Recombinant Expression of the Plasma Membrane Na⁺/Ca²⁺ Exchanger Affects Local and Global Ca²⁺ Homeostasis in Chinese Hamster Ovary Cells

Marisa Brini, Sabrina Manni, and Ernesto Carafoli

From the Department of Biochemistry and Center for the Study of Biomembranes of the National Research Council, University of Padova, Viale G. Colombo 3, 35121 Padova and the Venetian Institute of Molecular Medicine, Via Orus 2, 35129 Padova, Italy

The cardiac type Na⁺/Ca²⁺ exchanger (NCX1) has been transiently expressed in Chinese hamster ovary cells, which do not contain an endogenous exchanger, together with aequorin chimeras that are targeted to different intracellular compartments to investigate intracellular Ca²⁺ homeostasis. The expression of NCX decreased the endoplasmic reticulum Ca²⁺ concentration, [Ca²⁺]_{er}, in resting cells, showing that the exchanger was operative under these conditions. It induced a greater reduction in the height of the mitochondrial and cytosolic Ca²⁺ transients in agonist-stimulated cells than would have been expected from the \([Ca^{2+}]_{er}\) decrease. It also had a major effect on the sub-plasma membrane Ca²⁺ concentration, [Ca²⁺]_{pm} after a transient [Ca²⁺]_{pm} rise induced by the activation of capacitative Ca²⁺ influx, [Ca²⁺]_{pm} settled to a value about 3-fold higher than in controls. The sustained [Ca²⁺]_{pm} increase after the transient was due to the operation of the exchanger, either directly by operating in the Ca²⁺ entry mode, or indirectly by removing the Ca²⁺ inhibition on the capacitative Ca²⁺ influx channels.

The Na⁺/Ca²⁺ exchanger is the major Ca²⁺ extrusion system in cells characterized by large Ca²⁺ swings, e.g. cardiac myocytes and neurons. It is ideally qualified for the task of rapidly removing large amounts of Ca²⁺ from the cytosol, because its transport capacity is very large. Normally, the system exchanges one intracellular Ca²⁺ for three extracellular Na⁺ but can also operate in the opposite direction, i.e. it can mediate Ca²⁺ influx, depending on the membrane potential and the gradients of Na⁺ and Ca²⁺. The physiological role of the exchanger and the mechanism of the transport reaction in the Ca²⁺ efflux mode are well understood. By contrast, the role and the mechanism of the Ca²⁺ influx mode are less well defined and still somewhat controversial. Another area of uncertainty is the regulation of the exchanger activity by Ca²⁺. Experiments on excised membrane patches indicate that half-maximal activity of the exchanger requires about 2 μM Ca²⁺ on the cytosolic side (1), but experiments in cardiac myocytes and transfected CHO cells indicate half-maximal activity in a lower cytosolic Ca²⁺ concentration range, between 20 and 50 nM (2, 3). These differences are not trivial, because the former values would make the exchanger essentially inoperative in resting cells, whereas the latter would be compatible with exchanger activity in living cells at resting cytosolic Ca²⁺ concentration. It must also be considered that post-translational modifications, e.g. phosphorylations or other factors, could increase the Ca²⁺ affinity of the exchanger in vivo. Finally, it is likely that the Ca²⁺ concentration ([Ca²⁺]) sensed by the exchanger in the sub-plasma membrane zone is significantly higher than in the bulk cytoplasm.

In this report we have used transiently transfected CHO cells that do not normally express Na⁺/Ca²⁺ exchange activity. After transfection with an expression vector coding for the dog cardiac exchanger (NCX1, 4), high levels of expression of the protein in the plasma membrane were achieved. To evaluate the role of the exchanger with respect to that of the other mechanisms for cellular Ca²⁺ transport, we have followed the procedure previously used to study the interplay of the two Ca²⁺-removing pumps, the plasma membrane Ca²⁺ ATPase (PMCA) and the sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase (SERCA) (5). Targeted recombinant aequorins (6) were co-transfected with NCX1, and the handling of [Ca²⁺]_{pm} was monitored in the endoplasmic reticulum (ER), in the cytoplasm, in mitochondria, and in the restricted space beneath the plasma membrane. The results have shown that the transfected exchanger was active also in cells at rest, its activity contributing directly to the regulation of [Ca²⁺]_{pm} in the intracellular stores. NCX1 drastically reduced the amplitude of the cytosolic and mitochondrial [Ca²⁺] transients generated by cell stimulation with an inositol 1,4,5-trisphosphate (InsP₃)-generating agonist. The expressed NCX1 had an unexpected effect on the homeostasis of [Ca²⁺]_{pm} in the sub-plasma membrane domain. After the transient elicited by capacitative influx, [Ca²⁺]_{pm} settled at a significantly higher level than in control cells, suggesting that the operation of NCX1 enhanced Ca²⁺ entry into the restricted space beneath the plasma membrane.

