Phosphorylation of the C-terminal domain of the Na⁺/H⁺ exchanger by Ca²⁺/calmodulin-dependent protein kinase II

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The Na⁺/H⁺ exchanger is a pH-regulatory protein that extrudes one H⁺ ion in exchange for one Na⁺ ion when intracellular pH declines. A number of studies have shown phorbol ester stimulation of activity in intact cells, leading to the idea that the exchanger is regulated by protein kinase C-mediated phosphorylation *in vivo*. cDNA encoding the protein has been cloned, and a recent model suggests a large internal cytoplasmic C-terminal domain that may be a site of regulation of the exchanger [Sardet, Franchi & Pouyssegur (1989) *Cell* 56, 271–280]. We examined this region of the protein using a rabbit cardiac Na⁺/H⁺ exchanger cDNA clone. cDNA of the Na⁺/H⁺ exchanger, coding for the C-terminal 178 amino acid residues, was cloned into the expression vector pEX-1 and expressed as a fusion protein with β -galactosidase. The fusion protein reacted with an antibody produced against a synthetic peptide of the C-terminal 13 amino acid residues of the Na⁺/H⁺ exchanger, confirming the identity of the expressed protein. Control and experimental pEX-1–Na⁺/H⁺ exchanger protein was purified on a *p*-aminophenyl β -D-thiogalactopyranoside–agarose column. Purified Ca²⁺/calmodulin-dependent protein kinase II readily phosphorylated the Na⁺/H⁺ exchanger protein in a Ca²⁺- and calmodulin-dependent manner *in vitro*, but this region of the protein was not a substrate for purified protein kinase C or for the catalytic subunit of cyclic AMP-dependent protein kinase. Control-expressed β -galactosidase was phosphorylated to a maximal level of 0.77 ± 0.17 mol of P_i/mol (mean $\pm$ S.E.M., *n* = 6) whereas the fusion protein was phosphorylated to a maximal level of 4.09 ± 0.39 mol of P_i/mol (*n* = 6), suggesting one site of phosphorylation in β -galactosidase and three in the C-terminal domain of the Na⁺/H⁺ exchanger. Examination of the deduced amino acid sequence of this part of the exchanger reveals three consensus sequences for Ca²⁺/calmodulin-dependent protein kinase II. These results suggest that the exchanger may be directly regulated *in vivo* by calmodulin-dependent protein kinase II but not by protein kinase C or cyclic AMP-dependent protein kinase.

INTRODUCTION

The Na⁺/H⁺ exchanger is a pH-regulatory protein almost universally distributed in mammalian tissues. The exchanger extrudes one H⁺ ion in exchange for one Na⁺ ion when decreases in intracellular pH occur [1,2]. The exchanger is also involved in a number of other cellular functions, such as the regulation of cell volume and transport of salt and water, and may play a role in the onset and/or maintenance of the cellular proliferative cascade (see ref. [3] for a review).

Regulation of the Na⁺/H⁺ exchanger has generally been studied in intact cell systems by using measurements of internal pH with fluorescent compounds to determine Na⁺/H⁺ exchange activity [3–5]. Exposure of a number of cell types to hormones and growth factors, such as platelet-derived growth factor, thrombin, angiotensin II, [arginine]vasopressin and epidermal growth factor, causes rapid activation of the exchanger. Phorbol esters and diacylglycerol [4–6] and other agents such as Ca²⁺ ionophores, chemotactic stimuli, fertilization and osmotic shrinking have similar effects [5]. These effects are dependent on cell type and are generally believed to act either via direct activation of protein kinase C or through diacylglycerol after stimulation of phosphoinositide-specific phospholipase C [4,5].

Direct phosphorylation of the Na⁺/H⁺ exchanger was recently observed, suggesting a mechanism for its regulation. Sardet *et al.* [7] demonstrated phosphorylation of the antiporter in response to phorbol esters, suggesting that protein kinase C may phosphorylate the protein. They also suggested that the protein

exists in the plasma membrane with ten transmembrane segments and a large cytoplasmic domain. However, the exact region of the protein that was phosphorylated was not identified.

