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The co-presence of Na^+/Ca^{2+}-K^+ exchanger and Na^+/Ca^{2+} exchanger in bovine adrenal chromaffin cells

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Abstract

We have previously shown that there is high Na^+/Ca^{2+} exchange (NCX) activity in bovine adrenal chromaffin cells. In this study, by monitoring the $[Ca^{2+}]_i$ change in single cells and in a population of chromaffin cells, when the reverse mode of exchanger activity has been initiated, we have shown that the NCX activity is enhanced by K^+. The K^+-enhanced activity accounted for a significant proportion of the Na^+-dependent Ca^{2+} uptake activity in the chromaffin cells. The results support the hypothesis that both NCX and Na^+/Ca^{2+}-K^+ exchanger (NCKX) are co-present in chromaffin cells. The expression of NCKX in chromaffin cells was further confirmed using PCR and northern blotting. In addition to the plasma membrane, the exchanger activity, measured by Na^+-dependent $^{45}Ca^{2+}$ uptake, was also present in membrane isolated from the chromaffin granules enriched fraction and the mitochondria enriched fraction. The results support that both NCX and NCKX are present in bovine chromaffin cells and that the regulation of $[Ca^{2+}]_i$ is probably more efficient with the participation of NCKX.

Keywords: bovine chromaffin cell, Na^+/Ca^{2+} exchanger, Na^+/Ca^{2+}-K^+ exchanger.

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The Na^+/Ca^{2+} exchanger (NCX) in the plasma membrane is expressed in virtually all animal tissues and is involved in the maintenance of cytosolic Ca^{2+} homeostasis. NCX was first reported in the heart (Reuter and Seitz 1968) and squid giant axons (Baker *et al.* 1969). In bovine adrenal chromaffin cells, among the mechanisms known for the maintenance of intracellular Ca^{2+} homeostasis, NCX across the plasma membrane has been shown to be one of the major mechanisms involved in bringing the cytosolic free calcium ($[Ca^{2+}]_i$) back to the resting level (Liu and Kao 1990; Chern *et al.* 1992; Lin *et al.* 1994; Powis *et al.* 1994; Pan and Kao 1997; Tang *et al.* 2000).

There are two major types of mammalian NCXs: the NCX originally found in the heart, which has a stoichiometry of $3Na^+ : 1Ca^{2+}$ and the retinal rod type K^+-dependent Na^+/Ca^{2+} exchanger (NCKX), in which K^+ co-transports with Ca^{2+} with a stoichiometry of $4Na^+ : 1Ca^{2+} + 1K^+$ (Philipson and Nicoll 1992). Three members of the NCX gene family, NCX1–3 (Nicoll *et al.* 1990, 1996; Li *et al.* 1994) and five members of the NCKX gene family, NCKX1–5, have been identified (see a review by (Lytton 2007). There is very little sequence similarity between the NCX and NCKX families of molecules. Nevertheless, the putative molecular topology of the two molecular families is similar; there are two clusters of transmembrane segments and a large cytoplasmic loop between the two clusters. Both types of exchangers can function as in either the forward mode (extracellular Na^+ in exchange for intracellular Ca^{2+}) or the reverse mode (intracellular Na^+ in exchange for extracellular Ca^{2+}) depending on the membrane potential and the Na^+ and Ca^{2+} gradients across the plasma membrane (Philipson and Nicoll 1992; Reeves 1992).

Potassium-dependent exchange activity has been shown in cell types other than retinal rods such as rat brain synaptic plasma membrane vesicles (Dahan *et al.* 1991) and human platelets (Kimura *et al.* 1993, 1999). However, it is not known if the NCKX is present in the chromaffin cells. In this

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Abbreviations used: NCKX, Na^+/Ca^{2+}-K^+ exchanger; NCX, Na^+/Ca^{2+} exchanger; NMG, *N*-methyl-D-glucamine.

study, we have used both molecular and cell biology approaches to demonstrate that NCKX is co-present with NCX in bovine chromaffin cells.

Materials and methods

Preparation of the subcellular fractions

The membrane fractions of plasma membrane, granules and mitochondria from bovine chromaffin cells were prepared as previously described (Kao and Cheung 1990) except that the preparations were stored in 0.25 M sucrose and 10 mM 3-(N-morpholino) propanesulfonic acid, pH 7.3, buffer. Briefly, the medullae were homogenized and centrifuged. The pellet of 10 000 *g* centrifugation was used to obtain the chromaffin granules and mitochondria by a Percoll gradient (Kao and Westhead 1984a). For purified plasma membranes, the pellet of 28 000 *g* centrifugation was resuspended and passed through a discontinuous sucrose gradient as described previously (Kao and Cheung 1990).

To prepare the membrane fraction from cardiac cells, we isolated the myocytes with collagenase from the heart of Hartley guinea pigs (250–300 g) as described before (Williford *et al.* 1990). The isolated myocytes were resuspended in phosphate-buffered saline and sonicated. Unbroken cells and large organelles were pelleted and discarded by centrifugation at 2000 *g* for 20 min. The supernatant was centrifuged again at 100 000 *g*, 1 h; the pellet was recovered in 0.25 M sucrose and 10 mM 3-(N-morpholino) propanesulfonic acid, pH 7.3, buffer as the membrane fraction and this was then stored in a −80°C freezer.

$^{45}Ca^{2+}$ uptake by membrane vesicles

To load Na^+ into the membrane vesicles, the membrane fraction was pelleted down at 100 000 *g* (TLA 100.3 rotor; Beckman Coulter, Inc., Fullerton, CA, USA) in 10 volume of sodium buffer (145 mM NaCl and 10 mM HEPES, pH 7.3) and resuspended in sodium buffer with a protein concentration of about 0.2 mg/mL. Calcium uptake was initiated by adding 10 μL of the membrane fraction into 300 μL of either sodium buffer, NMG buffer (145 mM *N*-methyl-D-glucamine and 10 mM HEPES, pH 7.3), potassium buffer (145 mM KCl and 10 mM HEPES, pH 7.3), or other buffers with equilmolar monovalent cation. For Ca^{2+} uptake activity, $^{45}Ca^{2+}$ (0.3 Ci/mmol) was added into these buffers. The reaction solution was stopped by adding 1 mL of chilled stop buffer (145 mM NaCl, 5 mM $LaCl_3$, and 10 mM HEPES, pH 7.3) and filtered through Whatman GF/C membrane (Whatman Plc., Maidstone, UK) immediately. The GF/C membrane was washed with 10 mL chilled stop buffer to minimize non-specific binding. The isotope activity was counted by a β-counter (LS series; Beckman Coulter, Inc.).

