The Transport Activity of the Na\textsuperscript{+}-Ca\textsuperscript{2+} Exchanger NCX1 Expressed in HEK 293 Cells Is Sensitive to Covalent Modification of Intracellular Cysteine Residues by Sulphydryl Reagents*

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Membrane permeable N-ethylmaleimide (NEM) and (2-aminoethyl) methanethiosulfonatehydrobromide (MTSEA) inhibited the rat brain Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger RBE-2 (NCX1.5) expressed in HEK 293 cells in a dose dependent manner. 50% inhibition was obtained at 1 mM MTSEA and 1.65 mM NEM. External application of membrane impermeable [2-(trimethylammonium) ethyl] methanethiosulfonatebromide (MTSET) and sodium(2-sulfonatoethyl)methanethiosulfonate (MTSES) did not inhibit the transport activity in whole cells. Following reconstitution, however, of RBE-2 transfected cell proteins into proteoliposomes, external application of MTSET and MTSES led to a decrease in transport activity to 42.7 (S.D. = 9.1) and 51% (S.D. = 10.14), respectively. Similar results were obtained also when the rat heart isoform RHE-1 (NCX1.1) or the rat brain isoform RBE-1 (NCX1.4) was expressed. NEM and MTSEA inhibited Na\textsuperscript{+} gradient-dependent Ca\textsuperscript{2+} uptake also in HEK 293 cells expressing RBE-2/C14A/C20S/C122S/C780S (numbering corresponds to RBE-2), a mutant in which all putative extracellular cysteines were exchanged. To study the accessibility of different cysteines to covalent modification, surface biotinylation of cells expressing the wild type exchanger and its mutants was carried out using 3-(N-maleimidylpropionyl)biocytin. Surface biotinylation revealed immunoreactive protein derived from the wild type Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger only if the transfected cells were exposed to the reducing agent Tris(2-carboxyethyl)phosphine. No reduction was needed when the single cysteine mutants of RBE-2, C14A, C20S, and C780S, were expressed. Treatment of the cells expressing these mutants with MTSET before biotinylation, led to a decrease in the amount of exchange protein that was revealed. No immunoreactive protein was detected when the quadruple mutant RBE-2, C14A/C20S/C122S/C780S, was biotinylated, suggesting that no additional cysteines are accessible directly from the extracellular face of the membrane. Permeabilizing the cells expressing RBE-2/C14A/C20S/C122S/C780S with streptolysin O resulted in biotinylation of the exchanger protein. Its amount decreased if exposure to NEM preceded streptolysin O treatment. Our results suggest that Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange activity is inhibited by covalent modification with sulphydryl reagents of cysteine residues that are accessible from the cytoplasmic face of the membrane.

Sulphydryl reagents such as NEM, dithiothreitol, methylmethane thiosulfonate, p-chloromercuribenzoate, and mersalyl were shown to have considerable effects on Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange activity in membrane preparations derived from sarcolemma and synaptosomes (1–5). Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange activity in the squid giant axon could also be modulated by internal application of p-chloromercuriophenylsulfonic acid (4). Moreover, the effects of the thiol reagents on Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange depended on the ionic composition of the medium (3), which suggested that ion binding or translocation sites were involved.

Yet examination of the effects of different methanethiosulfonate (MTS) reagents in conjunction with cysteine-scanning mutagenesis, which was carried out to study the membrane topology of the dog heart Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger NCX1.1 (5, 6) expressed in Xenopus oocytes, suggested that none of the membrane permeable or impermeable sulphydryl reagents tested had any significant effect on transport activity. MTSET sometimes stimulated Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange (5). In CCL39 cells expressing the cloned dog cardiac Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger only MTSET was tested, but it also did not modulate transport activity. (7).

Recently, based on differences in electrophoretic mobility of the dog cardiac Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger in the presence and absence of reducing reagents and on site-directed mutagenesis, it was suggested (8) that disulfide bonds form between cysteine 792 and either cysteine 14 or 20 in the extracellular (9) N terminus of the protein. The existence of these disulfide bonds could potentially restrict access of different reagents to some residues at the extracellular face of the membrane. The possibility that the oxidation-reduction state of these bonds might differ in the different experimental preparations might explain some of the apparent differences between the various studies using sulphydryl reagents.