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‡ To whom correspondence should be addressed: Dept. of Biochemistry, University of Padova, Viale G. Colombo, 3, 35121 Padova, Italy. Tel.: 39-049-827-6167; Fax: 39-049-827-6125; E-mail: brini@mail.bio.unipd.it.

The abbreviations used are: CHO, Chinese hamster ovary cells; NCX1, cardiac type Na⁺/Ca²⁺ exchanger; ER, endoplasmic reticulum; InsP₃, inositol 1,4,5-trisphosphate; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine; eAEQ, ER-targeted aequorin; cytAEQ, cytosolic-targeted AEQ; mtAEQ, mitochondrially targeted AEQ; pmAEQ, plasma membrane-targeted AEQ; KRB, Krebs-Ringer buffer; CCE, capacitative Ca²⁺ entry; SR, sarcoplasmic reticulum.
Experimental Procedures

Cell Cultures and Transfection—CHO cells were grown in Ham’s F-12 medium, supplemented with 10% fetal calf serum, in 75-cm² Falcon flasks; before transfection, they were seeded onto 13 glass cover slips and allowed to grow to 50% confluence. At this stage transfection with 3 μg of plasmid DNA (or 1.5:1.5 μg in the case of co-transfection) was carried out as previously described (7). Aequorin measurements and immunocytochemistry were performed 36 h later.

Cultures of cardiomyocytes were prepared from ventricles of 1- to 3-day-old rats as previously described (8). The heart was removed and rapidly placed in saline containing 116 mM NaCl, 0.8 mM NaH₂PO₄, 5.3 mM KCl, 0.4 mM MgSO₄, 5 mM glucose, and 20 mM HEPES, adjusted to pH 7.35. Ventricles were submitted to four digestions with 0.45 mg/ml collagenase and 5% pancreatin (20 min each). The first supernatant containing the non-myocytes and blood cells was discarded. Cell suspensions from the three subsequent digestions were centrifuged, and the pellet was resuspended in 2 ml of newborn calf serum. Cells were pooled and resuspended in a complete medium (Dulbecco’s modified Eagle’s medium/Medium 199 (4:1), supplemented with 2 mM glutamine, penicillin (100 units/ml), streptomycin (100 mg/ml), 5% newborn calf serum, and 10% horse serum). They were then incubated for 1 h at 37 °C, to permit a selective attachment of non-myocytes. The unattached cells were plated at a density of 0.35 × 10⁶ cells/ml onto 24-well plates coated with laminin (1 μg/cm²). After the first day of culture, cells were washed and maintained in Dulbecco’s modified Eagle’s medium/Medium 199 (4:1) containing 5% horse serum and 0.5% newborn calf serum for 2 days.

Immunolocalization—36 h after transfection CHO cells were processed for immunofluorescence as follows: they were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) and permeabilized with 0.1% Triton X-100 in PBS, followed by 1 h wash with 50 mM NH₄Cl. Permeabilization of membranes was obtained with a 5-min incubation with 1% Triton X-100 in PBS, followed by 1 h wash with 1% gelatin (type IV, from calf skin) in PBS. Then the coverslip was processed for the NCX1 staining with a polyclonal antibody against the NCX1 isoform (Novus Biologicals) at a 1:1,000 dilution in PBS. Staining was performed with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:100 dilution in PBS, Roche Molecular Biochemicals, Denmark). After each incubation, cells were washed four times with PBS. Fluorescence was analyzed with a Zeiss Axiovert microscope equipped with a 12-bit digital cooled camera (Micromax-1300Y, Princeton Instruments, Inc.). The sample was illuminated alternatively at 340/380 nm, and the emitted light (filtered with an interference filter centered at 510 nm) was collected, 5 μm camera images were acquired using Metafluor software (Universal Imaging Corp.). The ratio values (1 ratio image/s) were calculated off-line, after background subtraction from each single image.