Measurements of $[Ca^{2+}]_i$

Isolated bovine chromaffin cells were loaded with fura-2AM and the reverse mode of NCX activity was measured. The measurement of $[Ca^{2+}]_i$ for the cell population was carried out as described previously (Liu and Kao 1990; Kimura *et al.* 1993). Briefly, the fura-2-loaded chromaffin cells were first loaded with Na^+ by incubation in a loading buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM glucose, 2.2 mM $CaCl_2$, and 10 mM HEPES, pH 7.3) containing 0.3 mM carbachol and 100 μM ouabain for 10 min at approximately 25°C. The Na^+-loaded cells were centrifuged (15 000 *g*, 10 s) to remove carbachol and ouabain and then resuspended in a Ca^{2+}-free, Na^+-free loading buffer (the same as loading buffer except that NaCl was replaced with equilmolar Li^+ or NMG and no $CaCl_2$ was added). To inhibit the Ca^{2+} influx induced by activation of nicotinic receptor and Ca^{2+} channels, 1 mM hexamethonium, 0.1 mM verapamil, and 10 μM nifedipine were included; these have been shown to inhibit completely the carbachol- and high K^+-induced $[Ca^{2+}]_i$ rise in preliminary experiments (data not shown). The reverse mode of NCX activity was initiated by adding Ca^{2+} (final concentration: 2.2 mM) into the reaction buffer. The $[Ca^{2+}]_i$ was measured by a dual-excitation fluorometer (CM system; HORIBA Jobin Yvon Inc., Edison, NJ, USA).

The single cell measurements were carried as described previously with some modifications (Pan and Kao 1997). The fura-2-loaded chromaffin cells were bathed in the loading buffer containing ouabain (100 μM) for at least 30 min to accumulate intracellular Na^+. To initiate the reverse mode NCX activity, Na^+-free NMG buffer with or without 5 mM KCl was puffed onto the cell, which was incubated in the Ca^{2+} containing loading buffer, by a micropipette with an opening of ∼2 μm. The micropipette was positioned at ∼20 μm from the target cell and the buffer was pressure ejected by an Eppendorf injector (Eppendorf AG, Hamburg, Germany). The excitation wavelength was provided by a Polychrome IV (TILL Photonics GmbH, Gräfelfing, Germany) and the fluorescent images were acquired by a CCD camera (MicroMax; Princeton Instrument, Trenton, NJ, USA). The Polychrome IV, the CCD camera, and image acquisition were controlled and analyzed by Image Workbench (ver 2.0; Molecular Devices, Sunnyvale, CA, USA).

Measurement of catecholamine secretion

For catecholamine secretion from a population of cells, freshly isolated chromaffin cells were first loaded with Na^+ by incubation with ouabain (100 μM) for 1 h in a Na^+-containing solution. Then, the solution was changed to a Na^+-free NMG solution with or without 5 mM K^+. Control experiments were carried out in the Na^+-containing solution. After the time indicated, the supernatant was collected and the catecholamine released was measured by HPLC (Kao and Westhead 1984a,b). Catecholamine secretion was shown as the percentage of total catecholamine released.

For single cell secretion, amperometric and $[Ca^{2+}]_i$ measurements described above were carried out simultaneously. For amperometirc measurements, a carbon fiber electrode (ProPFE; World Precision Instruments, Inc., Sarasota, FL, USA) was positioned at the top of a cell within a distance of ∼1 μm and a constant 650 mV was applied through an EPC10 amplifier (HEKA GmbH, Lambrecht, Germany). The currents were recorded by the same amplifier under the control of Pulse software (HEKA GmbH, Lambrecht, Germany). The sampling rate was 1 kHz and the lowpass filter was set at 2.9 kHz. Data were analyzed by Minianalysis software (Synaptosoft, Inc., Fort Lee, NJ, USA). NMG buffers with or without K^+ were applied through a puff pipette at 1 s after the start of recording for 60 s and total current recording time was 145 s.

Northern blot analysis and RT-PCR

Total RNA was isolated from bovine chromaffin cells using TRIzol (Invitrogen, Life Technologies, Inc., San Diego, CA, USA), and

cDNA was synthesized with Transcriptor First Strand cDNA Synthesis kit (Roche, Basel, Switzerland). The primers for NCKX were designed based on the bovine NCKXs nucleotide sequences (GenBank number: NCKX1 NM_174655, NCKX2 XM_603971, NCKX3 XM_868421, NCKX4 XM_001252265 and NCKX5 XM_001249737). The PCR conditions were 94°C for 30 s, 50°C or 53°C for 30 s, and 72°C for 50 s (30 cycles) for NCKX1 and 94°C for 30 s, 53°C for 30 s, and 72°C for 60 s (40 cycles) for various NCKX isoforms. The PCR primers and amplicon size were as follows: NCKX1, 5′-GCGACGCTGCATTAGGAGGTGA and 5′-TTCCTCCTCTTCCTCCTCCTCTTC, 674 bp and 5′-TACCTCATGGTTTGGTGGGCTCAC and 5′-CTCAGACAGATACAGGGCAGGATA, 445 bp; NCKX2, 5′-GGAGAGACAATTGGCATTAGT and 5′-TGTTCATCCGCCATTTGCAG, 316 bp; NCKX3, 5′-ACTGCATGGCCAGCCTCATT and 5′-GTTGAACTCCGTCATGATGG, 320 bp; NCKX4, 5′-TGGCAGTCTCTAACACCATCGGA and 5′-TCGTCTTCTCGGCACATTGGCA, 316 bp; NCKX5, 5′-TATATCCTGGTTTGGATGGTCAC and 5′-CTTTCTGTCTAGTTTCCAGCCA, 351 bp. The PCR products were further amplified with Ex Taq PCR kit (TaKaRa Bio, Inc., Otsu, Shiga, Japan) and then sequenced.

For northern blot analysis, total RNA prepared from both rat brain and bovine chromaffin cells were applied to a formaldehyde-denaturing agarose gel. Electrophoresed RNA was blotted onto nylon membrane and stabilized by UV cross-linking. The PCR product obtained above was used as the probe for hybridization at 65°C overnight. After washing, the blot was visualized on a phosphoimager (Molecular Dynamics, Sunnyvale, CA, USA).

Results

Potassium enhanced the reverse mode Na^+/Ca^{2+} exchanger activity

The reverse mode of NCX and NCKX activities were first examined in a population of intact chromaffin cells. Na^+ was loaded into the fura-2-loaded chromaffin cells by stimulation with carbachol in a Ca^{2+}-free, Na^+-containing solution, which contained ouabain to inhibit Na^+ pumping out by Na^+,K^+-ATPase. To generate the Na^+ gradient, the cells were then transferred to a Na^+-free solution with or without K^+. The Na^+-dependent Ca^{2+} uptake was initiated by adding Ca^{2+}. In the presence of K^+, in both Na^+-free Li^+ (Fig. 1a) and NMG (Fig. 1b) solution, the $[Ca^{2+}]_i$ in Na^+-loaded cells increased from a basal level of ~100 nM to 593 ± 80 and 501 ± 24 nM, respectively. In contrast, in the control Na^+-containing solution, the $[Ca^{2+}]_i$ elevated to 233 ± 20 nM (Fig. 1c). In the absence of extracellular K^+, the net $[Ca^{2+}]_i$ increase in Na^+-loaded cells was significantly lower than that measured in the presence of K^+; the $[Ca^{2+}]_i$ levels were elevated to 387 ± 45 and 340 ± 19 nM in Na^+-free Li^+ and NMG solution, respectively. The average changes in the $[Ca^{2+}]_i$ under different conditions are summarized in Fig. 1d.

