ELSEVIER

Biochimica et Biophysica Acta 1466 (2000) 361–371

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

# Characterization of Na/Ca exchange in plasmalemmal vesicles from zona fasciculata cells of the bovine adrenal gland

Christian Brunold [a], Annick Guyot [a], Denis Noble [b], Oger Rougier [a], André Bilbaut [a], Carlos Ojeda [c,*]

[a] *Université Claude Bernard, UMR CNRS 5578, Laboratoire de Physiologie des Eléments Excitables, 43 Boulevard du 11 Novembre, 69622 Villeurbanne Cedex, France*

[b] *University Laboratory of Physiology, South Parks Road, OX1 3PT Oxford, UK*

[c] *INSERM U 121, 18 Avenue du Doyen Lépine, 69500 Bron, France*

Received 9 December 1999; received in revised form 14 March 2000; accepted 15 March 2000

**Abstract**

The presence of an Na/Ca exchange system in fasciculata cells of the bovine adrenal gland was tested using isolated plasmalemmal vesicles. In the presence of an outwardly $Na^+$ gradient, $Ca^{2+}$ uptake was about 2-fold higher than in $K^+$ condition. $Li^+$ did not substitute for $Na^+$ and 5 mM $Ni^{2+}$ inhibited $Ca^{2+}$ uptake. $Ca^{2+}$ efflux from $Ca^{2+}$-loaded vesicles was $Na^+$-stimulated and $Ni^{2+}$-inhibited. The saturable part of $Na^+$-dependent $Ca^{2+}$ uptake displayed Michaelis–Menten kinetics. The relationship of $Na^+$-dependent $Ca^{2+}$ uptake versus intravesicular $Na^+$ concentration was sigmoid (apparent $K_{0.5} \approx 24$ mM; Hill number $\approx 3$) and $Na^+$ acted on $V_{max}$ without significant effect on $K_m$. $Na^+$-stimulated $Ca^{2+}$ uptake was temperature-dependent (apparent $Q_{10} \approx 2.2$). The inhibition properties of several divalent cations ($Cd^{2+}$, $Sr^{2+}$, $Ni^{2+}$, $Ba^{2+}$, $Mn^{2+}$, $Mg^{2+}$) were tested and were similar to those observed in kidney basolateral membrane. The above results indicate the presence of an Na/Ca exchanger located on plasma membrane of zona fasciculata cells of bovine adrenal gland. This exchanger displays similarities with that of renal basolateral cell membrane. © 2000 Elsevier Science B.V. All rights reserved.

*Keywords:* Adrenal cell; Na/Ca exchange; Membrane vesicle

## 1. Introduction

Most living cells use a large $Ca^{2+}$ gradient for the generation of an intracellular $Ca^{2+}$ signal which is the link between stimuli occurring at the cell surface and intracellular $Ca^{2+}$-mediated processes. During this $Ca^{2+}$ signal, the internal concentration can go up to $10^{-6}$ M, the source being either internal stores and/or influx of external $Ca^{2+}$ via different pathways at the cell surface membrane. To maintain this large $Ca^{2+}$ gradient, cells must extrude not only the $Ca^{2+}$ that enters during activity but also that which leaks continuously into the cell. This is accomplished by $Ca^{2+}$ pump and a powerful $Ca^{2+}$ extrusion via an Na/Ca exchanger. Although this last system has been extensively described in numerous cell preparations (see for review [1]), very few data are available

Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N′,N′,N′,N′*-tetraacetic acid; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane

* Corresponding author. Fax: +33-04-78-94-68-20;
E-mail: ojeda@physio.univ-lyon1.fr

0005-2736/00/$ – see front matter © 2000 Elsevier Science B.V. All rights reserved.
PII: S0005-2736(00)00202-9

about the existence of an Na/Ca exchanger in the cortical cells of the adrenal gland [2–4]. Preliminary experiments performed in our laboratory using patch-clamp in whole cell configuration suggested the presence of such an exchanger in fasciculata cells isolated from bovine adrenal gland. In this work, we used plasmalemmal vesicles prepared from these cells to demonstrate directly the presence of Na/Ca exchange. We then attempted to characterize this system and compare its biochemical properties with those already described in other preparations. Part of the results has been published in abstract form [5].

## 2. Materials and methods

### 2.1. Chemicals

All chemicals were of the highest purity available from commercial sources. $^{45}CaCl_2$ (59 mCi/ml) was obtained from ICN Biomedicals (Irvine, CA, USA). Prolabo (Paris, France) supplied sucrose, $BaCl_2$, KCl, LiCl, $MnCl_2$, NaCl and $SrCl_2$. Glucose-6-phosphate, ouabain, *para*-nitrophenylphosphate, saponin, tris (hydroxymethyl) aminomethane (Tris), ATP, A23187, ethylenediaminetetraacetic acid (EDTA), ethylene glycol bis(β-aminoethyl ether)-*N′,N′,N′,N′*-tetraacetic acid (EGTA), 3-(*N*-morpholino)propanesulfonic acid (MOPS) and TEA were purchased from Sigma (St. Quentin Fallavier, France). $MgCl_2$, $NaN_3$ and $NiCl_2$ were obtained from Merck (Darmstadt, Germany). $CaCl_2$ (volumetric solution; 1 M) was purchased from BDH Laboratory Supplies (Pool, UK). The water used in this study was purified on ion-exchange column and was checked for contaminating $Ca^{2+}$ (about 0.4 μM). Nitrocellulose membrane filters (0.45 μm; type HAWP) were provided by Millipore (Bedford, MA, USA).

### 2.2. Isolation of plasma membrane vesicles

The bovine adrenal glands were taken from a local slaughter-house. Cortical slices from zona fasciculata were obtained by use of a microtome as previously described [6]. The centrifugation scheme used to purify plasma membrane was a modification of the method described by Rosenthal and Narasimhulu [7] and Kao and Cheung [8]. All of the following procedures were carried out at 4°C and the centrifugal forces were those at the middle of the test tube ($g_{av}$). Typically, 15 g of tissue (30 glands) was suspended in 50 ml of medium A (20 mM MOPS adjusted to pH 7.4 with Tris, 5 mM NaCl, 0.32 M sucrose and 0.5 mM EDTA) and minced with scissors. This suspension was homogenized by five strokes in a loose-fitting Potter (glass homogenizing vessel) followed by three passes in a tight-fitting Potter, filtered through two layers of gauze and then diluted with medium A to give a 5% (w/v) homogenate. The homogenate was centrifuged at $11\,000 \times g$ (11 000 rpm; rotor JA 14 Beckman, Palo Alto, CA, USA) for 20 min. This gave a supernatant S 1 and a pellet P 1, which was discarded. S 1 was then centrifuged at $145\,500 \times g$ (40 000 rpm; rotor Ti 50.1 Beckman) during 60 min to yield a pellet P 2 (the microsomal fraction). Therefore, the microsomal pellet was resuspended in buffer containing 20 mM MOPS/Tris and 160 mM KCl mixed with 2 M sucrose to give a final concentration of 0.91 M sucrose (28% w/w). 10 ml of this suspension was placed in the bottom of a centrifuge tube and overlaid with 5 ml of medium A. Centrifugation at $111\,000 \times g$ (35 000 rpm; rotor Ti 50.1 Beckman) for 150 min yielded a top layer (fraction F I) at the 10/28% interface and a pellet (fraction F II) at the bottom of the tube. Fraction F I, which was enriched in plasma membrane markers, was collected with a syringe, diluted five times in NaCl or KCl media (20 mM MOPS/Tris, pH 7.4, 160 mM NaCl or KCl) and spun down at $145\,500 \times g$ for 60 min. The sediment was resuspended in NaCl or KCl media in small samples (200 μl), at a protein concentration of 10 mg/ml and stored in liquid nitrogen. For marker enzyme measurements, all the fractions obtained at the different steps of this procedure were suspended in medium A and stored at −20°C.