In this work, we report that the membrane permeable reagents NEM and MTSEA inhibited Na\textsuperscript{+} gradient-dependent Ca\textsuperscript{2+} influx in HEK 293 cells expressing the cloned rat brain exchangers RBE-1 (NCX1.4) and RBE-2 (NCX1.5) and the rat heart isoform RHE-1 (NCX1.1). As reported previously in oocytes (5, 6), the membrane impermeable reagents MTSET and MTSES also did not inhibit transport activity in HEK 293 cells when applied from the outside. These reagents did, however,

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The abbreviations used are: NEM, N-ethylmaleimide; RBE-1 (NCX-1.4) and RBE-2 (NCX1.5), rat brain exchanger 1 and 2 (GenBank accession no. X68812 and x68813, respectively); RHE-1 (NCX1.1), rat heart exchanger 1 (GenBank accession no. X68191); WT, wild type exchanger; MTS, methanethiosulfonate; MTSEA, (2-aminoethyl) methanethiosulfonatehydrobromide; MTSET, [2-(trimethylammonium) ethyl] methanethiosulfonatebromide; MTSES, sodium(2-sulfonatoethyl)methanethiosulfonate; FN, N-terminal Flag (DYKDDDDK)-tagged; MB, 3-(N-maleimidylpropionyl)biocytin; TCEP, Tris(2-carboxyethyl)phosphine; SLO, streptolysin O.
inhibit transport activity when proteins derived from cells expressing RBE-1, RBE-2, or RHE-1 were reconstituted into proteoliposomes. We show that in the wild type exchanger the putative external cysteine residues were not directly accessible to covalent modification. Reduction, however, of the protein by Tris(2-carboxyethyl)phosphine (TCEP), or exchange of one of the three cysteine residues Cys-14, Cys-20, and Cys-780 with either alanine or serine, resulted in surface biotinylation with 3-(N-maleimidylpropionyl)biocytin (MB). Pretreatment of the cells expressing C14A, C20S, or C780S with MTSET considerably reduced surface biotinylation by MB but did not result in inhibition of the transporter. Permeabilizing the cells expressing a mutant in which all putative extracellular cysteines were exchanged revealed that cysteines facing the cytoplasmic side of the membrane and derived from the Na+-Ca2+ exchanger (NCX1.5).
are biotinylated by the reagent. The amount of the biotinylated Na\(^+\)-Ca\(^{2+}\) exchanger was considerably reduced when NEM treatment of cells expressing this mutant preceded biotinyla-
tion. Based on these and other results, we suggest that cyste-
teines accessible from the cytoplasmic face of the membrane
can be modified covalently by sulfhydryl reagents, which in
turn might be responsible for the impaired transport activity.
Some of the preliminary results were reported in a previously
published abstract (10).