Similar K^+ enhancement of the Na^+ gradient-dependent catecholamine secretion was also observed. The catecholamine secretion from Na^+-loaded cells in Na^+-free NMG solution was significantly higher in the presence of extracellular K^+ than that in the absence of extracellular K^+ (Table 1).

The Na^+ gradient-dependent $[Ca^{2+}]_i$ rise increased with the concentration of K^+; the maximum rise was reached at around 15 mM K^+ (Fig. 1e). The $[Ca^{2+}]_i$ levels were elevated to 417 ± 58 and 534 ± 59 nM for 1 and 15 mM K^+, respectively. For cells with no carbachol stimulation, that is without Na^+ loading, there was only a small increase in $[Ca^{2+}]_i$ when Ca^{2+} was added. The increase in $[Ca^{2+}]_i$ was enhanced by K^+ in Na^+-free Li^+ buffer but not in NMG buffer.

The above results show that both NCX and NCKX activities are present in bovine chromaffin cells. To further confirm whether both NCX and NCKX activity are co-present in chromaffin cells, the $[Ca^{2+}]_i$ elevation elicited by reverse mode exchange activity was monitored in single chromaffin cells. Each cell was first treated with ouabain to increase the intracellular Na^+ concentration and the extracellular Na^+ was replaced by NMG to initiate the reverse mode NCX activity. Similar results to those obtained using a population of cells was obtained (Fig. 2). The results showed that for cells stimulated with control solutions without generating the Na^+ gradient, there was little change in $[Ca^{2+}]_i$ in the presence or absence of K^+. When a cell was stimulated with Na^+-free NMG solution, the $[Ca^{2+}]_i$ was elevated after a lag period and returned to basal only when the puffing stopped. The averaged result showed that the peak $[Ca^{2+}]_i$ was not changed when cells were puffed with control solutions (data not shown), while it was significantly elevated by puffing with NMG and NMG + K^+ solutions, respectively (Fig. 2a and b). The presence of K^+ also significantly shortened the time needed to reach the peak level, from 69.5 ± 3.9 s in the absence of K^+ to 39.7 ± 3.0 s in the presence of K^+ (Fig. 2d). These results show that the presence of K^+ outside the cell enhanced not only the amplitude but the rate of the Na^+ gradient-dependent $[Ca^{2+}]_i$ increase.

The catecholamine secretion from single cells, measured by amperometry, also showed similar trends as the changes in $[Ca^{2+}]_i$ (Fig. 2a and b). In the presence of K^+, the spikes appeared after a shorter time lag upon puffing NMG buffer and more exocytic events occurred than that in the absence of K^+ (Fig. 2c). The average size of the spikes was similar both in the presence and absence of K^+ (data not shown).

Potassium enhancement in the membrane vesicles isolated from plasma membrane, granules and mitochondria

The above results support the existence of both NCX and NCKX activities in intact chromaffin cells. NCX activity was then examined in plasma membrane isolated from bovine adrenal medulla. The isolated membrane vesicles were loaded with Na^+ first and then Na^+-dependent $^{45}Ca^{2+}$ uptake in Na^+-free NMG and Na^+-free K^+ solution were measured.