Our population of vesicles was a mixture of inside-out and outside-out ones. We did not test the percentage of each in this population but as assessed by Philipson and Nishimoto [9], it is likely that, in these in vitro experiments, the Na/Ca exchange responded in a symmetrical manner in both populations of vesicles.

### 2.3. Miscellaneous assays

The following marker enzymes were assayed according to previously published methods: Na/K ATPase [10], 5′ nucleotidase [11] and $K^+$-stimulated *para*-nitrophenylphosphatase [12] for plasma membrane; azide-sensitive ATPase [13] for mitochondria and glucose-6-phosphatase [14] for endoplasmic reticulum. Protein was assayed by the method of Lowry et al. [15] using serum albumin as the standard.

### 2.4. Na/Ca exchange activity

For $Na^+$-dependent $Ca^{2+}$ uptake experiments, vesicles were thawed at room temperature and then prepared by incubation at 35°C for 45 min [16] in their respective suspension medium to allow ions to passively equilibrate across plasmalemmal membranes. Unless otherwise indicated, uptake was routinely assayed in media containing 20 mM MOPS/Tris, pH 7.4, 160 mM NaCl or KCl and 50 μM $^{45}Ca^{2+}$ (2 μCi/ml). A 2 μl bead of vesicles (20 μg protein) was placed on the side of a test tube which contained 98 μl of incubation medium. The reaction was started by stirring with a truck-mixer and terminated at the desired time by addition of 4 ml ice-cold stop solution (1 mM $LaCl_3$, 160 mM KCl, 20 mM MOPS/Tris). Vesicles were collected on 0.45 μm filter. The filter was washed twice with 4 ml of stop solution. The radioactivity on the filter was determined by liquid scintillation counting and compared with 5 μl of the incubation mixture. The $K_m$ and $V_{max}$ values of the $Ca^{2+}$ uptake were determined by measuring the initial rate (3 s time points) of $Ca^{2+}$ uptake at different concentrations (1–1000 μM) of $Ca^{2+}$. $Ca^{2+}$ buffers were not used for this purpose because $Ca^{2+}$ chelators are known to alter the properties of the Na/Ca exchange system [17].

For $Ca^{2+}$ efflux experiments, vesicles were loaded passively with 50 μM $^{45}CaCl_2$ in a mixture containing 20 mM MOPS/Tris, pH 7.4, and 160 mM TEA. The suspension was allowed to equilibrate for 4 h at 35°C [16]. The $Ca^{2+}$ efflux was measured after diluting 60 times the loaded vesicles in a buffer containing 20 mM MOPS/Tris, pH 7.4, 0.5 mM EGTA to prevent back flow [18] and 160 mM of the desired counter ions. The reaction was stopped by 4 ml of ice-cold solution and the radioactivity associated with the vesicles was assayed by the filtration technique as described above.

### 2.5. Data analysis

Results are expressed as 'absolute uptake' (nmol/mg of protein) for time course studies or as 'absolute velocities' (nmol/mg of protein/s), measured in initial velocity condition, for saturation kinetic experiments. The data are presented as mean (±S.E.M.) when indicated in the text from triplicate experiments performed with at least 2–3 different membrane preparations.

Velocity data as a function of $Ca^{2+}$ concentration were fitted using a non-linear regression algorithm (Sigma Plot version 4.11; Jandel Corporation) according to the following equation which is the sum of a saturable and a non-saturable component:

$$V = \{V_{max}[Ca^{2+}]/(K_m + [Ca^{2+}])\} + K_{Diff}[Ca^{2+}] \quad (1)$$

where $V_{max}$ is the maximal velocity of uptake, $K_m$ the Michaelis constant and $K_{Diff}$ the apparent diffusional term.

$IC_{50}$ values for the different inhibitors were estimated as described by Trosper and Philipson [19].

### 2.6. Conventions

In the text and in the figures, the incubation conditions are described using the following convention: the labels on the left hand of the fraction bar indicate the intravesicular medium while those on the right hand represent the extravesicular one (incubation medium).

## 3. Results

### 3.1. Plasma membrane characterization

The procedure for plasma membrane isolation was monitored using marker enzymes (Table 1). The fraction found at the 10/28% interface (fraction F I) showed a large enrichment in plasma membrane markers. The 5′ nucleotidase was enriched around

Table 1
Marker enzyme activities in homogenate and F I fractions

| | Protein | 5′ Nucleotidase | K-pNPPase | Na/K ATPase | $N_3$ ATPase | G6Pase |
|---|---|---|---|---|---|---|
| Homogenate | 1116.1 ± 74.2 | 2.1 ± 0.2 | 0.9 ± 0.1 | 28.1 ± 3.5 | 75.1 ± 2.1 | 14.5 ± 0.7 |
| Fraction F I | 3.8 ± 0.1 | 37.6 ± 1.8 | 11.6 ± 0.2 | 273.5 ± 35.7 | 44.5 ± 2.0 | 23.9 ± 0.7 |
| Enrichment | – | 17.9 | 13.4 | 9.7 | 0.6 | 1.7 |
| Yield | 0.3 | 6.2 | 4.6 | 3.3 | 0.2 | 0.6 |

Tissue from zona fasciculata was disrupted to give the homogenate. Fraction F I was separated by differential and sucrose density gradient centrifugations. The procedure was monitored by enzyme activities representative of the different membrane types. Proteins are in mg and the specific activities of 5′ nucleotidase, potassium-stimulated *para*-nitrophenylphosphatase (K-pNPPase), Na/K ATPase, azide-sensitive ATPase ($N_3$ ATPase) and glucose-6-phosphatase (G6Pase) are in nmol/mg protein/min. Enrichment was calculated as the ratio of the specific activity measured in fraction F I to the specific activity measured in the homogenate. Yields are expressed in % of total activity relative to the homogenate.

