pH 7.4), with or without nystatin (50 μM or 125 nmoles/mg protein), $(221)C_{10}$ (10 μM or 27 nmoles/mg protein), $(222)C_{10}$ (10 μM or 27 nmoles/mg protein), valinomycin (1 μM or 3 nmoles/mg protein) and FCCP (10 μM or 26 nmoles/mg protein), in the presence or absence of 3 mM barium or 1 mM amiloride. Each value (±SEM) is the mean of four–six experiments (in duplicate) for the control, $(221)C_{10}$, $(222)C_{10}$ and valinomycin, and of two–four experiments (in duplicate) for FCCP and nystatin. The statistical significance (at least $P < 0.05$) of the ionophore effect (*), i.e., ionophore vs. appropriate control, and of the inhibitor effect (+), i.e., control plus inhibitor vs. control standard, are indicated on the figure.

the effects of $(222)C_{10}$ and valinomycin on the ATPi content were similar, being significantly greater than that of $(221)C_{10}$ and significantly lower than that of FCCP.

In the presence of 3 mM barium, 1 mM amiloride or 1 mM ouabain, the effects of ionophores resembled those observed under standard conditions, except that: (i) $(222)C_{10}$ no longer caused a significant decrease in the ATPi content in the presence of barium. As indicated above, the irreversible binding of Ba^{2+} ions to the $(222)C_{10}$-cryptand prevented the formation of K^+-complexes; (ii) the magnitude of the $(222)C_{10}$ and valinomycin effects on the ATPi content did not differ significantly from that of $(221)C_{10}$ in the presence of amiloride and (iii) the effect of $(221)C_{10}$ was not found to be significant in the presence of ouabain, probably because of the limited number of experiments performed under these conditions ($n = 2$).

3.4. ^{86}Rb efflux

In an attempt to quantify the effect of the ionophores at the plasma membrane, the efflux of ^{86}Rb from OK cells was measured after loading the cells for 1 h in HEPES buffer (pH 7.4), with or without inhibitors. Fig. 4 compares the effects of cryptands to those of the other ionophores when acting on Rb efflux for 5 min.

Under standard conditions, Rb efflux was stimulated to a high and significant extent by nystatin (32%), was unmodified by valinomycin, and was significantly inhibited by (221)C_{10} (19%), (222)C_{10} (19%) and FCCP (10%).

The control Rb efflux was significantly decreased by 3 mM barium (39%) and 1 mM amiloride (32%). In the presence of these inhibitors, the significant stimulation of Rb efflux by nystatin persisted, whereas the other ionophores were without effect.

When lowering the pH from 7.4 to 6.4, control Rb efflux was decreased insignificantly, by 14% of its value at pH 7.4, i.e., nearly the magnitude of the inhibition induced here by cryptands at pH 7.4 (19%) (Table 2). The Rb efflux was still stimulated by nystatin and unchanged by valinomycin, while the inhibition by (221)C_{10}, (222)C_{10} and FCCP vanished.

Table 2
pH-Dependence of the effect of ionophores on ^{86}Rb efflux from OK cells

^{86}Rb Efflux (%)	pH 7.4	pH 6.4
Control	60.6 ± 2.8	52.3 ± 4.3
(221)C_{10}	48.9 ± 2.9[a]	51.0 ± 2.0
(222)C_{10}	48.9 ± 3.2[a]	52.6 ± 0.6
Valinomycin	60.7 ± 2.8	53.9 ± 1.2
Nystatin	80.0 ± 2.6[a]	72.0 ± 4.8[a]
FCCP	54.3 ± 3.3[a]	51.6 ± 1.2

OK cells were preloaded for 1 h in HEPES buffer (pH 6.4 and 7.4) containing 0.4 μCi/ml ^{86}RbCl. The Rb efflux was measured after a 5-min incubation period in the glucose-free buffer (pH 6.4 and 7.4), with or without nystatin (50 μM or 125 nmoles/mg protein), (221)C_{10} (10 μM or 27 nmoles/mg protein), (222)C_{10} (10 μM or 27 nmoles/mg protein), valinomycin (1 μM or 3 nmoles/mg protein) and FCCP (10 μM or 26 nmoles/mg protein).