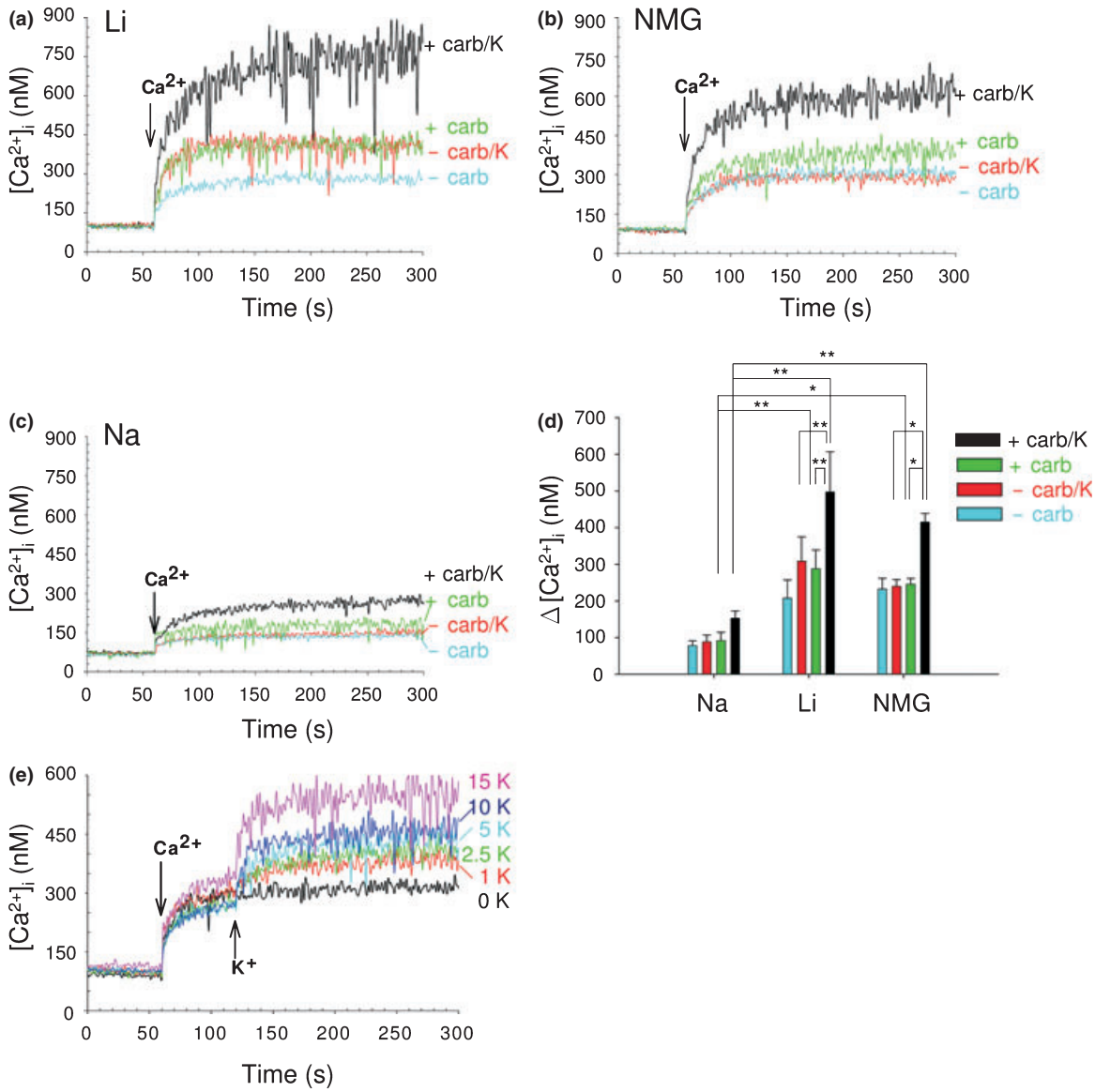

Fig. 1 Potassium enhancement of the Na^+/Ca^{2+} exchange activity in bovine chromaffin cells. Fura-2 loaded chromaffin cells were loaded with Na^+ by incubation with carbachol and ouabain for 10 min in Ca^{2+}-free, Na^+-containing solution (black and green traces). Control experiments without carbachol stimulation were carried out in parallel (red and cyan traces). Then the cells were transferred to a Ca^{2+}-free, Na^+-free Li^+ (a), NMG (b), or control Na^+-containing (c) solution with (black and red traces) or without 5 mM K^+ (green and cyan traces). $CaCl_2$ (2.2 mM) was added as indicated by the black arrow. Changes in $[Ca^{2+}]_i$ upon adding $CaCl_2$, obtained by subtracting the basal $[Ca^{2+}]_i$ values from the $[Ca^{2+}]_i$ of the plateau level, under various conditions were averaged from three experiments and shown in (d). Data shown are mean ± SEM. Statistical tests are performed by one-way ANOVA followed by LSD as a *post hoc* analysis; $*p < 0.05$ and $**p < 0.01$. (e) Different concentrations of K^+ were added (black, 0 mM; red, 1 mM; green 2.5 mM; cyan, 5 mM; blue, 10 mM; and purple, 15 mM), 1 min after $CaCl_2$ was added. The experiments were repeated at least three times and results from one representative experiment were shown.

The results showed that there was a time-dependent Na^+-gradient-dependent $^{45}Ca^{2+}$ uptake by the plasma membrane (Fig. 3). In Na^+-free K^+ solution the $^{45}Ca^{2+}$ uptake was higher than that in the Na^+-free NMG solution (see below).

We further examine whether K^+ was used to compensate for the electrogenic effects of the exchanger. The results show that by adding K^+ to NMG solution, the Na^+ gradient-dependent $^{45}Ca^{2+}$ uptake was also enhanced (Table 2). Furthermore, the $^{45}Ca^{2+}$ uptake activity was not significantly affected by valinomycin. Valinomycin is a K^+ ionophore that carries K^+ across the membrane without counter ion and thus keeps the K^+ concentration on both sides of the membrane vesicle the same and shunts the negative charge accumulate inside the vesicles.

Table 1 Potassium enhancement of the Na^+ gradient-induced catecholamine secretion from bovine chromaffin cells

	Control (% of total)		NMG (% of total)		NMG/K^+ (% of total)	
	E	NE	E	NE	E	NE
0 min	1.53 ± 0.85	1.46 ± 0.91	1.72 ± 0.70	1.65 ± 0.78	1.63 ± 0.55	1.56 ± 0.65
1 min	1.80 ± 0.94	1.82 ± 1.06	1.72 ± 0.43	1.74 ± 0.45	4.30 ± 0.62	5.65 ± 1.03
10 min	1.75 ± 0.62	1.59 ± 0.68	5.38 ± 0.55	4.17 ± 0.87	26.78 ± 3.99	22.75 ± 4.65

Chromaffin cells were loaded with Na^+ and then incubated in a Na^+-containing solution (Control), Na^+-free NMG solution (NMG), or Na^+-free NMG solution with 5 mM K^+ (NMG/K^+). At the times indicated, the supernatant was collected and the epinephrine (E) and norepinephrine (NE) released were measured by HPLC. Catecholamine secretion was shown as the percentage of total catecholamine released. Data shown are mean ± SEM from four experiments.

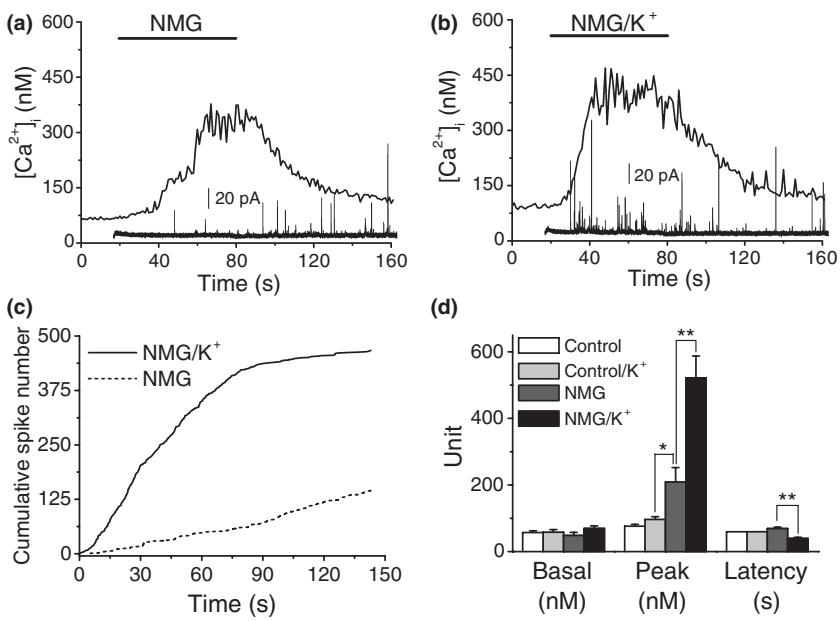

Fig. 2 Potassium could enhance the rate of Na^+-dependent $[Ca^{2+}]_i$ elevation and catecholamine secretion. Fura-2 loaded single chromaffin cell was treated with ouabain to elevate the intracellular Na^+ concentration. The $[Ca^{2+}]_i$ elevation of each single chromaffin cell was initiated by puffing Na^+, Na^+-free solution with (NMG/K^+) or without (NMG) 5 mM K^+ onto the cell for 60 s as indicated by the black bar. (a and b) Representative $[Ca^{2+}]_i$ and amperometric traces from a single cells. (c) Cumulative spike number. (d) Averaged changes in $[Ca^{2+}]_i$ and the latency. Basal is the $[Ca^{2+}]_i$ before stimulation. Peak is the maximal $[Ca^{2+}]_i$ obtained after the start of NMG solution application. Latency is the time required to reach the maximal $[Ca^{2+}]_i$ upon stimulation. Time zero in (c) is set at the start of NMG application. Data shown are mean ± SEM. Statistical tests are performed by Student's *t*-test; $*p < 0.05$ and $**p < 0.001$.