20 times over the homogenate while the Na/K ATPase and $K^+$-stimulated *para*-nitrophenylphosphatase (two different activities carried by the same enzyme) were enriched around 10 times. The discrepancy between these two plasma membrane markers may reflect sequestration of these activities in different plasma membrane domains or a selective denaturation of Na/K ATPase during the purification [8]. By contrast, glucose-6-phosphatase (a reticulum marker) shows little enrichment and the specific activity of azide-sensitive ATPase (a mitochondria marker) measured in fraction F I is lower than that of the homogenate. Electron microscopy of fraction F I showed membrane fragments which resided in a closed vesicular configuration with diameters ranging from 0.2 to 0.5 μm. Taken together, these results indicate that fraction F I consists of vesicles of plasma membrane origin with little mitochondria or reticulum contamination. This purification procedure yielded 0.63 mg of plasma membrane protein/g of tissue (0.4% of the protein measured in the homogenate) and 7.3% of the total 5′ nucleotidase activity found in the homogenate was recovered in the F I fraction.

The permeability of the plasma membrane vesicles was studied by incubation with saponin. Assuming that sealed vesicles are impermeable to ATP and ouabain, the unmasking of latent Na/K ATPase activity reflects the degree of resealed vesicles [10]. When plasma membranes were submitted to 0.12% of saponin, the Na/K ATPase activity increased from 263.5 nmol/mg protein/min to 700 nmol/mg protein/min. This suggested that 60% of the vesicle population was resealed.

Since organelles like mitochondria are known to contain a Na/Ca exchange system, it was important to assess that the Na/Ca exchange activity was of plasma membrane origin. The relationship between the degree of purification of $Na^+$-dependent $Ca^{2+}$ uptake (which corresponds to the Na/Ca exchanger activity, as it will be shown later) with the relative enrichment of plasma membrane markers was followed in order to determine the localization of the exchanger. As shown in Fig. 1, for separate fractions corresponding to different steps of plasmalemmal preparation (pellet P 1, microsomal fraction P 2, fractions F I and F II), the $Na^+$-dependent $Ca^{2+}$ uptake closely correlated with 5′ nucleotidase activity. This result indicated copurification of $Na^+$-dependent $Ca^{2+}$ uptake and 5′ nucleotidase with increasing purification of plasmalemmal vesicles and that, in our experimental conditions using fraction F I, the plasma membranes were the source of the $Na^+$-dependent $Ca^{2+}$ uptake.

### 3.2. Evidence for $Na^+$-dependent $Ca^{2+}$ accumulation

Fig. 2 shows an experiment where vesicles were incubated for variable times in a solution containing 50 μM $^{45}Ca^{2+}$ either in the presence of an outwardly directed $Na^+$ gradient (Na/K conditions) or with $K^+$ equally distributed (K/K conditions). Whatever the conditions, the $Ca^{2+}$ uptake was linear during the first 4 s (Fig. 2, insert). In the presence of intravesicular $K^+$, 0.5 nmol of $Ca^{2+}$/mg protein was taken up after 15 s and an equilibrium level, 1 nmol of $Ca^{2+}$/mg protein, was reached after 360 s. When $K^+$ was replaced by $Na^+$ (Na/K conditions), a net stimula-

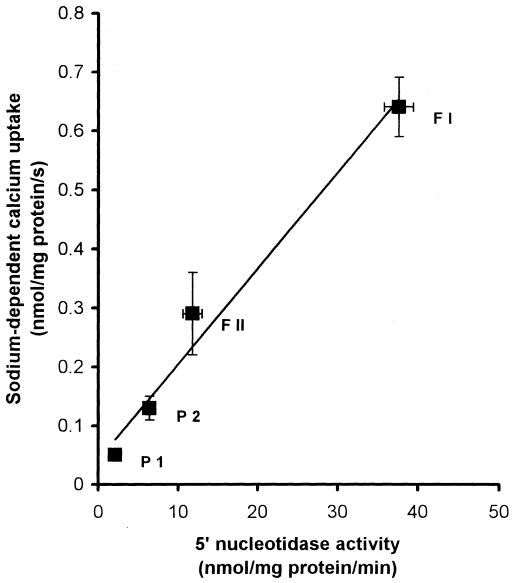


Fig. 1. Relationship between 5′ nucleotidase activity and $Na^+$-dependent $Ca^{2+}$ uptake in various fractions obtained during the isolation procedure. $Na^+$-dependent $Ca^{2+}$ uptake (expressed in nmol/mg protein/s) and 5′ nucleotidase activity (expressed in nmol/mg protein/min) are determined in four fractions: pellet P 1, pellet P 2 (microsomal fraction), fraction F I and fraction F II. Note the correlation between 5′ nucleotidase activity used as plasma membrane marker and $Na^+$-dependent $Ca^{2+}$ uptake.

tion of $Ca^{2+}$ uptake was observed. At 15 s, 1 nmol of $Ca^{2+}$/mg protein was accumulated, a 2-fold higher uptake compared with K/K conditions and this stimulation was observed throughout the time course of the experiment.

To verify that the $^{45}Ca^{2+}$ taken up by the vesicles in Na/K condition had been accumulated against its gradient into the intravesicular space, we tested the effect of the $Ca^{2+}$ ionophore A23187. Thus when $Na^+$-loaded vesicles had accumulated $Ca^{2+}$ for 2 min and were then treated by A23187, vesicle-associated $Ca^{2+}$ dropped to the level observed in K/K conditions (Fig. 2). This efflux of $Ca^{2+}$ demonstrated that this ion was concentrated above the level of the extravesicular space [20].

$Ni^{2+}$ was described by Kimura et al. [21] as a potent inhibitor of Na/Ca exchange. In the presence of 5 mM $Ni^{2+}$, the $Ca^{2+}$ uptake was less than 0.4 nmol of $Ca^{2+}$/mg protein after 120 s incubation and the uptake curve could be fitted by a single exponential (Fig. 2). Furthermore, it must be noted that the $Ca^{2+}$ uptake in the absence of $Na^+$ (K/K conditions, Fig. 2, or Li/K condition, Table 2) was also inhibited by 5 mM $Ni^{2+}$.