**EXPERIMENTAL PROCEDURES**

Expression of Rat Isoforms RBE-1 (NCX1.4), RBE-2 (NCX1.5), and
RBE-1 (NCX1.1) or Their Mutants in HEK 293 Cells—Transfection of
HEK 293 cells and determination of Na\(^+\)-dependent Ca\(^{2+}\) uptake was
done exactly as described (11). Transfected cells were preloaded with a
solution containing 0.16 M NaCl, 0.02 M Tris HCl, pH 7.4, 0.02 M MgCl\(_2\),
25 μM nystatin, and 1 mM ouabain for 10 min. The Na\(^+\) preloading
solution was replaced by a solution of identical composition except that
it did not contain nystatin. When added, NEM (dissolved in Me\(_2\)SO) and
MTS reagents (Toronto Research Chemicals Inc.) (dissolved in H\(_2\)O) at
10−50× concentrations were added directly to this solution and kept at
25 °C for 30 min. The concentration of Me\(_2\)SO was kept below 1%, which
by itself had no effect on the transport assays. The cells were washed
with the same solution (without the sulfhydryl reagents) twice, and
[Na\(^+\)]-dependent \(^{45}\)Ca\(^{2+}\) uptake was measured as described (11). In
some control experiments, the MTS reagents were added to the cells
expressing the transporter in buffered choline chloride prior to the Na\(^+\)}
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The Effects of Sulfhydryl Reagents on Na\textsuperscript{+} Gradient-dependent Ca\textsuperscript{2+} Uptake—Fig. 1 shows the effects of membrane permeable MTSEA (panel A) and NEM (panel B) on Na\textsuperscript{+} gradient-dependent Ca\textsuperscript{2+} uptake in HEK 293 cells transfected with RBE-2. It can be seen that the inhibition with both reagents is concentration-dependent. Exposure of cells expressing RBE-2 to 2.5 mM MTSEA reduces Na\textsuperscript{+} gradient-dependent Ca\textsuperscript{2+} uptake to about 25% relative to that measured in untreated cells, and 50% inhibition is obtained at about 1 mM MTSEA. A higher concentration of NEM is required to inhibit the transporter. Although the inhibition of Na\textsuperscript{+} gradient-dependent Ca\textsuperscript{2+} uptake is detectable already at 0.5 mM NEM and 5 mM NEM reduces Na\textsuperscript{+} gradient dependent Ca\textsuperscript{2+} uptake to below 20% relative to that of the untreated cells, 50% inhibition is reached when cells are exposed to 1.65 mM NEM.

The inhibition of Na\textsuperscript{+} gradient-dependent Ca\textsuperscript{2+} uptake with the membrane permeable reagents is obtained both when the WT exchanger is expressed (black squares in Fig. 1) and when the glycosylation mutant FN-RBE-2, in which the Flag epitope replaces Asn-9, the single glycosylation site of the protein (11,

**Fig. 4.** Expression of surface biotinylated proteins and total cell proteins derived from HEK 293 cells expressing FN-RBE-2 and its cysteine mutants. HEK 293 cells grown in 12-well plates were transfected with FN-RBE-2 plasmid DNA (A) and with some of its cysteine mutants (B and C). 24 h post-transfection, cells were surface-biotinylated and isolated by binding to immobilized streptavidin, and following release from the beads, Western analysis was carried out. Total immunoreactive protein derived from parallel transfections (40 µg of cell protein/lane) is shown (see "Experimental Procedures"). A, immunoreactive protein derived from surface-biotinylated cells expressing FN-RBE-2 without reduction and with 500 µM or 750 µM TCEP. Immunoreactive protein derived from total cell extracts prepared in parallel is also shown. B, immunoreactive biotinylated protein derived from cells expressing single cysteine mutants C14A, C20S, and C780S (lanes marked A) and from parallel total cell lysates derived from transfections with the same mutants (lanes marked B). C, the same as B but with different double, triple, and quadruple cysteine mutants.

**Fig. 5.** Surface expression of the cysteine mutants RBE-2/C122S and RBE-2/C14A/C20S/C122S/C780S. HEK293 cells were transfected with the WT Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger RBE-2 and its cysteine mutants C122S and C14A/C20S/C122S/C780S. 24 h post-transfection, the cells were biotinylated with NHS-SS-biotin, biotinylated immunoreactive protein was derived from streptavidin beads, biotinylated immunoreactive protein was derived from the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger, and its mutants was analyzed by SDS-polyacrylamide gel electrophoresis (lanes marked A). Total immunoreactive protein derived from parallel transfection of cells expressing the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger and its mutants is also shown (lanes marked B).
18) (black triangle in Fig. 1A and black circle in Fig. 1B), is expressed. Transport activity of the FN-tagged RBE-2 was similar to the WT exchanger bearing no tag.

The membrane impermeant reagents MTSET and MTSES (Fig. 2) did not inhibit Na\(^+\) gradient-dependent Ca\(^{2+}\) uptake when added to transfected cells in situ (Fig. 2A). In some experiments, as reported by Nicoll et al. (5), addition of the reagents led to stimulation of Na\(^+\)-dependent Ca\(^{2+}\) uptake.