It has been shown that there is NCX activity in the chromaffin granules and mitochondria (Phillips 1981; Krieger-Brauer and Gratzl 1982; Gunter and Pfeiffer 1990). The level of NCX and NCKX activity were also examined in the mitochondrial- and chromaffin granule membrane-enriched fractions isolated from bovine adrenal medulla (Fig. 3). When extravesicular Na^+ was replaced by NMG, $^{45}Ca^{2+}$ uptake was increased significantly in the three membrane fractions. The $^{45}Ca^{2+}$ uptake reached plateau in about 10 min with the three membrane fractions. After subtracting the background activity (the $^{45}Ca^{2+}$ taken up by the control membrane vesicles without Na^+ loading), the highest $^{45}Ca^{2+}$ uptake activity in the absence of K^+ was that of the plasma membrane with 11.4 ± 0.4 pmol $^{45}Ca^{2+}$/μg protein was taken up in 10 min; and the lowest activity was that of the mitochondrial fraction with 1.5 ± 0.2 pmol $^{45}Ca^{2+}$/μg protein. The level for the chromaffin granule membrane fraction was intermediate at 7.1 ± 0.6 pmol $^{45}Ca^{2+}$/μg protein. When K^+ was used to replace Na^+, the activity was significantly higher than that measured in NMG

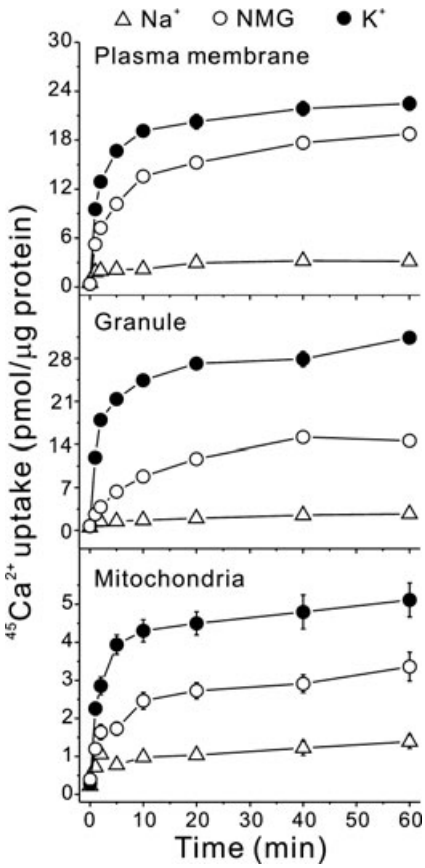

Fig. 3 Potassium-dependence of the Na^+/Ca^{2+} exchange activity of plasma membrane, chromaffin granule membrane fraction, and mitochondrial membrane fraction. The three membrane fractions were loaded with Na^+ by incubation in a Na^+-containing solution. The Na^+-loaded membrane vesicles were then transferred to Na^+ (△), Na^+-free NMG (○), or 145 mM K^+ (●) solutions in the presence of $^{45}Ca^{2+}$. The $^{45}Ca^{2+}$ uptake at various times was then measured. Data shown are mean ± SEM of three experiments.

solution; with the plasma membrane, the activity was increased by ~50% to 16.9 ± 0.7 pmol $^{45}Ca^{2+}$/μg protein and that in granule and mitochondrial membrane fractions, it increased by about two- to threefold, 22.7 ± 0.3 pmol $^{45}Ca^{2+}$/μg protein and 3.3 ± 0.3 pmol $^{45}Ca^{2+}$/μg protein, respectively. The results show that Na^+-dependent $^{45}Ca^{2+}$ uptake activity existed in all three membrane fractions and became higher in K^+ solution.

No K^+ enhancement in cardiac plasma membrane

To show the specificity of the K^+ effect, similar experiment was carried out using plasma membrane vesicles prepared from cardiac tissue where only NCX is known to be present. The results showed that the enhancement of $^{45}Ca^{2+}$ uptake activity by K^+ could only be observed in plasma membrane vesicles isolated from bovine chromaffin cell but not in those from cardiac cells (Table 3). The results further support the idea that the $^{45}Ca^{2+}$ uptake activity enhanced by K^+ in chromaffin cells is due to NCKX.

Rb^+ had similar effect as K^+ in enhancing the $^{45}Ca^{2+}$ uptake

The ion selectivity of the Na^+-dependent $^{45}Ca^{2+}$ uptake by the plasma membrane and chromaffin granule membrane vesicles with respect to the extravesicular monovalent cation was then characterized (Table 4). When the plasma membrane fraction was loaded with Na^+, the replacement of the extravesicular Na^+ with Li^+ or Cs^+ caused about a 3.5-fold increment in the $^{45}Ca^{2+}$ uptake over the background; while K^+ or Rb^+ replacement caused about a 7.5-fold increment. Other commonly used Na^+ replacement such as NMG, sucrose, Tris, and choline caused a similar 2.5-fold increase in the $^{45}Ca^{2+}$ uptake. The K^+ enhancement was larger with the granule membrane fraction than with the plasma membrane fraction. For the granule membrane fraction, the

Table 2 Potassium enhancement of the Na^+/Ca^{2+} exchange activity in the plasma membrane isolated from bovine chromaffin cells was not affected by valinomycin

	$^{45}Ca^{2+}$ uptake (pmol/μg protein)
Na	2.64 ± 0.06
NMG	12.09 ± 0.91
NMG/K	18.04 ± 1.09
NMG/K + valinomycin	19.81 ± 1.36

Plasma membrane vesicles isolated from bovine adrenal medulla were loaded with Na^+ and then incubated in a Na^+-containing solution (Na), or a Na^+-free NMG solution (NMG) containing $^{45}Ca^{2+}$ (100 μM) for 1 min in the absence or presence of 10 mM K^+ (NMG/K) or 10 mM K^+ plus valinomycin (2.5 μM) as indicated. Data shown are mean ± SEM from three experiments.

Table 3 Comparison of the K^+ enhancement of the Na^+/Ca^{2+} exchange activity in the plasma membrane isolated from bovine chromaffin cells and rat cardiac cells

	$^{45}Ca^{2+}$ uptake (pmol/μg protein)	
	Chromaffin cells	Cardiac cells
Control	0.56 ± 0.02	0.67 ± 0.03
NMG	2.81 ± 0.04	2.51 ± 0.12
K^+	4.64 ± 0.10	1.89 ± 0.17

Plasma membrane vesicles were isolated from bovine adrenal medulla and rat cardiac tissue. For Ca^{2+} uptake measurements, the isolated membrane vesicles were loaded with Na^+ and then incubated in a Na^+-containing solution (control), Na^+-free NMG solution (NMG), and Na^+-free K^+ (K^+) solution for 10 min. All solution contained $^{45}Ca^{2+}$ (100 μM). Data shown are mean ± SEM from three experiments.