The results presented in Table 2 show the effects of various monovalent cations on the $Ca^{2+}$ accumulation by vesicles. When $Na^+$-loaded vesicles were incubated in media which contain an increased $Na^+$ concentration, the $Ca^{2+}$ uptake stimulation was decreased ($Na^+$ 160 mM/$Na^+$ 60 mM conditions) and reached the level observed in K/K conditions when $Na^+$ was equilibrated ($Na^+$ 160 mM/$Na^+$ 160 mM condition). The stimulation loss is probably due to $Na^+$ gradient dissipation or *cis*-$Na^+$ inhibition [22]. $Li^+$-loaded vesicles incubated in $K^+$ media did not accumulate $Ca^{2+}$ (same level as K/K condition), suggesting that $Na^+$ could not be substituted by $Li^+$. An unexpected effect was observed when the incubation took place in TEA buffer. The $Ca^{2+}$ accumulation was greater either in the $Na^+$-stimulated condition (Na/TEA) or control condition (TEA/TEA condition) when compared with experiments realized in the presence of $K^+$.

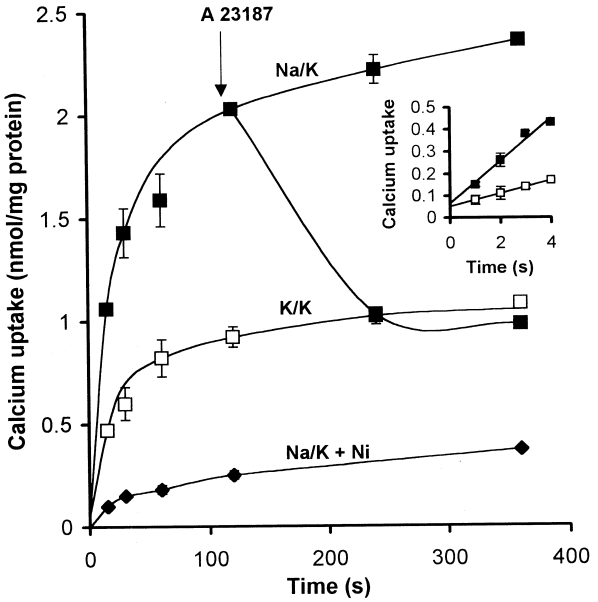


Fig. 2. Time course of $Na^+$-dependent $Ca^{2+}$ uptake. Vesicles preloaded with 160 mM NaCl (solid squares) or 160 mM KCl (open squares) were incubated in a medium containing 50 μM $^{45}Ca^{2+}$ and 160 mM KCl. After 120 s of $Ca^{2+}$ accumulation in $Na^+$-dependent condition, 2 μM of A23187 was added (arrow) and $Ca^{2+}$ uptake was followed. In inhibitory experiments, $Na^+$-loaded vesicles were incubated in medium containing 50 μM $^{45}Ca^{2+}$, 160 mM KCl and 5 mM $NiCl_2$ (diamonds). Insert: $Ca^{2+}$ uptake during the first 4 s.

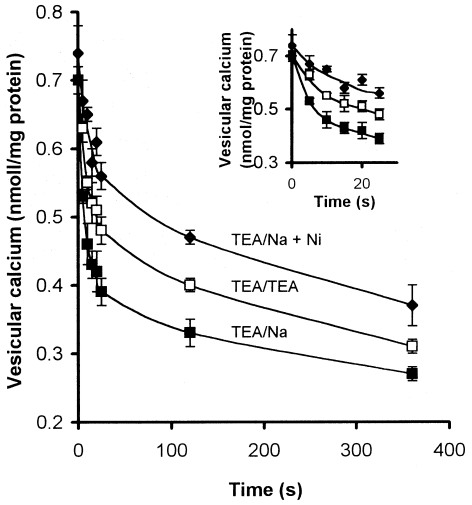


Fig. 3. Effect of $Na^+$ on $Ca^{2+}$ efflux. Vesicles were passively loaded with 50 μM $^{45}CaCl_2$. Efflux was initiated by diluting the vesicle suspension with solution of either 160 mM NaCl (solid squares), 160 mM TEA (open squares) or 155 mM NaCl plus 5 mM $NiCl_2$ (diamonds). Insert shows the first 25 s of the efflux. At early time, the stimulatory effect of $Na^+$ is clearly evidenced as well as the inhibitory effect of 5 mM $Ni^{2+}$.

Therefore, $Ca^{2+}$ uptake appeared to be mediated by three different processes: a passive diffusion (observed in the presence of 5 mM $Ni^{2+}$), a $Na^+$-independent uptake (observed in K/K or Li/K conditions) and a $Na^+$-dependent $Ni^{2+}$-inhibited uptake (observed in Na/K conditions). Taken together, these data suggest that the $Na^+$-stimulated $Ca^{2+}$ uptake is a process mediated by a Na/Ca exchange system.

### 3.3. $Ca^{2+}$ efflux

In order to study the properties of this Na/Ca exchange in the 'normal' mode, $Na^+$-dependent $Ca^{2+}$ efflux from vesicles was measured. To improve this assay, TEA was used as $Na^+$ substitute. Vesicles containing 160 mM TEA were passively loaded with 50 μM of $^{45}Ca^{2+}$ and then diluted in a medium containing either 160 mM TEA (control) or 160 mM $Na^+$ (stimulated condition) and EGTA to avoid back flow. In Fig. 3, a clear $Na^+$-stimulated $Ca^{2+}$ efflux can be detected when we compared TEA/Na with TEA/TEA conditions. This is better seen in the insert (Fig. 3) where the first 25 s are shown on an expanded scale. Five mM $Ni^{2+}$ was able to inhibit the efflux in all cases. These results show that, as in uptake mode, the efflux mode also presents three components: a $Na^+$-dependent, a $Na^+$-independent one and a passive leak observed in the presence of 5 mM $Ni^{2+}$.

### 3.4. External $Ca^{2+}$ concentration effect on initial velocity of $Ca^{2+}$ uptake

Fig. 4A shows a typical experiment of $Ca^{2+}$ uptake as function of extravesicular $Ca^{2+}$ concentration in $Na^+$-dependent and $Na^+$-independent conditions. A clear $Na^+$ stimulation can be observed when comparing Na/K and K/K conditions. At low $Ca^{2+}$ concentration, the relationship between rate of uptake and solute concentration was not linear, suggesting a partial saturability, but at high $Ca^{2+}$ concentration, the velocity of uptake continued to increase. These data

Table 2
Effect of monovalent cations on $Ca^{2+}$ uptake

| Intravesicular medium | Extravesicular medium | $Ca^{2+}$ uptake (nmol/mg protein) | | |
|---|---|---|---|---|
| | | 3 s | 30 s | 120 s |
| $Na^+$ 160 mM | $K^+$ 160 mM | 1.06 ± 0.05 | 1.43 ± 0.02 | 2.03 ± 0.13 |
| $Na^+$ 160 mM | $Na^+$ 60 mM+$K^+$ 100 mM | 0.75 ± 0.11 | 1.17 ± 0.05 | 1.38 ± 0.07 |
| $Na^+$ 160 mM | $Na^+$ 160 mM | 0.58 ± 0.11 | 0.89 ± 0.04 | 0.92 ± 0.05 |
| $Li^+$ 160 mM | $K^+$ 160 mM | 0.67 ± 0.08 | 0.92 ± 0.06 | 1.05 ± 0.09 |
| $K^+$ 160 mM | $K^+$ 160 mM | 0.57 ± 0.02 | 0.60 ± 0.02 | 1.02 ± 0.05 |
| $Na^+$ 160 mM | TEA 160 mM | 0.77 ± 0.08 | 1.89 ± 0.03 | 2.72 ± 0.13 |
| TEA 160 mM | TEA 160 mM | 0.57 ± 0.05 | 1.25 ± 0.10 | 1.71 ± 0.05 |