Because membrane permeable reagents inhibited Na\(^+\)-Ca\(^{2+}\) exchange activity in whole cells and membrane impermeant reagents applied from the outside did not, we solubilized transfected cell proteins and reconstituted them into proteoliposomes. Fig. 2B shows the inhibition by 2 mM MTSET and 10 mM MTSES of Na\(^+\)-dependent Ca\(^{2+}\) uptake in proteoliposomes derived from RBE-2-transfected HEK293 cells. The addition of 0.1, 0.5, and 1 mM MTSET and 3 and 5 mM MTSES to the reconstituted preparation inhibited Na\(^+\)-dependent Ca\(^{2+}\) uptake in a concentration-dependent manner. However, increasing the concentration of MTSET to 2 mM and above (2–10 mM) and MTSES to 5 mM and above (5–15 mM) did not further increase the extent of inhibition that was reached in each preparation, which was between 40 and 60% relative to the transport activities measured in the absence of the sulfhydryl reagents.

Because none of the sulfhydryl reagents had a significant effect on the transport activity of the dog sarcolemmal Na\(^+\)-Ca\(^{2+}\) exchanger NCX1.1 expressed in oocytes (5–7) and because the dog heart isoform has an extra cysteine in position 879 instead of a serine in the corresponding rat isoform, we prepared a mutant of RHE-1, RHE-1/S879C. RHE-1/S879C was expressed in HEK 293 cells, and Na\(^+\)-gradient-dependent Ca\(^{2+}\) uptake activity of this mutant was determined in the absence and in the presence of the sulfhydryl reagents. Inhibition of transport activity was obtained by the membrane permeable reagents NEM and MTSEA added to whole cells from the outside and by MTSET and MTSES only following reconstitution, in a manner similar to the results presented in Figs. 1 and 2 for the rat isoforms.

**Accessibility of Cysteines to Sufhydryl Reagents**—To further examine the functional and structural implications that the effects of membrane permeable and impermeable sulfhydryl reagents had on the Na\(^+\)-Ca\(^{2+}\) exchanger, we determined the accessibility of the different cysteines to covalent modification. This was done by surface biotinylation of adherent cells expressed transfection with FN-RBE-2 or its single cysteine mutants C14A, C20S, and C780S. A, 24 h post-transfection, transport activity was measured without and with 5 mM MTSET. B, immunoreactive biotinylated protein of cells expressing the WT exchanger and the same single cysteine mutants without and with preincubation with 5 mM MTSET before biotinylation. 1 mM TCEP was also included in the biotinylation mixture of the WT exchanger.

**Fig. 6.** Na\(^+\) gradient-dependent Ca\(^{2+}\) uptake and immunoblot of biotinylated surface expressed cysteine mutants expressed in HEK 293 cells. HEK 293 cells were transfected with FN-RBE-2 or its single cysteine mutants C14A, C20S, and C780S. A, 24 h post-transfection, transport activity was measured without and with 5 mM MTSET. B, immunoreactive biotinylated protein of cells expressing the WT exchanger and the same single cysteine mutants without and with preincubation with 5 mM MTSET before biotinylation. 1 mM TCEP was also included in the biotinylation mixture of the WT exchanger.
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pressing the $\text{Na}^+\cdot\text{Ca}^{2+}$ exchanger and some of its cysteine mutants with MB directly or following the permeabilization of these cells in situ with SLO. Biotinylated proteins were isolated from lysed cells on immobilized streptavidin; Western analysis of the proteins released from the beads was carried out to detect the $\text{Na}^+\cdot\text{Ca}^{2+}$ exchanger or its mutants. To examine whether glycosylation of the exchanger played a role in determination of accessibility of the protein to covalent modification, transfection was carried out both with glycosylated and nonglycosylated (FN-tagged) RBE-2 and its cysteine mutants. As also seen with transport activity (see Figs. 1 and 2), no differences in surface expression were detected between the glycosylated and nonglycosylated exchangers.