Table 4 Sodium-gradient dependent $^{45}Ca^{2+}$ uptake in plasma membrane and granule membrane vesicles

Extravesicular Na^+ substitute	Plasma membrane	Chromaffin granule
	$^{45}Ca^{2+}$ uptake (pmol/μg protein)	
Na^+	2.79 ± 0.33	3.04 ± 0.34
Li^+	9.42 ± 0.63	5.01 ± 0.16
K^+	21.36 ± 1.16	37.12 ± 1.15
Rb^+	21.49 ± 1.51	37.06 ± 1.86
Cs^+	10.20 ± 0.37	8.67 ± 0.93
NMG	6.90 ± 0.50	6.15 ± 0.18
Sucrose	7.41 ± 0.56	5.44 ± 0.08
Tris	6.95 ± 0.82	5.15 ± 0.05
Choline	6.74 ± 0.70	5.37 ± 0.25

Vesicles isolated from bovine chromaffin plasma membrane or granules were loaded with Na^+ and then transferred to solutions containing different monovalent cations and $^{45}Ca^{2+}$ (100 μM) for 1 min. Data shown are mean ± SEM from three experiments.

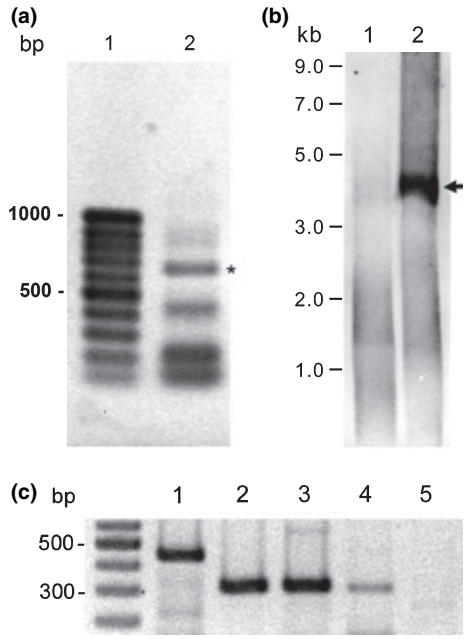

Fig. 4 The presence of NCKX in chromaffin cells by RT-PCR and northern blot analysis. (a) A specific primer pair against the C-terminal of the intracellular loop of NCKX1 was used to amplify the NCKX1 in bovine chromaffin cells by RT-PCR as described in Materials and methods. The individual bands were further amplified and sequenced. Only the band marked *, with a size of 674 bp as predicted, showed the same sequence as that of bovine NCKX1 (lane 2). Lane 1: 100 bp DNA ladder. (b) Total RNA isolated from rat brain (lane 1) and bovine chromaffin cells (lane 2) were separated in a formaldehyde-denaturing gel. Specific primers against the C-terminal of the NCKX1 intracellular loop were used to amplify the NCKX1 expressed in bovine chromaffin cell by RT-PCR and this was used as the northern probe. DNA size markers in kb are indicated. (c) Specific primer pairs against various NCKX isoforms was used to amplify the NCKX in bovine chromaffin cells by RT-PCR. The size of bands for each isoform was as predicted (1, NCKX1, 445 bp; 2, NCKX2, 316 bp; 3, NCKX3, 320 bp; 4, NCKX4, 316 bp; and 5, NCKX5). Left lane: 100 bp DNA ladder.

replacement of extravesicular Na^+ with K^+ or Rb^+ caused about a 12-fold increment in $^{45}Ca^{2+}$ uptake; Li^+ and the other commonly used Na^+ replacement caused a similar one- to twofold increase.

In another series experiments to examine whether Na^+ can be replaced by other ions, the plasma membrane vesicles were pre-loaded with various monovalent cations including Li^+, Cs^+, K^+, and Rb^+ to replace Na^+ and the $^{45}Ca^{2+}$ uptake was performed in Na^+-free NMG solution. The results showed that the intravesicular Na^+ can only be partially replaced by Li^+ and not at all by other monovalent ions including Cs^+, K^+, and Rb^+ (data not shown).

NCKX gene is expressed in bovine chromaffin cell

The above results strongly support the hypothesis that a K^+-dependent NCX activity is present in intact chromaffin cells and in the vesicle fractions isolated from plasma membrane and intracellular organelles. To confirm the presence of the NCKX in chromaffin cells, we designed several pairs of primers based on the sequence of bovine NCKX1. These covered the DEGEIQA repeat motifs in the cytoplasmic loop. RT-PCR was then performed using mRNA from freshly isolated chromaffin cells as template. A PCR product was obtained with the size as that expected based on the bovine NCKX1 sequence (674 bp, Fig. 4a). The sequence of the PCR product was identical to the reported bovine NCKX1 sequence (Reiländer *et al.* 1992). A northern blot of the chromaffin cell RNA using the PCR product as a probe shows that a 4.2 kb band was present (Fig. 4b). The results support the presence of NCKX1 in bovine chromaffin cells. To further check whether there are other NCKX isoforms present in the chromaffin cells, primers were designed based on the bovine sequences of individual isoforms. The results showed that four isoforms of NCKX, NCKX1–4, are present in the bovine chromaffin cells (Fig. 4c). The sequences of the PCR products were identical to the predicted sequences. As a positive control, RT-PCR has been carried out using mRNA isolated from mouse brain and bands with the predicted sizes have been obtained for the five NCKX isoforms (data not shown).

Discussion

In this study, the results obtained using both biochemical and molecular approaches support the co-presence of NCX and NCKX in bovine adrenal chromaffin cells. In intact chromaffin cells, the $[Ca^{2+}]_i$ elevation elicited by the reverse mode exchange was significantly enhanced in the presence of

extracellular K^+. It appears that NCKX contributes at least equally with NCX to the NCX activity in intact cells. A similar K^+ enhancement of NCX activity was also demonstrated in the isolated plasma membrane. In addition, PCR and northern blot analysis showed the presence of several NCKX isoforms in bovine chromaffin cells. Therefore, the results support the co-presence of NCX and NCKX in bovine chromaffin cells.