Statistical Analysis—Data are reported as means ± S.D. Statistical differences were evaluated by Student’s two-tailed t test for impaired samples, with a p value 0.05 being considered statistically significant.

Results

Expression and Membrane Targeting of Recombinant NCX—

Prior to analyzing Ca²⁺ homeostasis, the level of expression and the membrane distribution of recombinant NCX1 were verified. CHO cells were transfected with the cDNA of NCX1 cloned in the mammalian expression vector pSG5 (Stratagene, La Jolla, CA) and analyzed 36 h later under the epifluorescence microscope. A polyclonal antibody specific for NCX1 (9) yielded a strong signal, with a clear decoration of the cell surface. The pattern was that of cells expressing plasma membrane proteins, showing that the transfected exchanger had been correctly targeted. A weak reticular positivity throughout the cytoplasm was also visible, indicating that a (minor) portion of the transfected NCX was retained in the ER (Fig. 1A). The co-expression of NCX1 and the aequorin probe were demonstrated by dual labeling immunocytochemistry. (In the experiment shown in Fig. 1A, NCX1 was revealed by FITC staining, and mitochondrially targeted aequorin, mtAEQ, by TRITC staining. As expected, the two stains did not overlap and showed the expected patterns.)

The level of recombinant exchanger expression in CHO cells was compared with that of the endogenous NCX in primary cultures of atrial myocardium by Western blotting and densitometric analysis (Fig. 1B). Crude membrane extracts were obtained from these samples (as described under “Experimental Procedures”) and loaded on SDS-PAGE. The nitrocellulose membrane was probed with the NCX isofrom 1 specific antibody (9). Fig. 1B shows that in NCX-transfected CHO cells (lane 1) and cardiac myocytes (lane 2) the antibody recognized mainly two proteins migrating with apparent molecular masses of about 160 and about 120 kDa. A 70-kDa proteolytic product was also frequently present. The 120-kDa band corresponds to the mature protein (14).

The level of NCX expression (the 120- and 160-kDa bands) in CHO cells was estimated to be about 15- to 20-fold lower than...
in cardiac myocytes. Because the average transfection efficiency was in the 15–25% range, the amount of exogenous exchanger, corrected for the whole cell population, would be about 3- to 4-fold lower than that of cardiac myocytes.

**Endoplasmic Reticulum Ca\(^{2+}\) in NCX-expressing Cells**—Endoplasmic reticulum-targeted aequorin, erAEQ, was used to measure the Ca\(^{2+}\) concentration in the ER lumen. Functional aequorin was reconstituted as previously described (11) with a modified prosthetic group, coelenterazine n, to decrease the Ca\(^{2+}\) affinity of the photoprotein and thus permit the measurement of the high concentration of Ca\(^{2+}\) in the ER lumen (12). After aequorin reconstitution in a Ca\(^{2+}\)-free medium, the cells were transferred to the luminometer chamber, and store refilling was initiated by supplementing the Krebs-Ringer buffer (KRB) perfusion medium with a concentration of Ca\(^{2+}\) corresponding to that of the extracellular space (1 m\(\text{M}\)). The experiment in Fig. 2A shows parallel batches of CHO cells transfected with erAEQ alone or co-transfected with erAEQ and NCX1. Approximately 1 min after Ca\(^{2+}\) addition, \([\text{Ca}^{2+}]_{\text{ER}}\) reached a steady-state level in control cells, which, in agreement with previous reports, was about 680 m\(\text{M}\) (617.1 ± 65.35 m\(\text{M}\), \(n = 13\)). In NCX-expressing cells \([\text{Ca}^{2+}]_{\text{ER}}\) was about 15% lower (523.54 ± 39.26 m\(\text{M}\), \(n = 13\)). The rate of Ca\(^{2+}\) release from the ER store induced by the InsP\(_3\)-generating agonist ATP was slightly slower in NCX-expressing cells. In principle, this could have been due to the activity of the fraction of NCX retained in the ER membrane, which could have counteracted the activity of the InsP\(_3\)-sensitive channels by transporting Ca\(^{2+}\) back to the ER lumen from a microdomain of high [Ca\(^{2+}\)] generated in its proximity. This, however, seems very unlikely.

On one hand, proteins destined to a given membrane system are normally inactive when retained somewhere else. On the other hand, the transport of Ca\(^{2+}\) into the ER would demand a significant Na\(^+\) gradient across the membrane of the latter, which to our knowledge has never been reported.