Several different pathways of activation of the Na⁺/H⁺ exchanger may exist. One of these may involve intracellular Ca²⁺ possibly acting through Ca²⁺/calmodulin-dependent protein kinase II (CaM kinase II). In various cell types a rise in intracellular [Ca²⁺] precedes intracellular alkalization induced by growth factors [8] and may be necessary for activation of the exchanger [9–11]. Other studies suggested that Ca²⁺ acts through CaM and is the key regulator of the Na⁺/H⁺ exchanger [12–18]. Brush-border membrane vesicles from rabbit ileal villus cells do show CaM-dependent inhibition of Na⁺/H⁺ exchange activity, in contrast with studies *in vivo*, which suggest CaM-dependent stimulation of activity [14,15,19,20]. Evidence also shows that the exchanger can be activated in the absence of protein kinase C or in protein kinase C-down-regulated cells [12,13,15]. Cyclic AMP-dependent effects on the Na⁺/H⁺ exchanger also occur: an elevation of cyclic AMP concentration was reported to activate [21], inhibit [21–23] or have no effect [24] on Na⁺/H⁺ exchange. Weinman *et al.* [25] have noted that in reconstituted proteoliposomes Na⁺/H⁺ exchange activity is inhibited by phosphorylation with cyclic AMP-dependent protein kinase. In the same type of study it was shown that CaM kinase II-mediated phosphorylation also inhibited exchange activity, but protein kinase C-mediated phosphorylation enhanced Na⁺/H⁺ exchange [26].

To help clarify the role of different kinases in the regulation of

Abbreviations used: CaM, calmodulin; CaM kinase II, Ca²⁺/calmodulin-dependent protein kinase II.

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the cardiac Na^+/H^+ exchanger, we have studied the direct phosphorylation of the cardiac protein *in vitro*. Since there is no readily available preparation of the protein, and studies concerning its purification and identification in intact tissue have produced some conflicting results (see ref. [5] for review), we chose to produce and purify the protein from a bacterial expression system *in vivo* using cDNA encoding the cytoplasmic domain of the rabbit cardiac Na^+/H^+ exchanger that we described earlier [27]. We chose to study this region of the protein because, being cytoplasmic and therefore accessible to intracellular kinases, it has been suggested to be a probable site of regulation of Na^+/H^+ exchange activity [5,28]. Furthermore, an analysis of putative phosphorylation sites based on the deduced amino acid sequence [7,27,28] showed that this region of the protein contains three possible sites of phosphorylation (RXXS) for CaM kinase II, while the remainder of the protein contains only two such sites and only one of these is predicted to be on the cytosolic side of the plasma membrane [7,28]. We studied the phosphorylation *in vitro* by three protein kinases: CaM kinase II, protein kinase C and cyclic AMP-dependent protein kinase. We show that, of the three kinases, only CaM kinase II phosphorylates this region of the protein, suggesting that this kinase, through phosphorylation of the intracellular cytoplasmic domain of the protein, may be an important regulator of the exchanger *in vivo*.

EXPERIMENTAL

Materials

Restriction endonucleases and DNA-modifying enzymes were obtained from Boehringer Mannheim Canada Ltd. (Laval, Quebec, Canada) and Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.). Peroxidase-conjugated goat anti-(rabbit IgG) antibody was from Bio/Can Scientific (Mississauga, Ont., Canada). Nitrocellulose membranes were from Schleicher and Schuell (Keene, NH, U.S.A.) or Bio-Rad Laboratories

(Richmond, CA, U.S.A.). pEX-1 was from Boehringer Mannheim. SDS/PAGE gels were prepared from reagents of Bio-Rad Laboratories or Boehringer Mannheim. *p*-Aminophenyl β -D-thiogalactopyranoside-agarose was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The synthetic peptide was obtained from the Alberta Peptide Institute (Edmonton, Alberta, Canada). [γ - ^{32}P]ATP (20–40 Ci/mmol) was purchased from Amersham (Oakville, Ont., Canada). *L*- α -Phosphatidyl-L-serine (bovine brain) was purchased from Serdary Research Laboratories (London, Ont., Canada) and 1,3-diolein from Sigma Chemical Co. General laboratory reagents used were of analytical grade or better.