Vesicles were allowed to equilibrate at 35°C during 45 min in the presence of the cations listed in the table (intravesicular medium). Aliquots (2 μl) were rapidly diluted in 98 μl of incubation medium (extravesicular medium) with 50 μM $^{45}CaCl_2$. At the time points listed in the table, the reaction was quenched by addition of stop solution and $Ca^{2+}$ uptake was determined.

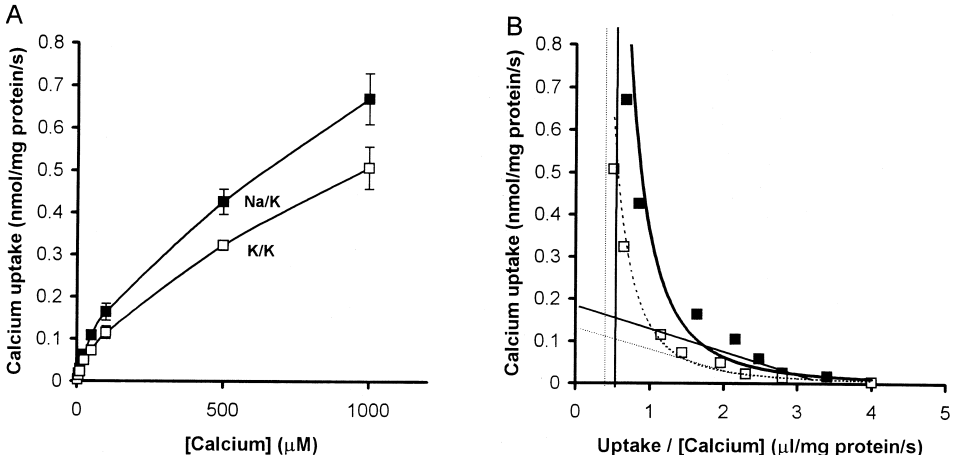


Fig. 4. Dependence of $Ca^{2+}$ uptake on extravesicular $Ca^{2+}$ concentration. (A) Vesicles preloaded with 160 mM NaCl (solid symbols) or 160 mM KCl (open symbols) were incubated in a medium containing 160 mM KCl and the indicated $Ca^{2+}$ concentration. $Ca^{2+}$ uptake was measured after 3 s incubation to determine the initial velocity of uptake. (B) Eadie–Hofstee transformation from data shown in A. The $Na^+$-dependent component was symbolized by thick lines and the $Na^+$-independent one by dotted lines. The vertical lines indicate the values of the non-saturable component as determined by non-linear regression and the straight lines show the saturable components.

suggest that the uptake contains two components, a saturable term and a non-saturable one corresponding to diffusion process or non-specific binding of $Ca^{2+}$ on the external surface of the vesicles. This is shown in Fig. 4B where the same experiment is represented in an Eadie–Hofstee plot. The graph displays a characteristic upward concave pattern. The $K_{Diff}$ value, the non-saturable component, was obtained by non-linear regression and is represented in Fig. 4B by the vertical lines. The $K_{Diff}$ values are not significantly different for Na/K and K/K conditions ($0.51 \pm 0.02$ μl/mg proteins/s and $0.48 \pm 0.01$ μl/mg proteins/s, respectively). From this plot, after correction for the non-saturable component, the comparison between Na/K and K/K conditions shows two nearly parallel lines (Fig. 4B). It turns out that the saturable part either of $Na^+$-dependent or $Na^+$-independent $Ca^{2+}$ uptake obeys simple Michaelis–Menten kinetics and the $Na^+$ effect is on the $V_{max}$ which increases from 0.08 to 0.16 nmol/mg protein/s (data extracted from the graph) without affecting the $K_m$. Results obtained from Eadie–Hofstee plot agree well with those calculated by non-linear regression which gave similar $K_m$ values for $Na^+$-independent and $Na^+$-dependent conditions ($59.21 \pm 6.93$ μM and $70.17 \pm 7.60$ μM, respectively) and a $V_{max}$ value which increased from $0.09 \pm 0.01$ nmol/mg proteins/s in K/K conditions to $0.19 \pm 0.01$ nmol/mg proteins/

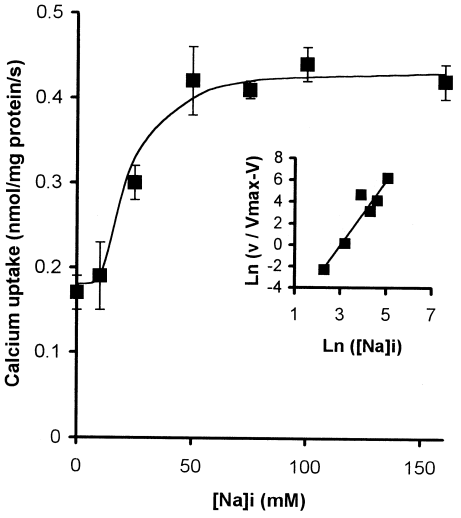


Fig. 5. Dependence of $Ca^{2+}$ uptake on intravesicular $Na^+$ concentration. Vesicles were preloaded with 160 mM TEA or with different concentrations of NaCl replacing TEA at constant osmolarity. Uptake of 50 μM $^{45}CaCl_2$ was measured at 3 s in a medium containing 160 mM TEA. The line was drawn according to a non-linear regression realized with the Hill model with the following parameters: $V_{max} = 0.25 \pm 0.02$ nmol/mg protein/s; $K_{0.5} = 24 \pm 2$ mM; $n = 3.5 \pm 0.7$. The inset shows a ln–ln transformation (Hill plot) of the data. Kinetic parameter values were obtained graphically: $K_{0.5} = 20$ mM and $n = 2.9$.