Means ± SEM are the result of four–six experiments (in duplicate) at pH 7.4 and of two experiments (in duplicate) at pH 6.4 (ongoing experiments).

[a]The ionophore effect is statistically significant at least at the $P < 0.05$ level, i.e., ionophore vs. appropriate control, is indicated in the table.

4. Discussion

The following discussion is based on the data and comments presented in Table 1. With the exception of Ba^{2+} ions blocking the (222)C_{10} cryptand cavity, all of the other cations were alternative substrates of the two cryptands. Also, due to the high affinity of the (221)C_{10} for Ca^{2+} ions, which are present in the buffer at a low concentration and are almost absent from the cellular media (0.1 μM), it is likely that Ca^{2+} was easily carried inside by (221)C_{10} and, to a lesser extent, by (222)C_{10}. In efflux experiments, it was thought likely that Rb^+ traced the K^+ exit by the two cryptands.

In the present study, about 90% of the Rb influx in OK cells was found to be ouabain-sensitive and, therefore, attributable to the functioning of the Na,K-ATPase. At pH 7.4 and 37°C, the control ouabain-sensitive Rb influx value (Fig. 1) was in agreement with previous data [32,33]. This value was decreased by barium, as already found by Sjodin and Ortiz [34], and by amiloride, two inhibitors that have no effect on the ATPi content (Fig. 3). The K^+-channels blockade by barium leads to down-regulation of the Na,K-ATPase by mechanisms that have not yet been elucidated [34–37]. Amiloride blocks sodium entry through Na^+-channels and via the Na/H antiporter. The latter has been shown to be very active in regulating the internal pH (pH_i) [26] and its blockade by amiloride reduces both the Na_i and pH_i [25]. An inhibition of the Na,K-ATPase activity was therefore expected because (i) Na_i is one of the major determinant of the pump's activity [1]; (ii) intracellular acidosis directly blocks the Na,K-ATPase [38]; (iii) K^+ permeability in OK cells is down-regulated when the pH_i decreases [21]. This, in turn, reduces the pump's activity [35] and (iv) amiloride directly inhibits the Na,K-ATPase [39]. Independent of the presence of inhibitors, all of the ionophores significantly modified the ouabain-sensitive Rb influx

in OK cells, except for (222)C_{10} in the presence of barium. Nystatin increased the control value (in agreement with the data of Middleton et al. [32]) by its ability to increase the Na_i (and K_i) [10]. The inhibition of the ouabain-sensitive Rb influx by the other ionophores (mobile carriers) was independent of their ionic selectivity.

The control V_{max} of the Na,K-ATPase phosphatase activity (20.5 ± 2.9 μmole Pi/mg protein/h) was in agreement with previous data [40], and was not modified by the ionophores under investigation (Fig. 2). Hence, direct interactions with the Na,K-ATPase or its lipidic environment could probably be excluded. Besides, ouabain-sensitive Rb influx inhibition by (222)C_{10} was entirely removed when Ba^{2+} ions irreversibly bound to the cryptand, confirming the absence of any effect of the cryptand's aliphatic chain within the Na,K-ATPase.

Another mechanism for the ouabain-sensitive Rb influx inhibition by the ionophores was their potential uncoupling activity [12] (Fig. 3). ATP synthesis in mitochondria is driven by an electrochemical proton gradient within the inner mitochondrial membrane [2,6]. ATP synthesis blockade may arise from a decrease in the membrane potential (ΔV) and/or of the proton concentration gradient (ΔpH). It is now clearly established that FCCP dissipates the ΔpH [6,41], whereas valinomycin decreases the ΔV [6]. The cryptands' effects on ATP synthesis were expected to reflect a valinomycin-like effect on ΔV, combined with an FCCP-like effect on ΔpH (see below).