Production and purification of the Na^+/H^+ exchanger protein

To produce the truncated Na^+/H^+ exchanger, a portion of the previously described [27] rabbit cardiac cDNA clone was cloned in the appropriate reading frame into the expression vector pEX-1 by the use of standard cloning techniques. This cDNA encodes the C-terminal 178 amino acid residues of the protein, which was expressed as a fusion protein with β -galactosidase. Control (no insert) and experimental plasmids containing the cDNA encoding the terminal 178 amino acid residues of the Na^+/H^+ exchanger were transformed into *Escherichia coli* N4830-1. Overnight cultures were grown at 30 °C and transient expression was induced by shifting the temperature to 42 °C. Cells were harvested by centrifugation (5000 *g* for 5 min) and lysed by passage through a French press. The homogenate was centrifuged at 20000 *g* for 20 min at 4 °C and the supernatant and pellet were collected. To solubilize the expressed proteins from the pellet, this fraction was made to 1% with respect to sodium *N*-lauroylsarcosine, incubated at room temperature for 1 h and pelleted at 100000 *g* for 1 h. The supernatant was used for subsequent purification procedures.

Purification of the experimental and control solubilized proteins utilized a *p*-aminophenyl β -D-thiogalactopyranoside-agarose column. Samples (in 0.137 M-NaCl/8.1 mM-sodium phosphate/1.5 mM-potassium phosphate buffer, pH 7.4) were loaded at 4 °C, the column was washed repeatedly and samples were eluted with 0.1 M-sodium borate buffer, pH 10. Fractions eluted from the column were dialysed and examined by SDS/PAGE.

SDS/PAGE, transfer of protein and immunostaining

SDS/PAGE (10% acrylamide or 7.5–20% acrylamide) was carried out by the procedure of Laemmli [29] and transfer of protein to nitrocellulose by the method of Towbin *et al.* [30]. Immunoblotting was carried out in the presence of 1% milk powder as described earlier [31] and proteins reactive with antibodies were detected by developing a peroxidase reaction. Standards on immunoblots were Bio-Rad prestained markers: phosphorylase *b* (M_r 135000), BSA (M_r 75000), ovalbumin (M_r 50000), carbonic anhydrase (M_r 39000) and soya-bean trypsin inhibitor (M_r 27000). Other standards are (Fig. 5) high- M_r and low- M_r markers: a = myosin heavy chain (M_r 205000); b = β -galactosidase (M_r 116000); c = phosphorylase *b* (M_r 97400); d = BSA (M_r 66000); e = ovalbumin (M_r 45000); f = carbonic anhydrase (M_r 31000); g = soya-bean trypsin inhibitor (M_r 21500); h = lysozyme (M_r 14400). In some experiments, Ca^{2+} overlay was performed on both purified proteins [32] with calsequestrin as a control as described previously [33].

Preparation of antibodies

A synthetic peptide composed of the C-terminal 13 amino acid residues of the rabbit cardiac Na^+/H^+ exchanger [27] (EPGEGEPFIPKGQ) was supplied by the Alberta Peptide

Fig. 1. Expression of the rabbit cardiac Na^+/H^+ exchanger in *E. coli*

A portion of the rabbit cardiac Na^+/H^+ exchanger was cloned into the expression vector pEX-1 and produced as a fusion protein with β -galactosidase. The cells were induced and lysed, and the pellet and supernatant fractions prepared as described in the Experimental section. Lane M is Bio-Rad prestained markers. Lanes 1 and 2 represent *E. coli* transfected with pEX-1 (control plasmid) alone. Lanes 3 and 4 represent the expression of Na^+/H^+ exchanger- β -galactosidase fusion protein (experimental). Lanes 1 and 3 are supernatant and lanes 2 and 4 are pellet fractions of the lysed cells. The positions of β -galactosidase and the fusion protein are indicated.