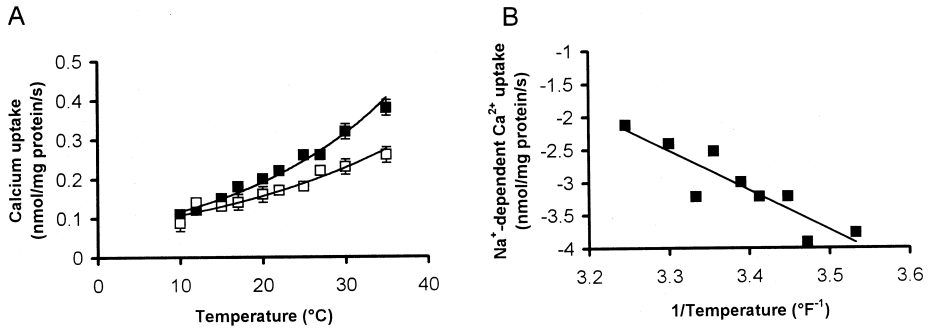


Fig. 6. Temperature dependence of $Na^+$-dependent $Ca^{2+}$ uptake. (A) Vesicles filled with 160 mM NaCl (solid symbols) or with 160 mM KCl (open symbols) were incubated in a medium containing 160 mM KCl and 50 μM $^{45}CaCl_2$ during 3 s at the indicated temperature. The lines were drawn by non-linear regression according to the Arrhenius equation. (B) Arrhenius plot of $Na^+$-dependent $Ca^{2+}$ uptake (difference of $Ca^{2+}$ uptake between $Na^+$- or $K^+$-loaded vesicles).

s in Na/K conditions. Alltogether, these results show that the saturation function for $Ca^{2+}$ uptake displayed three systems: a non-saturable component which predominated at high $Ca^{2+}$ concentration, a $Na^+$-independent saturable component and a $Na^+$-stimulated $Ca^{2+}$ uptake expressed by Na/Ca exchange system.

### 3.5. *Internal $Na^+$ concentration effect on initial velocity of $Ca^{2+}$ uptake*

The kinetic relationship between intravesicular $Na^+$ and $Ca^{2+}$ uptake was seemingly complex and difficult to measure with precision in the presence of $K^+$. This was probably due to the large $Na^+$-independent $Ca^{2+}$ uptake (a similar problem was found in renal basolateral vesicles [23]). To overcome this problem, we used TEA as $Na^+$ substitute. In order to measure the $Na^+$ dependence of the $Ca^{2+}$ uptake, vesicles were loaded with different concentrations of $Na^+$, with the above-mentioned precautions. The results of a typical experiment are shown in Fig. 5 where the initial rate of $Ca^{2+}$ uptake was plotted versus internal $Na^+$ concentration. The relationship between rate of uptake and internal $Na^+$ concentration was sigmoid [22] and the exchange system saturated with respect to intravesicular $Na^+$ at a concentration around 75 mM. The data points were fitted with a Hill model. This fitting procedure gave a $V_{max}$ value of $0.25 \pm 0.02$ nmol/mg/s, an apparent $K_{0.5}$ of

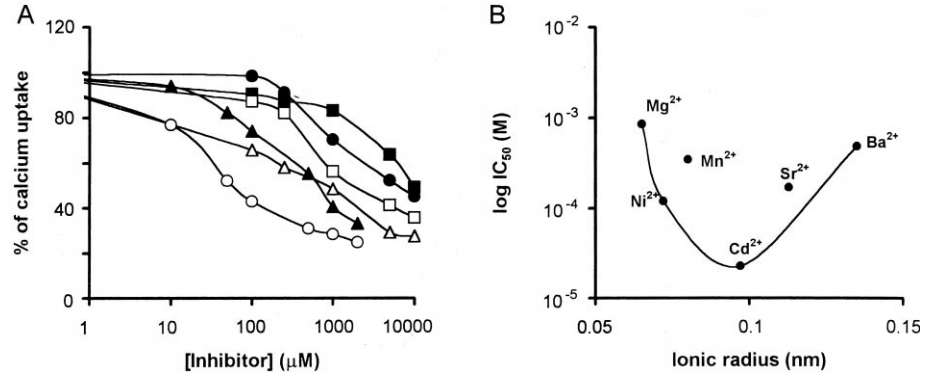


Fig. 7. Inhibition of $Na^+$-dependent $Ca^{2+}$ uptake by divalent cations. (A) Vesicles preloaded with 160 mM NaCl were incubated in a medium containing 160 mM KCl and 50 μM $^{45}CaCl_2$ during 3 s. Inhibitors were present in the incubation media at the concentration indicated, added at zero time. The following inhibitors were tested: $Ba^{2+}$ (solid circles), $Cd^{2+}$ (open circles), $Mg^{2+}$ (solid squares), $Mn^{2+}$ (open squares), $Ni^{2+}$ (open triangles) and $Sr^{2+}$ (solid triangles). Results are expressed as % of remaining $Ca^{2+}$ uptake. 100% values were estimated in the absence of inhibitors and were 0.70+0.02 nmol/mg protein/s. (B) Relation between the ionic radius of the inhibitory divalent cations and the logarithms of the $IC_{50}$. $IC_{50}$ (μM) was $485.2 \pm 64.2$ for $Ba^{2+}$, $22.8 \pm 2.1$ for $Cd^{2+}$, $857.4 \pm 205.5$ for $Mg^{2+}$, $350.1 \pm 8.3$ for $Mn^{2+}$, $119.5 \pm 33.75$ for $Ni^{2+}$ and $171.4 \pm 55.2$ for $Sr^{2+}$.

about 24 ± 2 mM and a Hill number of 3.5 ± 0.7. The Hill plot of these data (Fig. 5, insert) yields a $K_{0.5}$ of 20 mM and a Hill number of about three. We can see that parameter values obtained by graphical or numerical methods are rather similar.

### 3.6. Temperature dependence on initial velocity of Na/Ca exchange

The experiment presented in Fig. 6A shows the effect of temperature on $Na^+$-independent and $Na^+$-dependent $Ca^{2+}$ uptake. It can be noticed that both components are markedly temperature-dependent (apparent $Q_{10}$ of about 2 and 1.3, respectively, apparent $Q_{10}$ of 2.2 for the difference presented in Fig. 6B). These values are in agreement with what is known in preparations where Na/Ca exchange has been well characterized [18,20,21].

### 3.7. Inhibition of Na/Ca exchange

Seven inhibitors, six divalent cations ($Ba^{2+}$, $Cd^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Ni^{2+}$ and $Sr^{2+}$) and one organic (*N*-benzamilamiloride) were tested on the initial rate of $Na^+$-dependent $Ca^{2+}$ uptake. Fig. 7A shows the inhibition curves where the results are expressed in % of remaining $Ca^{2+}$ uptake, 100% values were determined from control conditions. It should be noted that all the tested compounds present a maximum inhibitory effect of around 80% of the control values. The remaining $Ca^{2+}$ uptake (20%) should be attributed to passive diffusion. $IC_{50}$ was evaluated for all the ionic compounds and plotted against ionic radii (Fig. 7B). *N*-Benzamiloride also was a potent inhibitor of the $Na^+$-dependent $Ca^{2+}$ uptake and presented an $IC_{50}$ value of 250 μM.