**Cytosolic and Mitochondrial Ca\(^{2+}\) in NCX-expressing Cells**—Because the differences in the \([\text{Ca}^{2+}]_{\text{ER}}\) level were presumably due to the reduction in the cytosolic [Ca\(^{2+}\)], we decided to evaluate the latter in control and NCX-expressing cells. The steep response curve of aequorin makes it difficult to accurately estimate \([\text{Ca}^{2+}]_{\text{cyt}}\) at concentration <200–300 n\(\text{M}\), thus computerized image analysis with Fura-2 was used instead in cells expressing the NCX, which were identified by co-transfection with the green fluorescent protein. The resting fluorescence values (i.e., the 340/380 nm fluorescence values minus the background) were slightly reduced in NCX-expressing cells: 0.357 ±
The effects of NCX on the cytosolic and mitochondrial \([\text{Ca}^{2+}]\) transients (\([\text{Ca}^{2+}]_\text{c}\) and \([\text{Ca}^{2+}]_\text{m}\)) were then studied following agonist stimulation. Fig. 2B shows that the average peak value of 3.03 ± 0.30 \(\mu\)M (\(n = 17\)) for the \([\text{Ca}^{2+}]_\text{c}\) transient (measured with cytosolic-targeted aequorin, cytAEQ), in control cells, was drastically reduced in the cells expressing NCX1 (to 0.93 ± 0.46 \(\mu\)M, \(n = 16\)). Similar results were also obtained in fura-2 experiments (data not shown). Fig. 2C shows a similar experiment on CHO cells co-transfected with the low affinity variant of mtAEQ (15) and mtAEQ/NCX plasmids. The \([\text{Ca}^{2+}]_\text{m}\) peak induced by ATP was 63.07 ± 25.27 \(\mu\)M (\(n = 22\)) in control cells and much lower (17.00 ± 7.18 \(\mu\)M, \(n = 19\)) in NCX-expressing cells.

The decrease of the cytosolic and mitochondrial \([\text{Ca}^{2+}]\) transients in NCX-expressing cells could either have been due to the approximate 15% reduction of \([\text{Ca}^{2+}]_\text{c}\) in the ER lumen, or, alternatively, to the direct removal of \(\text{Ca}^{2+}\) from the cytoplasm by the expressed NCX, which would reduce the \([\text{Ca}^{2+}]_\text{c}\) “hotspots” at the mouth of the InsP3-sensitive and the plasma membrane channels. To discriminate between the two alternatives, the degree of \(\text{Ca}^{2+}\) filling of the ER lumen was titrated-down. CHO cells transfected with erAEQ were re-exposed to KRB supplemented with 0.7 \(\text{mM}\), instead of 1 \(\text{mM}\) CaCl\(_2\). Fig. 3A shows that the procedure lowered the plateau of \([\text{Ca}^{2+}]_\text{er}\) to 526.05 ± 23.60 \(\mu\)M, \(n = 8\) (as compared with 617.15 ± 65.35 \(\mu\)M, \(n = 13\) in cells exposed to 1 \(\text{mM} \text{CaCl}_2\), see above), a value which was similar to that in cells expressing NCX in KRB supplemented with 1 \(\text{mM} \text{CaCl}_2\) (523.54 ± 39.26 \(\mu\)M, \(n = 13\), see above). The same protocol of ER empting and refilling was applied to CHO cells transfected with cytAEQ and then challenged with ATP (Fig. 3B). The height of the \([\text{Ca}^{2+}]_\text{c}\) peak was similar in cells exposed to 0.7 (2.50 ± 0.46 \(\mu\)M, \(n = 6\)) or 1 \(\text{mM} \text{CaCl}_2\) (2.57 ± 0.28 \(\mu\)M, \(n = 5\)), showing that the 15–20% reduction of \([\text{Ca}^{2+}]_\text{c}\) could not account for the 70% reduction in \([\text{Ca}^{2+}]_\text{c}\). Evidently, the activity of NCX directly affected the height of the peak transient. Accordingly, the replacement of extracellular \(\text{Na}^+\) with \(\text{Li}^+\) during the generation of the \(\text{Ca}^{2+}\) transients with ATP abolised the effect (data not shown).