Fig. 2. Purification of β -galactosidase and β -galactosidase-Na⁺/H⁺ exchanger fusion protein

Control and experimental plasmids containing the terminal 178 amino acid residues of the Na⁺/H⁺ exchanger were transformed into *E. coli* N4830-1. Overnight cultures were grown, harvested and lysed by passage through a French press as described in the Experimental section. Solubilized pellet fractions were purified with a *p*-amino-phenyl- β -D-thiogalactopyranoside-agarose column. Fractions eluted from the column were examined by SDS/PAGE. Lane C, control (β -galactosidase); lane E, experimental (β -galactosidase-Na⁺/H⁺ exchanger); lane M, Bio-Rad prestained standards.

Institute. It was coupled to keyhole-limpet haemocyanin [34] and used to immunize rabbits as described previously [31].

Preparation of protein kinases

Protein kinase C was prepared from rat brain as described by Wolf *et al.* [35] and the catalytic subunit of type II cyclic AMP-dependent protein kinase from bovine heart by the method of Demaille *et al.* [36]. CaM kinase II, purified from bovine brain, was generously provided by Dr. R. K. Sharma, Department of Medical Biochemistry, University of Calgary.

Phosphorylation of β -galactosidase and Na⁺/H⁺ exchanger- β -galactosidase fusion protein

The standard phosphorylation conditions were as follows. For protein kinase C, the liposomal assay described by Kikkawa *et al.* [37] was used under the following conditions: 20 mM-Tris/HCl buffer pH 7.5, 5 mM-MgCl₂, 0.1 mM-CaCl₂, 40 μ g of phosphatidylserine/ml, 0.8 μ g of 1,3-dioclein/ml, 10% (v/v) protein kinase C, 16 μ g of β -galactosidase/ml or 19.7 μ g of fusion protein/ml, and 0.1 mM- $[\gamma$ -³²P]ATP (approx. 200 c.p.m./pmol). For cyclic AMP-dependent protein kinase, the following conditions were used: 20 mM-Tris/HCl buffer, pH 7.5, 5 mM-MgCl₂, 1 mM-EGTA, 2 μ g of catalytic subunit of cyclic AMP-dependent protein kinase/ml, 16 μ g of β -galactosidase/ml or 19.7 μ g of fusion protein/ml, and 0.1 mM- $[\gamma$ -³²P]ATP (approx. 200 c.p.m./pmol). For CaM kinase II, the following reaction conditions were used: 20 mM-Tris/HCl buffer, pH 7.5, 5 mM-MgCl₂, 0.2 mM-CaCl₂, 10 μ g of calmodulin/ml, 5 μ g of CaM kinase II/ml, 16 μ g of β -galactosidase/ml or 19.7 μ g of fusion protein/ml, and 0.1 mM- $[\gamma$ -³²P]ATP (approx. 200 c.p.m./pmol). Reactions were initiated by addition of ATP,

Fig. 3. Immunoreactivity of β -galactosidase (lane C) and β -galactosidase-Na⁺/H⁺ exchanger fusion protein (lane E)

Pellet fractions from *E. coli* N4830-1 were prepared as described in the Experimental section, and proteins were separated by SDS/PAGE and, after transfer to nitrocellulose, examined by immunostaining with antibody against a synthetic peptide of the Na⁺/H⁺ exchanger. Lane M, Bio-Rad prestained standards. (a) Pre-immune serum; (b) immune serum.