The control ATPi content of OK cells had a slightly higher value than that determined by Li et al. [42,43]. Under standard conditions (Fig. 3), the marked decrease in the ATPi content by valinomycin and FCCP is typical of those expected from mitochondrial uncouplers [12]. The K-selective (222)C_{10} reduced the ATPi content to the same level as valinomycin, whereas (221)C_{10} was less efficient. Several reasons may have accounted for this result: (i) (221)C_{10} possesses a Na^+ over K^+ selectivity and, therefore, its ability to decrease the ΔV may have been less than that of (222)C_{10}; (ii) the ionization constants of (221)C_{10} are higher than those of (222)C_{10} [14]. (221)C_{10}, therefore, may have reduced the ΔpH to a lesser extent than (222)C_{10} and (iii) (221)C_{10} can bind and transport Ca^{2+} ions [14,44]. (221)C_{10} may have carried Ca^{2+} ions into the mitochondria, thus stimulating ATP synthesis [45]. Due to the linear ouabain-sensitive Rb influx vs. ATPi content relationship [46,47], the large inhibition of ouabain-sensitive Rb influx by (221)C_{10} was not mediated entirely through an effect on ATP synthesis. In addition, (221)C_{10} may have reduced the ouabain-sensitive Rb influx (i) by exchanging K^+ for H^+ ions across the plasma membrane (see Rb efflux discussion), an event which decreased the pH_i and, in turn, the Na,K-ATPase activity [38] and (ii) possibly by increasing the free Ca_i, thus decreasing the Na,K-ATPase activity [48–50]. In summary, it is likely that the inhibition of ouabain-sensitive Rb influx by (222)C_{10} was due mainly to its effect on ATP synthesis, whereas that by (221)C_{10} was mediated through its combined effects on ATP synthesis and on the pH_i- and Ca_i-dependent Na,K-ATPase activity.

In an attempt to quantify the effect of the ionophores at the plasma membrane, a study of Rb efflux from OK cells was performed (Fig. 4). In agreement with previous data [12,19], the large nystatin effect arose from the dissipation of the K^+ (and Na^+) gradient. Independent of their ionic selectivities, cryptands inhibited Rb efflux, an effect reproduced to a lesser extent by FCCP. This H^+-carrier has been shown to acidify the intracellular pH of OK cells [51]. Cryptands were expected to mimic concomitantly the behavior of the Na/H and K/H antiporters of OK cells. However, due to their internal interface localization ($\sim 90\%$) [17,18], they would have behaved here mainly like K^+–H^+ exchangers. The results at variable pH values (Table 2) strongly suggested that Rb efflux from OK cells was pH-sensitive, as already shown by Graber [52], and that inhibition of Rb efflux by cryptands and FCCP at pH 7.4 arose from the ionophores ability to decrease the pH_i. Besides, no effects of (221)C_{10} and of FCCP could be observed with barium, indicating that these carriers did not modify the K/H antiporter activity of OK cells (Fig. 4). Hence, cryptands and FCCP may have acted mainly on the pH-sensitive barium-blockable K^+-channels of OK cells.

In agreement with the data of Graber [52], barium decreased the control values of Rb efflux by blocking the barium-sensitive K^+-channels [21]. Nystatin increased this efflux, whereas the other ionophores were without effect. This suggested that (221)C_{10} and FCCP could not induce an additional pH effect on the

remaining K^+-conductance, i.e., according to Graber [52], the K/H antiporter. Due to the decrease in the pH_i induced by amiloride, inhibition of the pH_i-sensitive barium-blockable K^+-channels was expected, as occurred in the presence of barium. Hence, the ionophore effects were similar with amiloride and barium.