Effect of NCX Activity on the Sub-plasma Membrane \(\text{Ca}^{2+}\) Pool—One controversial issue on the exchanger is its activity at the resting cellular \([\text{Ca}^{2+}]\) values, which are well below its apparent \(K_m\) for \(\text{Ca}^{2+}\), and thus in principle inadequate to significantly activate the exchanger. Fig. 2A had nevertheless shown that NCX was active in un-stimulated cells, because its expression modulated the level of \([\text{Ca}^{2+}]_\text{c}\) filling of the ER lumen. One convenient way to explain this paradoxical observation would be by postulating that the concentration of \(\text{Ca}^{2+}\) in the sub-plasma membrane region, which is in immediate contact with the exchanger, is significantly higher than in the bulk cytosol, as previously reported, for example, in A7r5 smooth muscle cells (16). It was thus decided to measure directly \([\text{Ca}^{2+}]_\text{pm}\) in CHO cells by co-expressing the NCX and a low affinity plasma membrane-targeted aequorin chimera, pmAEQ. The measurements were performed in monolayers of cells after 60 min of exposure to coelenterazine to reconstitute aequorin. To increase the efficiency of the reconstitution no \(\text{Ca}^{2+}\) was added to the KRB, which was supplemented with 100 \(\mu\)M EGTA. When added back to the medium, \(\text{Ca}^{2+}\) entered into the cells through plasma membrane channels, generating a transient \([\text{Ca}^{2+}]_\text{pm}\) rise (Fig. 4A), which peaked at 78.78 ± 37.63 \(\mu\)M (\(n = 17\)) in control cells and at 71.56 ± 31.15 \(\mu\)M (\(n = 23\)) in NCX-expressing cells. After the transient, resting \([\text{Ca}^{2+}]_\text{c}\) conditions were re-established in approximately 1 min, after which time \([\text{Ca}^{2+}]_\text{pm}\) was maintained at a plateau of about 1.93 ± 0.89 \(\mu\)M (\(n = 17\)) in control cells. Surprisingly, resting \([\text{Ca}^{2+}]_\text{pm}\) was maintained at a value that was about 3-fold higher (5.90 ± 1.16 \(\mu\)M, \(n = 23\)) in NCX-expressing cells.

The unexpected difference suggested that, under the experimental conditions, there was an increase in \(\text{Ca}^{2+}\) entry related to NCX expression and function. The possibility of a direct effect of the exchanger, which would mediate \(\text{Ca}^{2+}\) entry, was tested by replacing \(\text{Na}^+\) in the KRB medium with \(\text{Li}^+\) (or with choline chloride, data not shown), which is not transported by the exchanger (17). In the presence of extracellular \(\text{Ca}^{2+}\), the switch from \(\text{Na}^+\) to \(\text{Li}^+\) was expected to favor the \(\text{Ca}^{2+}\) entry operation of the exchanger, whereas in its absence the operation of the NCX was expected to be blocked. Prior to performing the measurements of \([\text{Ca}^{2+}]_\text{pm}\), ER, cytosolic, and mitochondrial \([\text{Ca}^{2+}]\) measurements were carried out. As would have been expected from the reverse operation of the exchanger, when \(\text{Na}^+\) was rapidly replaced by \(\text{Li}^+\), the ER \(\text{Ca}^{2+}\) content indeed increased in NCX-expressing cells but not in control cells (from 523.54 ± 39.26 \(\mu\)M, \(n = 13\), to 681.00 ± 79.36 \(\mu\)M, \(n = 8\)). The height of the cytosolic and mitochondrial \([\text{Ca}^{2+}]\) transients generated by agonist stimulation also increased (data not shown).
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![Graph](https://via.placeholder.com/150)

**Fig. 4. Monitoring of the sub-plasma membrane Ca\(^{2+}\) concentration with low affinity pmAEQ.** A, effects of CaCl\(_2\) re-addition (1 mM) to control (black trace) and NCX-expressing (gray trace) CHO cells maintained in a Ca\(^{2+}\)-free medium. After 60 min of reconstitution in the EGTA-supplemented medium, the coverslip with the transfected cells was transferred to the luminometer chamber and perfused with KRB containing 100 \(\mu\)M EGTA. Where indicated, KRB was supplemented with 1 mM CaCl\(_2\). The inset shows resting levels of the \([Ca^{2+}]_{pm}\) on an expanded scale. B, monitoring of the sub-plasma membrane Ca\(^{2+}\) concentration in NCX-expressing cells in a Na\(^+\) containing KRB medium (KRB-NaCl) or in Na\(^--\)free KRB medium (NaCl was replaced by LiCl, KRB-LiCl). C, effects of the reverse mode inhibitor of the Na\(^+/\)Ca\(^{2+}\) exchanger KB-R7943 on the resting level of \([Ca^{2+}]_{pm}\) following Ca\(^{2+}\) influx in NCX-expressing CHO cells. The inhibitor was applied where indicated. The inset shows the resting levels of \([Ca^{2+}]_{pm}\) on an expanded scale. D, effects of CaCl\(_2\) re-addition (0.7 mM or 1 mM) to control cells, whose ER was depleted of Ca\(^{2+}\).