the mixtures were incubated at 30 °C, and samples were withdrawn at times indicated in the text, added to an equal volume of SDS/polyacrylamide gel sample buffer and boiled before SDS/PAGE and autoradiography with Kodak X-Omat AR film in Dupont Cronex cassettes fitted with Dupont Quanta III intensifying screens. Films were allowed to develop for 3 days or less at room temperature. Densitometry of destained gels and autoradiograms was carried out in an LKB model 2202 Ultrosan laser densitometer equipped with a Hewlett-Packard model 3390A integrator. In one series of experiments, the maximal levels of phosphorylation of β -galactosidase and fusion protein by CaM kinase II were obtained by withdrawing 0.1 ml samples of the phosphorylation reactions, quenching the reaction by addition to 0.5 ml of 25% trichloroacetic acid/2% sodium pyrophosphate, and [³²P]P_i incorporation into precipitated protein was quantified as described by Walsh *et al.* [38].

Protein concentration determinations

Protein concentrations were determined by the method of Bradford [39] with reagents purchased from Bio-Rad Laboratories, except for the bacterially expressed β -galactosidase and β -galactosidase-Na⁺/H⁺ exchanger fusion protein, which were determined by amino acid analysis with norleucine as an internal standard.

RESULTS

Expression and purification of the Na⁺/H⁺ exchanger

To study phosphorylation of the Na⁺/H⁺ exchanger, the protein was expressed in *E. coli* from cloned cDNA of the cytoplasmic domain of the cardiac protein. Fig. 1 shows the results of expression of both control β -galactosidase and experimental β -galactosidase-Na⁺/H⁺ exchanger fusion protein. The addition of the terminal 178 amino acid residues of the Na⁺/H⁺ exchanger as expected resulted in the production of a larger protein (*M_r* 135000) than β -galactosidase alone (*M_r* 116000). For unknown reasons most of the control β -galactosidase was found in the pellet fraction whereas the experimental β -galactosidase-Na⁺/H⁺ exchanger protein was present in both pellet and supernatant fractions. The identity of β -galactosidase in both experimental and control fractions was confirmed by using an antibody (Promega) against β -galactosidase (results not shown).

LQKTRQRLRS YNRHTLVADP YEEAWNQMLL RRQKARQLEQ
KINNYLTVPA HKLDSPTMSR ARIGSDPLAY EPKADLPVIT
IDPASQSPSE SVDLVNEELK GKVLGLSREP RVAEEAAEED
EDGGIVMRPK EPSSPGTDDV FSPAPSDSPS SQRMQRCLSD
PGPHPEFGEG EPFIPKGQ

Fig. 4. Sequence of the C-terminal 178 amino acid residues of the rabbit cardiac Na⁺/H⁺ exchanger [27]

The three putative CaM kinase II recognition sites are underlined.
The C-terminal 13 amino acid residues, which were used as a synthetic peptide, are boxed.

Purification of the expressed proteins was achieved with a *p*-aminophenyl β-D-thiogalactopyranoside-agarose column. The one-step procedure resulted in highly enriched proteins (Fig. 2). Despite the use of an extensive cocktail of proteinase inhibitors throughout purification [40], some evidence of proteolysis was evident in the purified product, most notably in the fusion protein. When examining the purified fusion protein, it was found that antibody against β-galactosidase recognized these proteolytic fragments, suggesting that β-galactosidase remained intact or that the immunoreactive part of β-galactosidase remained. Antibody against the C-terminal region of the Na⁺/H⁺ exchanger did not recognize the slightly degraded purified fusion protein, suggesting that this region of the Na⁺/H⁺ exchanger protein had been removed (results not shown).

Characterization of the expressed proteins

To confirm that we have correctly expressed the C-terminal region of the Na⁺/H⁺ exchanger, we produced an antibody against a synthetic peptide composed of the C-terminal 13 amino acid residues of the rabbit cardiac Na⁺/H⁺ exchanger. Fig. 3 shows the immunoreactivity of control (C) expressed β-galactosidase and experimental (E) fusion protein crude pellet fractions from *E. coli* N4830-1 prepared as described above. The pre-immune sera showed no immunoreactivity with Western blots of these fractions, whereas immune sera at high dilution (1:3000)