It is highly likely that the K^+ enhancement of the NCX activity is due to the presence of NCKX. In this study, the cell was treated with ouabain to elevate the intracellular Na^+ concentration and the reverse-mode exchanger activity was elicited by lowering the extracellular Na^+ concentration. The results showed that the $[Ca^{2+}]_i$ could be elevated by this protocol and this elevation was greatly enhanced by the K^+. The increase in $[Ca^{2+}]_i$ was not through nicotinic receptors or Ca^{2+} channels because the experiments were carried out in the presence of inhibitors of the nicotinic receptors as well as Ca^{2+} channel blockers. In addition, K^+ must be present on the same side as Ca^{2+} to exert this enhancement; K^+ could not replace intravesicular Na^+ and induce $^{45}Ca^{2+}$ uptake. The presence of valinomycin did not significantly affect the $^{45}Ca^{2+}$ uptake. Rb^+ could replace K^+ in enhancing the $^{45}Ca^{2+}$ uptake; other ions had much smaller effect (Table 4). $^{86}Rb^+$ was taken up by the plasma membrane vesicles and the amount of Rb^+ transported is about the same as that of Ca^{2+} (data not shown). Furthermore, no K^+-enhanced $^{45}Ca^{2+}$-uptake activity was detected in the plasma membrane isolated from cardiac cells. Therefore, our evidence supports the idea that the K^+ enhanced $[Ca^{2+}]_i$ elevation in intact cells or the $^{45}Ca^{2+}$ uptake by the plasma membrane are through NCKX and not just by passive diffusion of the K^+ to compensate for the electrogenic effect of NCX.

In the presence of K^+, the exchange activity was significantly enhanced not only in plasma membrane, but in the membrane fractions isolated from chromaffin granules and mitochondria. It has been shown that NCX activity is present in granules and mitochondria to modulate the $[Ca^{2+}]_i$ homeostasis (Phillips 1981; Krieger-Brauer and Gratzl 1982; Gunter and Pfeiffer 1990); however, little is known about the molecular identity of the exchangers present in these subcellular organelles. The physiological significance of the K^+ enhancement of the NCX activity in the chromaffin granule membrane is not clear. It is possible that NCKX participates in the removal of cytosolic Ca^{2+}. It has been reported that Ca^{2+} concentrations in the chromaffin granules is in the range of 20–40 mM, mostly in a bound state (Phillips 1981; Krieger-Brauer and Gratzl 1982; Mahapatra *et al.* 2004). When the $[Ca^{2+}]_i$ is increased upon stimulation, the K^+ enhanced NCX activity in the granule membrane may accelerate the removal of Ca^{2+} from the cytosol. The Ca^{2+} accumulated in the granules can then be released outside of the cells during exocytosis. In PC12 cells, it has been shown that stimulation of exocytosis induces an increase in both $[Ca^{2+}]_i$ and the Ca^{2+} concentration in the granules, estimated by using a granule-targeted aequorin fusion protein (Mahapatra *et al.* 2004). Although the results support the role of chromaffin granules in removing cytosolic Ca^{2+}, the role of NCX and NCKX in bovine chromaffin cells remains to be explored.

The NCX of mitochondria in the regulation of $[Ca^{2+}]_i$ has been recognized. The presence of both NCX and NCKX activities in the mitochondria fraction isolated from bovine chromaffin cells suggest the possibility that the elevation of the intracellular Na^+ may establish a gradient to mobilize Ca^{2+} out of the mitochondria through the NCX and NCKX systems; this is especially true of the NCKX, which could utilize the strong K^+ gradient to facilitate the driving force. Similar role for chromaffin granules cannot be excluded.

The exchanger activity in mitochondria fraction is the smallest of the three fractions. The specific activity of the exchanger detected in the mitochondrial membrane fraction was about 20% of the chromaffin granule membrane fraction (Fig. 3). Therefore, contamination with at most 20% of the membrane from the chromaffin granules would result in the activity detected. This is certainly a strong possibility given the membrane preparation procedure used for this study (Kao and Cheung 1990). Furthermore, a similar level of K^+ enhancement of the exchanger activity was detected in the two fractions, about twofold, in contrast to the 50% enhancement in the plasma membrane detected under the same experimental conditions. Thus, it is quite possible that the exchanger activity detected in the mitochondrial membrane was due to contamination with chromaffin granule membrane.

There are two groups of mammalian sodium-calcium exchange genes: the *ncx* (*SLC8*) and *nckx* (*SLC24*) (Quednau *et al.* 2004; Schnetkamp 2004). Our previous results have shown that alternative spliced isoforms of *ncx1* were expressed in bovine adrenal medulla cells (Pan *et al.* 1998). For the different *nckx* genes, *nckx1* has the most restricted tissue distribution and is found only in rods and platelets. Other types of *nckx* (2–4) have been more widely detected in brain, aorta, and lung (Schnetkamp 2004). The size of the transcript detected by northern blot analysis is ~4.2 kb, which is different from the 6 kb transcript detected in the bovine retina (Reiländer *et al.* 1992). Similar phenomena to this with NCKX1 transcripts being of different lengths to that of eyes have been found in other tissues including rat adrenal gland (Poon *et al.* 2000). Results from RT-PCR suggest that NCKX1–4 are present in the bovine chromaffin cells. The functional significance of the presence of more than one NCKX isoform requires further study.

Our previous results have suggested the importance of NCX in regulating the $[Ca^{2+}]_i$ homeostasis in bovine chromaffin cells (Pan and Kao 1997; Yang and Kao 2001). Inhibiting NCX activity enhances exocytosis and delay $[Ca^{2+}]_i$ recovery after depolarization. The reverse mode NCX could bring Ca^{2+} into the bovine chromaffin cell to induce

catecholamine secretion. Most of the previous studies of NCX using subcellular membrane vesicles or intact cells were carried out in a solution containing K^+. It is likely that the activity measured included both NCX and NCKX. The role of NCX and NCKX in chromaffin cells needs to be re-examined. In the brain, the expression of NCX and NCKX could be widely detected in many areas; however, it is not clear whether both exchangers are expressed in the same type of cells. Our results show that both types of exchangers are expressed in bovine chromaffin cells and it is possible that the same phenomenon occur in neurons.

This report is the first paper to demonstrate the co-existence of NCX and NCKX in bovine chromaffin cells. Little is known about the functions of NCKX except in rod photoreceptors, which has been studied extensively (Schnetkamp 2004). In the rod photoreceptor, NCKX removes cytosolic Ca^{2+} to maintain the $[Ca^{2+}]_i$ at low level both in the dark and in the light, which appears to account for the response of photoreceptor to a wide range of light levels. In chromaffin cells, it is possible that under resting conditions, with the help of K^+ gradient across the plasma membrane, NCKX may dominate Ca^{2+} export to maintain the $[Ca^{2+}]_i$ at a low level. When the cell is stimulated resulting in an influx of Na^+, the cell is depolarized and the direction of the NCX system could be reversed to increase $[Ca^{2+}]_i$. At this point, NCKX may help to export Ca^{2+} from the cytosol with the manifestation of the outward K^+ gradient. The two types of exchanger may contribute differently under different conditions towards the same aim of regulating $[Ca^{2+}]_i$ in chromaffin cells.