Fig. 3A shows the location of the different cysteines. Because the topology of the protein has not been fully established, we based the model in Fig. 3A on a combination of the hydropathy analysis model and possible changes suggested by the neural network algorithm-based analysis of secondary structure of the protein and experimental data based on immunocytochemistry (9) and cysteine-scanning mutagenesis (5, 7). We exchanged the 14 cysteines of the rat isoforms of NCX1 with either alanine or serine alone or in different combinations. Each one of the single cysteine mutants was functional. Based on the results shown in Figs. 1 and 2, we have studied in this work the accessibility of the four putative extracellular cysteines to surface biotinylation by MB. The location of these cysteines is marked by an arrow in Fig. 3A.

Fig. 3B shows the relative transport activities of the different putative extracellular single and multiple cysteine mutants that were generated. It can be seen that although relative transport activities of these mutants varied, all were functional.

Fig. 4 shows an immunoblot of surface biotinylated FN-RBE-2 (panel A) and some of its FN-tagged cysteine mutants (panels B and C). Biotinylation was carried out with MB, which botinylates cysteine residues. The amount of “total” immunoreactive protein derived from transfected cell extracts is also shown. It can be seen (Fig. 4A), that in the surface membrane, only traces of immunoreactive protein were detected when cells expressing the FN-RBE-2 were biotinylated. To examine whether disulfide bonds block the access of the biotinylating reagent MB to extracellular cysteines and thereby prevent the isolation of exchange protein from FN-RBE-2-transfected cells, we used the reducing agent TCEP. TCEP, unlike dithiothreitol or $\beta$-mercaptoethanol, can be added together with MB without quenching it (19–21) and used for the reduction of disulfide bonds. The addition of an equimolar concentration (500 $\mu$M) of TCEP to the biotinylating reaction mixture led to detection of immunoreactive exchanger protein. The addition of an excess of TCEP (750 $\mu$M) over the concentration of MB (500 $\mu$M) that was used led to detection of higher amounts of exchanger protein. Biotinylation of cells expressing the single cysteine mutants FN-RBE-2/C14A, FN-RBE-2/C20S, and FN-RBE-2/C780S (Fig. 4B) resulted in detection of immunoreactive exchanger protein without any reduction. No immunoreactive protein was detected when mutant FN-RBE-2/C122S was biotinylated, although the mutant was functional and protein derived from it was detected in total cell extracts.

Fig. 4C shows the immunoblot derived from the $\text{Na}^+\cdot\text{Ca}^{2+}$ exchanger proteins that were detected when cells expressing the double mutants FN-RBE-2/C14A/C20S and FN-RBE-2/C20S/C122S, the triple mutant FN-RBE-2/C14A/C20S/C122S, and the quadruple mutant FN-RBE-2/C14A/C20S/C122S/C780S were biotinylated. Immunoreactive protein was revealed when each of these mutants was biotinylated except for the quadruple mutant FN-RBE-2/C14A/C20S/C122S/C780S.

Because no immunoreactive exchanger protein was revealed by surface biotinylation of cells expressing FN-RBE-2/C122S (Fig. 4B) and FN-RBE-2/C14A/C20S/C122S/C780S (Fig. 4C) with MB and because both mutants exhibited Na$^+$ gradient-dependent Ca$^{2+}$ uptake in whole transfected cells (Fig. 3B), we carried out surface biotinylation of cells expressing these mutants with NHS-SS-biotin. This membrane impermeable reagent covalently labels free amino groups such as N-terminal amines and $\varepsilon$-amino lysines (11). Fig. 5 shows that biotinylated protein derived from cells transfected with both RBE-2/C122S and RBE-2/C14A/C20S/C122S/C780S is detected in the surface membrane (lanes A) and not only in total cell extracts (lanes B). The surface expression and total cell expression of exchanger protein derived from RBE-2 is also shown.