Fig. 6. Ca²⁺/calmodulin-dependence of phosphorylation of the Na⁺/H⁺ exchanger-β-galactosidase fusion protein by CaM kinase II

Purified β-galactosidase or Na⁺/H⁺ exchanger-β-galactosidase fusion protein were phosphorylated with CaM kinase II. The reaction conditions were as described in the Experimental section. Reactions were started with ATP, the mixtures were incubated for 30 min at 30 °C and the reactions were stopped by adding an equal volume (50 μl) of gel sample buffer and boiling for SDS-PAGE and autoradiography.

strongly reacted with the pellet fraction containing the expressed Na⁺/H⁺ exchanger protein. There were a number of degradation products of the fusion protein present despite the presence of a cocktail of proteinase inhibitors throughout preparation [40]. These did not co-purify with the fusion protein (Fig. 2 and note above), presumably because the β-galactosidase portion of the protein was degraded and the resultant molecules did not bind to the β-galactosidase affinity column.

We have noted previously [5] that the C-terminal 100-residue region of the Na⁺/H⁺ exchanger is relatively negatively charged.

Fig. 5. Phosphorylation of the Na⁺/H⁺ exchanger-β-galactosidase fusion protein by CaM kinase II

Lanes 1 and 10, *M*_r markers a-h as described in the Experimental section; lanes 2 and 6, CaM kinase II alone; lanes 3 and 7, CaM kinase II plus β-galactosidase; lanes 4 and 8, CaM kinase II plus fusion protein; lanes 5 and 9, blank. The experiments depicted in lanes 6-8 were carried out with a different CaM kinase II from that in lanes 2-4. The reaction conditions were as described in the Experimental section. Reactions were started with ATP, the mixtures were incubated for 30 min at 30 °C and the reactions were stopped by adding an equal volume (0.1 ml) of gel sample buffer and boiling for SDS/PAGE (a) and autoradiography (b).

Fig. 7. Time course of phosphorylation of β -galactosidase and Na⁺/H⁺ exchanger- β -galactosidase fusion protein

Conditions were as described for Fig. 5 and samples (50 μ l) were withdrawn from the phosphorylation reaction at the indicated times, added to gel sample buffer and boiled for SDS/PAGE and autoradiography. Quantitative data were obtained by scanning the gel and autoradiogram enabling calculation of the P_i incorporation to be expressed per unit of protein (given in arbitrary units).

In the rabbit there are 21 acidic residues compared with nine basic ones in the terminal 100 amino acid residues (Fig. 4). Because of the acidic nature of this region we hypothesized that this region could directly participate in Ca²⁺ binding and that Ca²⁺ could directly act to regulate the protein. When Ca²⁺ overlay was performed on both purified proteins [32], with calsequestrin as a control as described previously [33], neither the fusion protein nor β -galactosidase alone bound significant amounts of Ca²⁺ under these conditions (results not shown).

Phosphorylation of the Na⁺/H⁺ exchanger

Examination of the amino acid sequence of the putative cytoplasmic domain of the protein [27,28] shows that there are three putative sites of phosphorylation for CaM kinase II in these 178 terminal amino acid residues of the rabbit cardiac Na⁺/H⁺ exchanger (XRXXS*X, Fig. 4; see ref. [41] for a recent review). On the other hand, this region of the Na⁺/H⁺ exchanger does not contain any consensus sequences for cyclic AMP-dependent protein kinase (XRRXS*X) or protein kinase C (XRXXS*XRX).

Fig. 5 shows the CaM kinase II-dependent phosphorylation of expressed β -galactosidase and the fusion protein. Phosphorylation of the Na⁺/H⁺ exchanger fusion protein was substantially greater than that of β -galactosidase. When quantified by laser scanning densitometry of the autoradiogram, the fusion protein was found to contain 3.9 times more radioactivity. (The two

smaller radiolabelled bands represent autophosphorylation of the subunits of CaM kinase II.) Phosphorylation was dependent on the addition of both calmodulin and Ca²⁺ (Fig. 6). Other experiments (results not shown) showed that this region of the exchanger is not phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase or by protein kinase C.