## 4. Discussion

In this study, we reported a simple method to obtain plasma membrane from zona fasciculata cells of adrenal cortex. To our knowledge, this is the first time that plasmalemmal vesicles prepared from this tissue were used. The 5′ nucleotidase activity, a well defined plasma membrane marker, measured in plasmalemmal fraction indicated a relatively high degree of enrichment: 18-fold compared with homogenate. This result is in agreement with data reported by authors using plasma membrane from various tissues [8,10,13].

Here, we give the first experimental evidence for the presence of Na/Ca exchange in the plasmalemmal membrane of fasciculata cells of the bovine adrenal cortex. Previous work suggested the implication of a Na/Ca exchange process in glomerulosa cells through indirect evidence based on the effect of $Na^+$ deprivation on internal $Ca^{2+}$ or $Na^+$ concentration [2,3]. Our results demonstrated that in these vesicles, there is a $Na^+$-stimulated $Ca^{2+}$ uptake as in other preparations like vesicles obtained from heart [13], chromaffin cells [8], renal basolateral cells [10,23] or brain microsomes [20]. In the present study, extravesicular $Na^+$ induced a 2-fold stimulation of $Ca^{2+}$ uptake when compared to control condition and this stimulation was close to that reported in renal basolateral preparation [10,23]. The stimulation of $Ca^{2+}$ uptake was suppressed by intravesicular $Li^+$, indicating that as in heart muscle, this ion does not substitute for $Na^+$. In efflux mode, we could show that there was still a stimulatory effect of $Na^+$ inhibited by $Ni^{2+}$.

When the dependence of $Ca^{2+}$ uptake on extravesicular $Ca^{2+}$ concentration was studied, the comparison between $Na^+$-stimulated and control conditions displays two features. First, the $Na^+$ stimulation did not act on the apparent $K_m$, signifying that this stimulation had no effect on the affinity of Na/Ca exchange for $Ca^{2+}$ but doubled the $V_{max}$ values. We can conclude that as in heart muscle, $Na^+$ displays a *V* effect. The $K_m$ values determined in $Na^+$-dependent condition were in the range of values reported for other preparations [24] and the $V_{max}$ was similar to the value obtained in renal basolateral vesicles [10,23] but was lower than that found in cardiac sarcolemmal vesicles [13]. Second, even in the absence of $Na^+$ gradient, some saturable $Ca^{2+}$ uptake occurs. A similar $Na^+$-independent $Ca^{2+}$ uptake was found in kidney basolateral membrane [10,23]. The fact that this $Na^+$-independent $Ca^{2+}$ uptake was inhibited by $Ni^{2+}$ indicated that this process could correspond to a Ca/Ca exchange mode of the Na/Ca exchanger.

We found, as in other preparations, that the relationship between $Na^+$ intravesicular concentration and $Ca^{2+}$ uptake was sigmoid with a Hill number close to three and a $K_{0.5}$ of about 20–25 mM. These

values are in agreement with what is known in most of the preparations where the Na/Ca exchange system has been characterized [22,23]. The sigmoid pattern revealed that more than one $Na^+$ was bound per $Ca^{2+}$ transported and that $Na^+$ interacted in a cooperative fashion.

The temperature dependence of the $Na^+$-stimulated system characterized by its apparent $Q_{10}$, 2.2 in this work, is well within the range of the values known for Na/Ca exchange systems described in other preparations. Its temperature dependence is of the same order of magnitude when measured in sarcolemmal vesicles (apparent $Q_{10}$ of 2.06 [18]) and close to brain microsomes (apparent $Q_{10}$ about 2, calculated from [20]).

Inhibition of Na/Ca exchange processes by several divalent cations was investigated. The order of cation effectiveness in inhibiting initial rates of $Na^+$-dependent $Ca^{2+}$ uptake was: $Cd^{2+} > Sr^{2+} > Ni^{2+} > Ba^{2+} \approx Mn^{2+} > Mg^{2+}$. When $IC_{50}$ was plotted against ionic radii, we found the usual U-shaped curve [19] with $Cd^{2+}$ as the most efficient blocker ($IC_{50}$ 21 μM). The effectiveness of the divalent cations was related to their ionic radius as compared with that of $Ca^{2+}$, suggesting competition with $Ca^{2+}$. This result is consistent with Na/Ca exchange inhibition in renal basolateral membrane [23]. Another class of inhibitor belonging to the analogues of amiloride was tested, the *N*-benzylamiloride. It appeared to be a potent inhibitor ($IC_{50}$ of about 250 μM) as expected for genuine Na/Ca exchange system [24].

In order to determine the stoichiometry of Na/Ca exchange, we first followed the biochemical approach described by Reeves and Hale [25] but the properties exhibited by $K^+$ [5] on our system prevented an accurate determination. An electrophysiological study using patch-clamp in whole cell configuration is actually under investigation to determine the stoichiometry value of this exchanger.

Finally we have recently identified the type of Na/Ca exchanger using molecular biology methods. It belongs to the NaCX1 family NACA3 isoform, similar to the one identified on renal cortex from rat and from pig (respectively 92% and 94% homology at the nucleotide sequence level [26]).

From our data, we can conclude that the plasmalemmal membrane of zona fasciculata cells of the bovine adrenal gland contains a functional Na/Ca exchanger whose biochemical characteristics resemble those of renal basolateral membrane. This Na/Ca exchange mechanism could play a significant role in the $Ca^{2+}$ homeostasis of fasciculata cells. For example, the secretagogue angiotensin II induces a biphasic increase of internal $Ca^{2+}$ concentration [27,28]. This increase is composed of a large initial peak due to the release of $Ca^{2+}$ sequestered in internal compartments followed by a more or less sustained plateau which is dependent on extracellular $Ca^{2+}$. Therefore, we cannot exclude that this plateau phase could be regulated by the activity of the Na/Ca exchanger. However, much has to be done to analyze the possible involvement of Na/Ca exchange, and/or its hormonal regulation [29] during the secretory events undergone by these cells. Experiments are still needed to construct a possible mechanistic model [30] of the ion transfer system and its cellular regulation.

## Acknowledgements

We thank J.L. Andrieu for his technical assistance in membrane preparation. We also wish to thank Dr. V. Jacquemond and Dr. M. Rongeat for careful reading of the manuscript and helpful comments during the course of this work.

## References

[1] D.W. Hilgemann, K.D. Philipson and G. Vassort, Sodium-Calcium Exchange. Annals of the New York Academy of Sciences, Vol. 779, The New York Academy of Sciences, New York, 1996.