The addition of MTSET to cells expressing FN-RBE-2 (Fig. 2A) or its mutants FN-RBE-2/C14A, FN-RBE-2/C20S, FN-RBE-2/C122S, and FN-RBE-2/C780S from the outside (Fig. 6A) did not inhibit $\text{Na}^+\cdot\text{Ca}^{2+}$ exchange activity; this could result either from insensitivity of the FN-RBE-2 exchanger and the different cysteine mutants to covalent modification by MTSET or the fact that the cysteines were inaccessible to the reagent. To distinguish between these possibilities, we determined the cross-reactivity between MTSET and MB. Cells expressing FN-RBE-2 and the different cysteine mutants were biotinylated directly or incubated first with 5 mM MTSET for 30 min and biotinylated after appropriate washes with PBS/CM. Because protein derived from cells expressing FN-RBE-2 was not revealed by biotinylation without reduction (Fig. 4A), 1 mM TCEP was included in the biotinylating reaction mixture of cells expressing this protein. Fig. 6B shows the results of these
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In experiments. It can be seen that pretreatment of the cells with MTSET considerably reduced the amount of biotinylated protein that was revealed, suggesting that MTSET and MB bind to the same site. These experiments also suggest that MTSET binds to the remaining cysteine(s) after the mutation of the single cysteine in each mutant, but this binding does not result in inhibition of their transport activity.

To determine whether intracellular cysteines are accessible to covalent modification, we expressed mutant FN-RBE-2-RBE-2C14A/C20S/C122S/C780S, in which all the cysteines that could potentially be biotinylated by external addition of the reagent were exchanged. Biotinylation of cells expressing this mutant with MB (Fig. 4C) did not lead to detection of immunoreactive Na\(^+-\)Ca\(^{2+}\) exchanger protein. Fig. 7A shows an experiment in which cells expressing FN-RBE-2-RBE-2C14A/C20S/C122S/C780S were permeabilized with SLO and then biotinylated with MB. Following cell lysis (see “Experimental Procedures”) and isolation of the biotinylated proteins on streptavidin beads, Western analysis was carried out. It can be seen, that immunoreactive protein derived from the Na\(^+-\)Ca\(^{2+}\) exchanger was revealed. Treatment of the cells with either 5 mM MTSEA (not shown) or 5 and 10 mM NEM prior to the biotinylation reduced the amount of biotinylated immunoreactive exchanger protein that was detected. Fig. 7B shows a parallel experiment in which the effect of NEM on the transport activity of this mutant was examined. It can be seen that Na\(^+\) gradient-dependent Ca\(^{2+}\) uptake was reduced when cells expressing the mutant were treated with 5 mM NEM. A similar inhibition was obtained when 2.5 mM MTSEA was added to cells expressing FN-RBE-2/C14A/C20S/C122S/C780S.

**DISCUSSION**

In this work we have shown that the transport activity of the rat isoforms of the NCX1 gene expressed in HEK 293 cells is inhibited by membrane permeable sulfhydryl reagents such as NEM and MTSEA applied to adherent cells from the outside and with the membrane impermeable reagents MTSET and MTSES, following reconstitution. We used reconstitution of transfected cell proteins to gain access to the cytoplasmic face of the membrane, because this procedure “scrambles” the topology of membrane proteins and at least some of the transporters might assume “inside-out” orientation and undergo covalent modification with membrane impermeable reagents added from the outside. Applying the membrane impermeable sulfhydryl reagents to the reconstituted preparation resulted in a concentration-dependent inhibition of Na\(^+-\)dependent Ca\(^{2+}\) uptake up to about 2 mM MTSET and 5 mM MTSES. At these concentrations, a 40–60% inhibition of the transport activity was obtained, which did not increase with a further increase in the concentration of the reagents. This finding suggests that in our preparation of the reconstituted proteoliposomes, about half of the transporters have an “inside-out” orientation and only these transporters are amenable to covalent modification by membrane impermeable reagents. When all of the externally accessible cysteines are covalently modified, a further increase in the concentration of the membrane impermeable reagents cannot lead to further inhibition.