Fig. 7 shows the time course of phosphorylation of β -galactosidase and the fusion protein. The quantitative data were obtained by scanning the stained gel and autoradiogram, enabling calculation of the amount of phosphate incorporation to be expressed per unit of protein in arbitrary units. The phosphorylation of the Na⁺/H⁺ exchanger fusion protein proceeded more rapidly and reached a higher level than the control protein. The initial rate of phosphorylation of the Na⁺/H⁺ exchanger fusion protein was approx. 3-fold greater than that of β -galactosidase after 5 min and the final level of phosphorylation was 3–4-fold greater than the control.

In a separate series of experiments, the maximal levels of phosphorylation of β -galactosidase and fusion protein by CaM kinase II were determined as described in the Experimental section. β -Galactosidase was phosphorylated to the extent of 0.77 ± 0.17 mol of P_i /mol (mean $\pm$ S.E.M., $n = 6$) whereas the fusion protein was phosphorylated to a maximal level of 4.09 ± 0.39 nmol of P_i /mol ($n = 6$).

DISCUSSION

To help clarify the role of different kinases in the regulation of the cardiac Na⁺/H⁺ exchanger, we have studied the direct phosphorylation of the cardiac protein *in vitro*. Since no purified preparation of the protein was available and efforts at its identification and purification have met with conflicting results, we produced the protein *in vivo* by using the cDNA encoding the rabbit cardiac form of the protein.

We have examined the C-terminal 178 amino acid residues of the protein since this region contains a cytoplasmic domain of the protein [7,28] and is a likely site of regulation. This region of the protein contains three putative CaM kinase II phosphorylation sites and three of the five possible sites of phosphorylation by CaM kinase II in the human protein (XRXXS*X [41]). These phosphorylation-site motifs are conserved in the rabbit cardiac Na⁺/H⁺ exchanger (Fig. 4 and [27]). Of the other two putative phosphorylation sites on the protein, according to the best known model, Ser-56 is on the extracellular surface of the plasma membrane [7] and the other (Ser-324) is on the inner face of the membrane.

Figs. 1 and 2 show that we have produced and purified a fusion protein with β -galactosidase. Addition of the amino acid residues of the Na⁺/H⁺ exchanger resulted in the expected increase in size from M_r 116000 to 135000. To confirm that we have correctly expressed the protein, we used an antibody against a synthetic peptide of the terminal 13 amino acid residues of the sequence. Fig. 3 shows that the antibody against the synthetic peptide recognized the expressed protein but showed no reactivity at all towards β -galactosidase. These results confirm that we have expressed the protein correctly in the appropriate reading frame. We note also that, despite the relatively acidic nature of the terminal 100 amino acid residues, they do not bind Ca²⁺ when assayed by the Ca²⁺ overlay technique [32]. This and the results described below suggest that the actions of increased intracellular Ca²⁺ [8–11] on the Na⁺/H⁺ exchanger are probably through CaM kinase II and not through a direct effect of Ca²⁺ on the protein.

Fig. 5 shows that the fusion protein is a substrate for CaM kinase II-dependent phosphorylation. To our knowledge, this represents the first demonstration of direct phosphorylation of