In conclusion, comparing cryptand effects to those of commonly used ionophores has shown a variety of responses on K^+ transport in OK cells. It has been found that the ionophore's action is localized at the plasma membrane (nystatin), or at the mitochondrial membrane (valinomycin) or both [$(221)C_{10}$, $(222)C_{10}$ and FCCP]. Cryptands were shown to inhibit the pH-sensitive K^+-conductance of OK cells by inducing a K^+–H^+ exchange at the plasma membrane, and to uncouple oxidative phosphorylation by inducing the entry of K^+ and H^+ (and possibly Ca^{2+}) ions into the mitochondria, thereby inhibiting the Na,K-ATPase. Moreover, their ability to inhibit ATP synthesis to different levels, together with the specific removal of the $(222)C_{10}$ effects by barium, arose from their different ionic selectivities only. This study emphasizes the importance of understanding metabolic and transport effects of ionizable ionophores, such as cryptands. The reversal of Na^+ and K^+ gradients of proximal tubules by $(221)C_{10}$, which was previously interpreted as occurring by cation transport across the plasma membrane [19], thus appears to have arisen from inhibition of Na,K-ATPase, through changes at the mitochondrial and plasma membranes.

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References

[1] A.I. Katz, Am. J. Physiol. 242 (1982) F207–F219.
[2] B. Alberts, D. Bray, J. Lewis, M. Raff, K. Roberts, J.D. Watson, in: Molecular Biology of the Cell, Garland, New York, 1994, pp. 507–549.
[3] Y.A. Ovchinnikov, V.T. Ivanov, A.M. Shkrob, in: Membrane-Active Complexones, BBA Library, Vol. 12, Elsevier, Amsterdam, 1974, pp. 331–406.
[4] B.C. Pressman, Biological applications of ionophores, Annu. Rev. Biochem. 45 (1976) 501–530.
[5] W.C. McMurray, R.W. Begg, Arch. Biochem. Biophys. 84 (1959) 546–548.
[6] A.L. Lehninger, D.L. Nelson, M.M. Cox, in: Principles of Biochemistry, Worth, New York, 1993, pp. 542–597.
[7] J. Cunarro, M.W. Weiner, Biochim. Biophys. Acta 387 (1975) 234–240.
[8] H. Murer, U. Hopfer, R. Kinne, Biochem. J. 154 (1976) 597–604.
[9] A. Cass, A. Finkelstein, V. Krespi, J. Gen. Physiol. 56 (1970) 100–124.
[10] A. Cass, M. Dalmark, Nat. New Biol. 244 (1973) 47–49.
[11] S.J. Harris, R.S. Balaban, L. Barrett, L.J. Mandel, J. Biol. Chem. 256 (1981) 10319–10328.
[12] S.P. Soltoff, L.J. Mandel, J. Membr. Biol. 94 (1986) 153–161.
[13] J.M. Lehn, Struct. Bond. 16 (1973) 1–69.
[14] J.M. Lehn, J.P. Sauvage, J. Am. Chem. Soc. 97 (1975) 6700–6707.
[15] A. Loiseau, M. Hill, G.M. Mulliert, M. Castaing, Biochim. Biophys. Acta 1235 (1995) 21–32.
[16] A. Loiseau, M. Hill, L. René-Corail, M. Castaing, Biochim. Biophys. Acta 1238 (1995) 107–117.
[17] M. Castaing, F. Morel, J.M. Lehn, J. Membr. Biol. 89 (1986) 251–267.
[18] M. Castaing, J.M. Lehn, J. Membr. Biol. 97 (1987) 79–95.
[19] R.M. Rajerison, M. Faure, F. Morel, VIe Congrès Européen de Physiologie Rénale, 23–28 May 1988, Varna, Bulgaria.
[20] A. Hus-Citharel, F. Morel, VIe Congrès Européen de Physiologie Rénale, 23–28 May 1988, Varna, Bulgaria.
[21] J.S. Schwegler, A. Henner, S. Silbernagl, Pflüg. Arch. Eur. J. Physiol. 415 (1989) 183–190.
[22] T. Ohno-Shosaku, T. Kubota, J. Yamaguchi, M. Fukase, T. Fujita, M. Fujimoto, Pflüg. Arch. Eur. J. Physiol. 413 (1989) 562–564.
[23] T. Ohno-Shosaku, T. Kubota, J. Yamaguchi, M. Fujimoto, Pflüg. Arch. Eur. J. Physiol. 416 (1990) 138–143.
[24] H.A. Kolb, Renal Physiol. Biochem. 13 (1990) 26–36.
[25] M. Graber, E. Pastoriza-Munoz, Am. J. Physiol. 265 (1993) F773–F783.
[26] A. Moran, M.H. Montrose, H. Murer, Biochim. Biophys. Acta 969 (1988) 48–56.
[27] L. Cheval, A. Doucet, Am. J. Physiol. 259 (1990) F111–F121.
[28] C.J. Venglarik, R.J. Bridges, R.A. Frizzell, Am. J. Physiol. 259 (1990) C358–C364.