The Li\(^+\) replacement experiment was thus repeated to measure \([Ca^{2+}]_{pm}\) exactly under the conditions described for Fig. 4A. Cells were incubated in the absence of external Na\(^+\) for about 4–5 min in an Li-KRB medium supplemented with EGTA, and then 1 mM CaCl\(_2\) was added back. As shown in Fig. 4B (light gray trace), following Ca\(^{2+}\) re-addition, a \([Ca^{2+}]_{pm}\) transient, in all likelihood occurring via the store-operated Ca\(^{2+}\) influx route, was generated. In NCX-expressing cells, the amplitude of this \([Ca^{2+}]_{pm}\) transient was not affected by the Li\(^+/\)Na\(^+\) replacement (69.83 ± 16.05 \(\mu\)M, \(n = 6\), in Li-KRB, versus 71.56 ± 31.15 \(\mu\)M, \(n = 23\), in Na-KRB). Unexpectedly, however, and at variance with the results shown in Fig. 4A (and reported for comparison also in Fig. 4B, dark gray trace) the post-transient \([Ca^{2+}]_{pm}\) in NCX-expressing cells was unaffected: it returned to a level similar to that of control cells (1.77 ± 0.80 \(\mu\)M, \(n = 6\)). Because the only difference in the experiments of Fig. 4A and B, carried out in NCX-expressing cells was the absence of extracellular Na\(^+\), the suggestion that NCX activity in the Ca\(^{2+}\) extrusion mode was involved in it was plausible. The difference between the experiment in Fig. 4B and the results described in the text above could be rationalized by assuming that the prolonged exposition of the cells to a medium free of Na\(^+\), even if the exchanger was inoperative due to the absence of external Ca\(^{2+}\), could still have decreased the intracellular Na\(^+\) content, because Na\(^+\) could have left the cells by other means, e.g., the Na\(^+/\)K\(^+\) pump. This, however, seems unlikely. A more likely possibility was that a slight increase of the resting \([Ca^{2+}]_{pm}\) in cells incubated in the Li\(^--\)KRB (promoted by the reverse operation of the exchanger) could have gone undetected by the low affinity sequorin, which had been used to measure \([Ca^{2+}]\) that was expected to exceed 2–3 \(\mu\)M.

A reasonable scenario would thus be the following: as a consequence of Ca\(^{2+}\) influx (through the capacitative channels) and in the presence of extracellular Na\(^+\), the exchanger would initially operate in the canonical Ca\(^{2+}\) export mode, generating a local Na\(^+\) gradient. This could reverse the NCX operating mode (or slow down the NCX activity), promoting the influx of Ca\(^{2+}\). Under the experimental conditions, the local Na\(^+\) gradient could perhaps be further augmented by Na\(^+\) entry through Ca\(^{2+}\) influx channels (18). Following the prolonged application of Li\(^+\), the exchanger could no longer operate in the Ca\(^{2+}\) extrusion mode, preventing the formation of the Na\(^+\) gradient, and thus lessening the driving force for the reverse operation of the NCX. Alternatively, the NCX working in the Ca\(^{2+}\) extrusion mode in the presence of extracellular Na\(^+\) would promptly remove the sub-plasma membrane Ca\(^{2+}\), protecting the capacitative channels from Ca\(^{2+}\)-dependent inactivation (19–21) and thus generating a higher sub-plasma membrane [Ca\(^{2+}\)] steady state. In the absence of extracellular Na\(^+\), the activation of the NCX in the Ca\(^{2+}\) extrusion mode would become impossible, and NCX-transfected cells would behave as control cells. Additional evidence, suggesting that the reverse-mode activity of the exchanger could have been responsible for the higher \([Ca^{2+}]_{pm}\) level in NCX-expressing cells, is presented in the experiment of Fig. 4C. The inhibitor KB-R7943, claimed to be specific for the reverse mode of the Na\(^+\)/Ca\(^{2+}\) exchanger (22), abolished the sustained elevation of \([Ca^{2+}]_{pm}\) in NCX-expressing cells, reducing \([Ca^{2+}]_{pm}\) to a level comparable to that of control cells: 2.29 ± 0.85 \(\mu\)M (\(n = 23\)).