the Na^+/H^+ exchanger by a specific protein kinase. Phosphorylation was dependent on both Ca^{2+} and calmodulin, as expected. Quantification of the maximal levels of phosphate incorporation suggest one site of phosphorylation by CaM kinase II in β -galactosidase (0.77 ± 0.17 mol of P_i /mol) and four sites in the fusion protein (4.09 ± 0.39 mol of P_i /mol), i.e. three sites in the cytoplasmic domain of the Na^+/H^+ exchanger. Consistent with these results the expressed cytoplasmic domain of the Na^+/H^+ exchanger contains three CaM kinase II recognition sequences, whereas β -galactosidase itself contains two such sites (around Ser-169 and Ser-213 [42]). The most likely explanation to describe the phosphorylation results is that CaM kinase II phosphorylates β -galactosidase to some degree at both or one of its consensus sites and the Na^+/H^+ exchanger to a higher level at Ser-10, Ser-65 and Ser-159 of the expressed C-terminal of the fusion protein (see Fig. 4). Fig. 7 shows the comparison of the time courses of phosphorylation of the fusion protein and control β -galactosidase. It is evident that both the initial rate and peak level of phosphorylation of the exchange protein are much greater than that of the control. Thus, although β -galactosidase contains two consensus sequences for CaM kinase II, it serves as a poorer substrate. The Na^+/H^+ exchanger is more rapidly phosphorylated to a higher degree and with a stoichiometry of 1 mol of phosphate per mol of consensus site. This is consistent with the idea that this region of the exchanger is a regulatory domain and an endogenous substrate of CaM kinase II. It is possible that some of the additional phosphate incorporated into the fusion protein, as compared with β -galactosidase itself, may actually be incorporated into sites within β -galactosidase that are exposed because of an altered conformation of this fusion protein. However, this possibility seems unlikely, especially since only two potential sites of phosphorylation of β -galactosidase are present in the protein and these were already phosphorylated to approx. 0.4 mol of phosphate per site.

The rate of phosphorylation of the Na^+/H^+ exchanger- β -galactosidase was similar to that of known endogenous substrates of CaM kinase II such as caldesmon, synapsin I [43] and calponin [44]. This is again consistent with the idea that this region of the exchanger is a regulatory domain and an endogenous substrate of CaM kinase II. It is possible that the pattern of protein phosphorylation in the native exchanger may differ from that of the cytoplasmic domain of the exchanger fused to β -galactosidase. Further studies will require sequencing of the sites in the expressed native exchanger phosphorylated by CaM kinase II and examination of the effects of phosphorylation on the expressed Na^+/H^+ exchange activity.

The phosphorylation of the Na^+/H^+ exchanger fusion protein was specific to CaM kinase II. The expressed cytoplasmic domain of the Na^+/H^+ exchanger was not phosphorylated by cyclic AMP-dependent protein kinase or protein kinase C. This is consistent with the absence of any clear consensus sequence for either of these kinases. The results of our studies carried out *in vitro* with the three protein kinases suggest therefore that the rabbit cardiac Na^+/H^+ exchanger may be regulated *in vivo* by phosphorylation within its C-terminal cytoplasmic domain catalysed by CaM kinase II, which is activated after an increase in cytosolic free Ca^{2+} . This appears to run contrary to the indications from a number of studies in intact cell systems that demonstrate that a variety of hormones and growth factors, which activate protein kinase C, activate the exchanger (see ref. [4] for a review). However, some of these results can be explained by the fact that inositol 1,4,5-trisphosphate will be released in addition to diacylglycerol in response to various stimuli that activate protein kinase C, and this will induce release of intracellular Ca^{2+} , which could then activate the Na^+/H^+ exchanger via CaM kinase II. A number of studies have suggested that, at

least in some cell types or in some special circumstances [12–16], Ca^{2+} - or calmodulin-dependent pathways predominate.

It is, however, more difficult to explain the results of direct phorbol ester activation of the exchanger that have been observed in a number of studies [7] (see ref. [6] for a review). Despite the fact that protein kinase C recognition sequences (XRXXS*XRXX) do not occur in either the human [28] or rabbit [27] cardiac Na^+/H^+ exchanger, another site may be recognized. Alternatively, it has been suggested that a number of different isoforms of the exchanger exist in different tissues [5,45] and its regulation has been shown to vary with cell type. It may be that one form of the protein is regulated by CaM kinase II and other form(s) are regulated differently, depending on their level of expression. Alternatively, another protein regulating the Na^+/H^+ exchanger may be involved and such a regulatory cofactor for cyclic AMP-dependent protein kinase has recently been identified [46]. Further studies are required to explore this possibility.

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