[2] L. Hunyady, S. Kayser, E.J. Cragoe, I. Balla, T. Balla, A. Spät, $Na^+$-$H^+$ and $Na^+$-$Ca^{2+}$ exchange in glomerulosa cells: possible role in control of aldosterone production, Am. J. Physiol. 254 (1988) C744–C750.

[3] I. Kojima, E. Ogata, Na-Ca exchanger as a calcium influx pathway in adrenal glomerulosa cells, Biochem. Biophys. Res. Commun. 158 (1989) 1005–1012.

[4] V. VanderBent, C. Demole, E.I. Johnson, M.F. Rossier, C.P. Python, M.B. Vallotton, A.M. Capponi, Angiotensin-II induces changes in the cytosolic sodium concentration in bovine adrenal glomerula cells: involvement in the activation of aldosterone biosynthesis, Endocrinology 133 (1993) 1213–1220.

[5] C. Brunold, A. Guyot, O. Rougier, A. Bilbaut, C. Ojeda,

Characterisation of $Na^+$-$Ca^{2+}$ exchange in plasmalemmal vesicles from zona fasciculata cells of the bovine adrenal gland, J. Physiol. 513 (1998) 53P.
[6] A. Crozat, A. Penhoat, J.M. Saez, Processing of angiotensin II (A-II) and ($Sar^1$, $Ala^8$) A-II by cultured bovine adrenocortical cells, Endocrinology 118 (1986) 2312–2318.
[7] O. Rosenthal, S. Narasimhulu, Adrenal steroid hydroxylases, Methods Enzymol. 15 (1969) 596–638.
[8] L.S. Kao, N.S. Cheung, Mechanism of calcium transport across the plasma membrane of bovine chromaffin cells, J. Neurochem. 54 (1990) 1972–1979.
[9] K.D. Philipson, A.Y. Nishimoto, Na-Ca exchange in inside-out cardiac sarcolemmal vesicles, J. Biol. Chem. 257 (1982) 5111–5117.
[10] M.P.E. VanHeeswijk, J.A.M. Geertsen, C.H. VanOs, Kinetic properties of the ATP-dependent $Ca^{2+}$ pump and the $Na^+$/$Ca^{2+}$ exchange system in basolateral membranes from rat kidney cortex, J. Membr. Biol. 79 (1984) 19–31.
[11] P. Mansier, D. Charlemagne, B. Rossi, M. Preteseille, B. Swynghedauw, L. Lelievre, Isolation of impermeable inside-out vesicles from an enriched sarcolemma fraction of rat heart, J. Biol. Chem. 258 (1983) 6628–6635.
[12] H.R. Besch, L.R. Jones, A.M. Watanabe, Intact vesicles of canine cardiac sarcolemma. Evidence from vectorial properties of $Na^+$,$K^+$-ATPase, Circ. Res. 39 (1976) 586–595.
[13] J.P. Reeves, J.L. Sutko, Sodium-calcium ion exchange in cardiac membrane vesicles, Proc. Natl. Acad. Sci. USA 76 (1979) 590–594.
[14] E.S. Baginski, P.P. Foa and B. Zak, Glucose-6-phosphatase, in: H.U. Bergmeyer (Ed.), Methods of Enzymatic Analysis, Academic Press Inc., New York, 1974, pp. 876–880.
[15] O.H. Lowry, V.J. Rosebrough, A.L. Farr, R.L. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[16] J.P. Reeves, Measurement of sodium-calcium exchange activity in plasma membrane vesicles, Methods Enzymol. 157 (1988) 505–510.
[17] T.L. Trosper, K.D. Philipson, Stimulatory effect of calcium chelators on $Na^+$-$Ca^{2+}$ exchange in cardiac sarcolemmal vesicles, Cell Calcium 5 (1984) 211–222.
[18] D.K. Bartschat, G.E. Lindenmayer, Calcium movements promoted by vesicles in a highly enriched sarcolemma preparation from canine ventricle. Calcium-calcium countertransport, J. Biol. Chem. 255 (1980) 9626–9634.
[19] T.L. Trosper, K.D. Philipson, Effects of divalent and trivalent cations on $Na^+$-$Ca^{2+}$ exchange in cardiac sarcolemmal vesicles, Biochim. Biophys. Acta 731 (1983) 63–68.
[20] G.D. Schellenberg, P.D. Swanson, Sodium-dependent and calcium-dependent calcium transport by rat brain microsomes, Biochim. Biophys. Acta 648 (1981) 13–27.
[21] J. Kimura, S. Miyamae, A. Noma, Identification of sodium-calcium exchange current in single ventricular cells of guinea-pig, J. Physiol. 384 (1987) 199–222.
[22] J.P. Reeves, J.L. Sutko, Competitive interactions of sodium and calcium with the sodium-calcium exchange system of the cardiac sarcolemmal vesicles, J. Biol. Chem. 258 (1983) 3178–3182.
[23] A. Jayakumar, L. Cheng, C.T. Liang, B. Sacktor, Sodium gradient-dependent calcium uptake in renal basolateral membrane vesicles. Effect of parathyroid hormone, J. Biol. Chem. 259 (1984) 10827–10833.
[24] G.J. Kaczorowski, M.L. Garcia, V.F. King and R.S. Slaughter, Development and use of inhibitors to study sodium-calcium exchange, in: T.J.A. Allen, D. Noble and H. Reuter (Eds.), Sodium-Calcium Exchange, Oxford University Press, Oxford, 1989, pp. 66–101.
[25] J.P. Reeves, C.C. Hale, The stoichiometry of the cardiac sodium-calcium exchange system, J. Biol. Chem. 259 (1984) 7733–7739.
[26] A. Morales, J. Lachuer, A. Bilbaut and C. Ojeda, 1999. EMBL/Genbank European Bioinformatics Institute, Accession Number: Y18619.
[27] I. Viard, W.E. Rainey, A.M. Capponi, M. Begeot, J. Saez, Ovine adrenal fasciculata cells contain angiotensin-II receptors coupled to intracellular effectors but are resistant to the steroidogenic effects of this hormone, Endocrinology 127 (1990) 2071–2078.
[28] A. Chorvatova, A. Guyot, C. Ojeda, O. Rougier, A. Bilbaut, Activation by angiotensin II of $Ca^{2+}$-dependent $K^+$ and $Cl^-$ currents in zona fasciculata cells of bovine adrenal gland, J. Membr. Biol. 162 (1998) 39–50.
[29] D.W. Hilgemann, R. Ball, Regulation of cardiac $Na^+$, $Ca^{2+}$ exchange and $K_{ATP}$ potassium channels by PIP2, Science 273 (1996) 956–959.
[30] S. Matsuoka, D.W. Hilgemann, Steady-state and dynamic properties of cardiac sodium-calcium exchange. Ion and voltage dependencies of the transport cycle, J. Gen. Physiol. 100 (1992) 963–1001.