To study further the effects of sulfhydryl reagents on Na\(^+\) gradient-dependent Ca\(^{2+}\) uptake, we monitored the accessibility of different cysteines of the Na\(^+-\)Ca\(^{2+}\) exchanger to covalent modification. This was done by surface biotinylating transfected cells that express the protein with MB and detection of immunoreactive biotinylated Na\(^+-\)Ca\(^{2+}\) exchanger. Our studies show that only traces of immunoreactive protein derived from the FN-RBE-2 could be revealed by biotinylation with MB directly, although the transporter was functional. This result is puzzling for the following reasons. First, our previous studies indicated that surface-expressed FN-tagged Na\(^+-\)Ca\(^{2+}\) exchanger of similar transport activity is detected by indirect immunofluorescence using the anti-Flag antibody and also by surface biotinylation with NHS-SS-biotin, which reacts with the free N-terminal and e-amino lysines. Second, MB was successfully used to monitor extracellular cysteines in different expression systems and with different transporters.

Third, based on hydrophathy analysis (24, 25), the position of the glycosylation site at Asn-9 (18) and indirect immunofluorescence studies with N-Flag-tagged Na\(^+-\)Ca\(^{2+}\) exchanger (9), Cys-14 and Cys-20 are in the extracellular N terminus of the protein (Fig. 3A) and should be accessible to surface biotinylation. Fourth, Santacruz-Toloza et al. (8) suggested that, in addition to Cys-14 and Cys-20, Cys-780 also is extracellular and Cys-780 is connected by a disulfide bond to either Cys-14 or Cys-20. Yet, if this disulfide bond alternates between Cys-14 and Cys-20 to Cys-780, one of these three cysteines (or a fraction from each at a time) that is not involved in sulfhydryl bond formation should be free for biotinylation. Because exposure of the cells expressing the transporter to the reducing agent TCEP led to detection of biotinylated immunoreactive RBE-2, it is possible that an additional cysteine might be involved in disulfide bond formation, which together with Cys-14, Cys-20, and Cys-780 prevents free access to the extracellular cysteines. The existence of an additional cysteine that participates in disulfide bond formation is also consistent with the finding that immunoreactive protein was detected without reduction when Cys-14 alone, Cys-20 alone, or Cys-780 alone (Fig. 4B) was exchanged with either Ala or Ser. From current topological models, cysteine 122 could have been an appropriate candidate to interact with Cys-14 or Cys-20. Yet mutation of this residue alone did not result in detection of immunoreactive protein (Fig. 4B), suggesting that it was not involved in disulfide bond formation and it was not responsible for blocking the access of the biotinylating reagent to the WT exchanger.

The inhibition with membrane permeable reagents persisted also when all of the putative external cysteines were mutated. Taken together with the effects of the membrane impermeable reagents in the reconstituted preparation, our results suggest that covalent modification of intracellular cysteines was involved. Support for this proposal comes from the experiment shown in Fig. 7 in which we incubated cells expressing FN-RBE-2/C14A/C20S/C122S/C780S without and with NEM, permeabilized them with SLO, and then exposed them to MB. Permeabilization of cells expressing FN-RBE-2/C14A/C20S/C122S/C780S before biotinylation resulted in detection of biotinylated immunoreactive protein derived from the Na\(^+-\)Ca\(^{2+}\) exchanger. Pretreatment of the cells with NEM led to a considerable decrease in the amount of immunoreactive protein that was revealed.

These results suggest that intracellular cysteines are accessible to covalent modification by membrane permeable sulfhydryl reagents. Because these sulfhydryl reagents impair transport activity not only of the WT transporter but also of its RBE-2/C14A/C20S/C122S/C780S mutant, which has no cysteines accessible from the extracellular face of the membrane, we suggest that the covalent modification of intracellular cysteines is responsible for the impaired Na\(^+-\)Ca\(^{2+}\) exchange activity. Further studies are required to identify which and how many of the intracellular cysteines are sensitive to the sulfhydryl reagents.

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