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References

Baker P. F., Blaustein M. P., Hodgkin A. L. and Steinhardt R. A. (1969) The influence of calcium on sodium efflux in squid axons. *J. Physiol.* **200**, 431–458.

Chern Y. J., Chueh S. H., Lin Y. J., Ho C. M. and Kao L. S. (1992) Presence of Na^+/Ca^{2+} exchange activity and its role in regulation of intracellular calcium concentration in bovine adrenal chromaffin cells. *Cell Calcium* **13**, 99–106.

Dahan D., Spanier R. and Rahamimoff H. (1991) The modulation of rat brain Na^+-Ca^{2+} exchange by K^+. *J. Biol. Chem.* **266**, 2067–2075.

Gunter T. E. and Pfeiffer D. R. (1990) Mechanisms by which mitochondria transport calcium. *Am. J. Physiol.* **258**, C755–C786.

Kao L.-S. and Cheung N. S. (1990) Mechanism of calcium transport across the plasma membrane of bovine chromaffin cells. *J. Neurochem.* **54**, 1972–1979.

Kao L.-S. and Westhead E. W. (1984a) Binding of actin to chromaffin granules and mitochondrial fractions of adrenal medulla. *FEBS Lett.* **173**, 119–123.

Kao L.-S. and Westhead E. W. (1984b) Temperature dependence of catecholamine secretion from cultured bovine chromaffin cells. *J. Neurochem.* **43**, 590–592.

Kimura M., Aviv A. and Reeves J. P. (1993) K^+-dependent Na^+/Ca^{2+} exchange in human platelets. *J. Biol. Chem.* **268**, 6874–6877.

Kimura M., Jeanclos E. M., Donnelly R. J., Lytton J., Reeves J. P. and Aviv A. (1999) Physiological and molecular characterization of the Na^+/Ca^{2+} exchanger in human platelets. *Am. J. Physiol.* **277**, H911–H917.

Krieger-Brauer H. and Gratzl M. (1982) Uptake of Ca^{2+} by isolated secretory vesicles from adrenal medulla. *Biochim. Biophys. Acta* **691**, 61–70.

Li Z., Matsuoka S., Hryshko L. V., Nicoll D. A., Bersohn M. M., Burke E. P., Lifton R. P. and Philipson K. D. (1994) Cloning of the NCX2 isoform of the plasma membrane Na^+-Ca^{2+} exchanger. *J. Biol. Chem.* **269**, 17434–17439.

Lin L. F., Kao L. S. and Westhead E. W. (1994) Agents that promote protein phosphorylation inhibit the activity of the Na^+/Ca^{2+} exchanger and prolong Ca^{2+} transients in bovine chromaffin cells. *J. Neurochem.* **63**, 1941–1947.

Liu P. S. and Kao L. S. (1990) Na^+-dependent Ca^{2+} influx in bovine adrenal chromaffin cells. *Cell Calcium* **11**, 573–579.

Lytton J. (2007) Na^+/Ca^{2+} exchangers: three mammalian gene families control Ca^{2+} transport. *Biochem. J.* **406**, 365–382.

Mahapatra N. R., Mahata M., Hazra P. P., McDonough P. M., O'Connor D. T. and Mahata S. K. (2004) A dynamic pool of calcium in catecholamine storage vesicles. *J. Biol. Chem.* **279**, 51107–51121.

Nicoll D. A., Longoni S. and Philipson K. D. (1990) Molecular cloning and functional expression of the cardiac sarcolemmal Na^+-Ca^{2+} exchanger. *Science* **250**, 562–565.

Nicoll D. A., Quednau B. D., Qui Z., Xia Y. R., Lusis A. J. and Philipson K. D. (1996) Cloning of a third mammalian Na^+-Ca^{2+} exchanger, NCX3. *J. Biol. Chem.* **271**, 24914–24921.

Pan C. Y. and Kao L. S. (1997) Catecholamine secretion from bovine adrenal chromaffin cells: the role of the Na^+/Ca^{2+} exchanger and the intracellular Ca^{2+} pool. *J. Neurochem.* **69**, 1085–1092.

Pan C. Y., Chu Y. S. and Kao L. S. (1998) Molecular study of the Na^+/Ca^{2+} exchanger in bovine adrenal chromaffin cells. *Biochem. J.* **336**, 305–310.

Philipson K. D. and Nicoll D. A. (1992) Sodium-calcium exchange. *Curr. Opin. Cell Biol.* **4**, 678–683.

Phillips J. H. (1981) Transport of Ca^{2+} and Na^+ across the chromaffin-granule membrane. *Biochem. J.* **200**, 99–107.

Poon S., Leach S., Li X. F., Tucker J. E., Schnetkamp P. P. and Lytton J. (2000) Alternatively spliced isoforms of the rat eye sodium/calcium + potassium exchanger NCKX1. *Am. J. Physiol. Cell Physiol.* **278**, C651–C660.

Powis D. A., Clark C. L. and O'Brien K. J. (1994) Lanthanum can be transported by the sodium-calcium exchange pathway and directly triggers catecholamine release from bovine chromaffin cells. *Cell Calcium* **16**, 377–390.

Quednau B. D., Nicoll D. A. and Philipson K. D. (2004) The sodium/calcium exchanger family-SLC8. *Pflugers Arch.* **447**, 543–548.

Reeves J. P. (1992) Molecular aspects of sodium-calcium exchange. *Arch. Biochem. Biophys.* **292**, 329–334.

Reiländer H., Achilles A., Friedel U., Maul G., Lottspeich F. and Cook N. J. (1992) Primary structure and functional expression of the

Na/Ca,K-exchanger from bovine rod photoreceptors. *EMBO J.* **11**, 1689–1695.

Reuter H. and Seitz N. (1968) The dependence of calcium efflux from cardiac muscle on temperature and external ion composition. *J. Physiol.* **195**, 451–470.

Schnetkamp P. P. (2004) The SLC24 Na^+/Ca^{2+}-K^+ exchanger family: vision and beyond. *Pflugers Arch.* **447**, 683–688.

Tang Y. M., Travis E. R., Wightman R. M. and Schneider A. S. (2000) Sodium-calcium exchange affects local calcium signal decay and the rate of exocytotic secretion in single chromaffin cells. *J. Neurochem.* **74**, 702–710.

Williford D. J., Sharma V. K., Korth M. and Sheu S. S. (1990) Spatial heterogeneity of intracellular Ca^{2+} concentration in nonbeating guinea pig ventricular myocytes. *Circ. Res.* **66**, 241–248.

Yang D. M. and Kao L. S. (2001) Relative contribution of the Na^+/Ca^{2+} exchanger, mitochondria and endoplasmic reticulum in the regulation of cytosolic Ca^{2+} and catecholamine secretion of bovine adrenal chromaffin cells. *J. Neurochem.* **76**, 210–216.