In principle, the higher steady-state level of the post-transient \([Ca^{2+}]_{pm}\) rise in NCX-expressing cells could also have been due to a higher capacitative Ca\(^{2+}\) entry (CCE) triggered by the lower level of ER Ca\(^{2+}\) filling (23). Thus we decided to monitor \([Ca^{2+}]_{pm}\) in control cells first depleted of their ER Ca\(^{2+}\) content and then incubated in KRB containing only 0.7 mM CaCl\(_2\), instead of 1 mM, to artificially decrease ER Ca\(^{2+}\) replen-
lishment to a level similar to that found in NCX-expressing cells (see above). Fig. 4D shows that the average post-transient [Ca2+]pm was similar in 0.7 and in 1 mM CaCl2 1.40 ± 0.93 μM (n = 5) in 0.7 mM CaCl2 and 1.36 ± 0.26 μM (n = 5) in 1 mM CaCl2. Thus, the decreased ER Ca2+ content had apparently not increased CCE activity to produce the sustained elevation of [Ca2+]pm in NCX-expressing cells.

Experiments were also performed in which the Ca2+ mobilization from intracellular stores (via InsP3 formation) was dissected from the Ca2+ influx through plasma membrane channels to further explore the possibility that the sustained Ca2+ elevation in the sub-plasma membrane region was due to increased Ca2+ entry and to better understand its mechanism. Cells were first incubated in a Ca2+-free medium supplemented with EGTA to prevent the influx of Ca2+ and then stimulated with ATP. The expected [Ca2+] transient was generated. After it had subsided, the addition of Ca2+ caused a second peak, evidently due to Ca2+ penetration. In NCX-expressing cells transfected with cytAEQ or mtAEQ the Ca2+ entry was enhanced. Whether this additional Ca2+ entry was due to the reverse mode of operation of the NCX or to its ability to relieve the Ca2+-dependent inhibition of the Ca2+ influx channels cannot be decided at the moment. Attempts to selectively inhibit the Ca2+ influx channels with a blocker, SKF96365, were unsuccessful, because the inhibitor was ineffective in control cells as well.

**DISCUSSION**

In the present work we studied the fluxes of Ca2+ through the Na+/Ca2+ exchanger in the physiological milieu of living cells. The problem of the activity of NCX in unstimulated cells, where the average [Ca2+]i is well below the micromolar range necessary to activate the exchanger, has been considered first. Previous claims that the system could be active in resting cells (2, 24, 25) had been indirectly supported by the finding that the Ca2+ concentration in the rim of cytoplasm beneath the plasma membrane was higher (about 2 μM) than in the bulk cytosol (16). The present findings have directly documented that the exchanger was indeed active under resting cell conditions.

The next question was the function of the exchanger in the loading and unloading of the Ca2+ stores, a matter on which conflicting reports have appeared. Adachi-Akahane et al. (26) had predicted that the sarcoplasmic reticulum (SR) Ca2+ content in the hearts of transgenic mice overexpressing the exchanger would be lower than in control hearts. However, the size of the caffeine-sensitive Ca2+ pool was found to be unchanged in the transgenic myocytes. Another study (27) found that the reloading of the SR after Ca2+ depletion is faster in heart myocytes from transgenic mice overexpressing the Na+/Ca2+ exchanger, suggesting that the overexpressed NCX mediates the influx of extracellular Ca2+. Another study showed instead that the rate of store refilling in a stable clone of CHO cells expressing the NCX is reduced, in agreement with the prediction of Adachi-Akahane et al. (26) that the exchanger would counteract the rise in [Ca2+]i, produced by sustained Ca2+ entry (28). The problems raised by these conflicting reports have been solved in the present study by measuring directly the ER Ca2+ concentration. The results have shown that the steady-state level of [Ca2+]in in NCX-expressing cells was about 15% lower than in control cells, evidently due to the lower activity of the SERCA pump, linked to the decrease in [Ca2+]i.