[29] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[30] R.L. Post, A.K. Sen, Methods Enzymol. 10 (1967) 762–768.
[31] A. Atkinson, A.D. Gatenby, A.G. Lowe, Biochim. Biophys. Acta 320 (1973) 195–204.
[32] J.P. Middleton, W.A. Khan, G. Collinsworth, Y.A. Hannun, R.M. Medford, J. Biol. Chem. 268 (1993) 15958–15964.
[33] S.P. Soltoff, L.J. Mandel, J. Gen. Physiol. 84 (1984) 601–622.
[34] R.A. Sjodin, O. Ortiz, J. Gen. Physiol. 66 (1975) 269–286.
[35] B.C. Kone, H.R. Brady, S.R. Gullans, Proc. Natl. Acad. Sci. U.S.A. 86 (1989) 6431–6435.
[36] A.G. Ramsay, D.L. Gallagher, R.L. Shoemaker, G. Sachs, Biochim. Biophys. Acta 436 (1976) 617–627.
[37] W. Nagel, Biochim. Biophys. Acta 552 (1979) 346–357.
[38] D.C. Eaton, K.L. Hamilton, K.E. Johnson, Am. J. Physiol. 247 (1984) F946–F954.
[39] S.P. Soltoff, L.J. Mandel, Science 220 (1983) 957–959.
[40] B. Escoubet, K. Djabali, C. Amiel, Am. J. Physiol. 256 (1989) C322–C328.
[41] B. Alberts, D. Bray, J. Lewis, M. Raff, K. Roberts, J.D. Watson, in: Molecular Biology of the Cell, Garland, New York (1994) pp. 655–696.
[42] H.-Y. Li, L.-J. Dai, C. Krieger, G.A. Quamme, Biochim. Biophys. Acta 1181 (1993) 307–315.
[43] H.-Y. Li, L.-J. Dai, C. Krieger, G.A. Quamme, J. Lab. Clin. Med. 122 (1993) 260–272.
[44] M. Kirch, in: Thèse de Doctorat ès Sciences Physiques Strasbourg, France, 1980, pp. 38–95.
[45] R.M. Denton, J.G. McCormack, Am. J. Physiol. 249 (1985) E543–E554.
[46] T. Ikehara, H. Yamaguchi, K. Hosokawa, T. Sakai, H. Miyamoto, J. Cell Physiol. 119 (1984) 273–282.
[47] S.P. Soltoff, L.J. Mandel, J. Gen. Physiol. 84 (1984) 643–662.
[48] D.R. Yingst, Ann. Rev. Physiol. 50 (1988) 291–303.
[49] W. Huang, A. Askari, Arch. Biochem. Biophys. 216 (1982) 741–750.
[50] D.R. Yingst, R.M. Jones, D.M. Polasek, Biophys. J. 49 (1986) 549a.
[51] C. Knoblauch, M.H. Montrose, H. Murer, Am. J. Physiol. 256 (1989) C252–C259.
[52] M. Graber, Am. J. Kidney Dis. 22 (1993) 436–444.