Another important point is the activity of NCX in stimulated cells and its role in restoring [Ca2+] to the resting conditions. The amplitudes of the rise in [Ca2+]i and [Ca2+]in induced by the InsP3-linked agonist were reduced by about 70% in NCX-expressing cells. Similar results had been obtained by others on CCL3 fibroblasts stably transfected with NCX1. The [Ca2+]i transient induced by α-thrombin or thapsigargin in these cells was about 30% lower than in the controls (29).

The decrease of [Ca2+]i we observed was altogether limited. One could thus have expected a similarly limited decrease of [Ca2+]i under the influence of InsP3. The large reduction in [Ca2+]i, which was instead observed, could be due the efficient and rapid dissipation of the [Ca2+]i hotspots (created by the activated InsP3 receptors and/or the plasma membrane channels) by the increased activity of the NCX in the canonical Ca2+ extrusion mode. The low Ca2+ affinity and high transport capacity of the exchanger would facilitate the prompt removal of large amounts of Ca2+ from the cytosol. The ability of the NCX, which is located in the plasma membrane, to efficiently dissipate the presumably distant cytosolic microdomains of high [Ca2+]i at the mouth of the InsP3-activated channels was surprising. The possibility, that NCX units still retained in the ER membrane on their way to the plasma membrane (see Fig. 1A) are functional and transport Ca2+ back into the ER lumen, thus contributing to the decrease of [Ca2+]i, seems remote. More likely, NCX activity could lower resting cytosolic [Ca2+]i to values below the threshold necessary to open the InsP3 channels (30) or to activate Ca2+ regulated phospholipase C (31). As for the very large reduction of the amplitude of the mitochondrial [Ca2+]i transients, which are strictly dependent on the microdomains of high [Ca2+]i generated at the mouth of the InsP3 channels, it could be linked to the presence of mitochondria and ER structures in the cortical region of CHO cells. Some of the ER could thus generate [Ca2+]i hotspots, which would be dissipated by vicinal NCX units before mitochondria could sense them. Finally, physical connections, probably mediated by microtubuli, could exist between the NCX molecules and mitochondria, as suggested by Opuni and Reeves (32).

The analysis of the [Ca2+]i changes in the restricted area beneath the plasma membrane unexpectedly revealed that the mean [Ca2+]i after the transient induced by the re-addition of Ca2+ to the cells settled at a higher level in NCX-expressing cells. Several explanations rationalize the observation, e.g. the potentiation of the CCE mechanism by the reduced ER Ca2+ content, the reverse operation of the Na+/Ca2+ exchanger, or the decrease of Ca2+-dependent inactivation of the Ca2+ influx channels.

An effect of NCX on CCE linked to the reduction in [Ca2+]i is unlikely, because no enhanced Ca2+ entry was observed in control cells in which the degree of refilling of intracellular Ca2+ stores was artificially limited. The possibility that the NCX could initially operate in the Ca2+ extrusion mode generating a locally confined Na+ gradient that would then switch the exchanger to the Ca2+ entry mode had been already suggested, e.g. as a mechanism to refill the Ca2+ stores of cardiomyocytes to promote the next contraction (33-35). The reverse operation of the exchanger had also been claimed to contribute to the immediate rise in [Ca2+]i in neurons following glutamate receptor activation (36). As for the suggestion that Ca2+ extrusion by the NCX would protect the CCE channels from Ca2+-dependent inactivation, it would be reminiscent of a similar function of mitochondria in lymphocytes (37). The results in the present study are compatible both with the possibility that the exchanger might control the [Ca2+]i beneath the plasma membrane by acting itself as a Ca2+ influx system and with the possibility that it would be controlling the activity of the capacitative Ca2+ influx channels.

The finding that the exchanger reduced the ER Ca2+ content in unstimulated cells while at the same time it increased
[Ca\(^{2+}\)]\(_{\text{in}}\) beneath the plasma membrane remains somewhat puzzling. Possibly, NCX-mediated Ca\(^{2+}\) cycling across the plasma membrane could generate spatially confined Ca\(^{2+}\) gradients, rather than inducing global Ca\(^{2+}\) changes. As suggested by Leblanc and Hume (38) for the case of cardiac myocytes, the increased penetration of Ca\(^{2+}\) through the exchanger promoted by the elevation of intracellular [Na\(^{+}\)] could then trigger Ca\(^{2+}\)-induced Ca\(^{2+}\) release from the ER.

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Marisa Brini, Sabrina Manni and Ernesto Carafoli

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