Interaction of PIP$_2$ with the XIP region of the cardiac Na/Ca exchanger

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He, Zhaoping, Siyi Feng, Qiusheng Tong, Donald W. Hilgemann, and Kenneth D. Philipson. Interaction of PIP$_2$ with the XIP region of the cardiac Na/Ca exchanger. Am J Physiol Cell Physiol 278: C661–C666, 2000.—The sarcolemmal Na/Ca exchanger undergoes an inactivation process in which exchange activity decays over several seconds following activation by the application of Na to the intracellular surface of the protein. Inactivation is eliminated by an increase in membrane phosphatidylinositol 4,5-bisphosphate (PIP$_2$). Inactivation is also strongly affected by mutations to a basic 20-amino acid segment of the exchanger known as the endogenous XIP region. The hypothesis that PIP$_2$ directly interacts with the XIP region of the exchanger was tested. First, we investigated the ability of a peptide with the same sequence as the XIP region to bind to immobilized phospholipid vesicles. [125I]-labeled XIP bound avidly to vesicles containing only a low concentration (~3%) of PIP$_2$. The binding was specific, in that binding was not displaced by other basic peptides. The effects of altering the sequence of XIP peptides also indicated binding specificity. Second, we examined the functional response to PIP$_2$ of exchangers with mutated XIP regions. Outward Na/Ca exchange currents were measured using the giant excised patch technique. The mutated exchangers either had no inactivation or accelerated inactivation. In both cases, the exchangers no longer responded to PIP$_2$ or to PIP$_2$ antibodies. Overall, the data indicate that the affinity of the endogenous XIP region for PIP$_2$ is an important determinant of the inactivation process.

NCX1.1; calcium transport; inactivation; regulation

The sodium/calcium exchanger of cardiac sarcolemma is a potent Ca extrusion mechanism that may also play a role in Ca influx (reviewed in Refs. 1 and 22). The Na/Ca exchanger is thus an important regulator of myoplasmic Ca and cardiac contractility. The exchanger catalyzes the countertransport of three Na for one Ca. The cardiac exchanger (NCX1.1) has been cloned and expressed (20), and molecular information on the exchanger protein is available. Current topological models indicate that the exchanger protein has nine transmembrane segments (13, 21). Five amino-terminal transmembrane segments are separated from four carboxy-terminal transmembrane segments by a large intracellular loop (loop f). Regions of loop f are involved in regulation of exchange activity. The exchanger, for example, not only transports Ca but is also separately regulated by intracellular Ca acting at a binding site on loop f (14, 18).

When the Na/Ca exchanger was first cloned, it was recognized from the sequence that the initial portion of the large intracellular loop (the endogenous XIP region) might be involved in autoregulation of exchange activity through interaction with other portions of the exchanger (15). To test this hypothesis, a 20-amino acid peptide (XIP peptide) with the sequence of the endogenous XIP region was synthesized. This peptide turned out to be a relatively potent [inhibition constant ($K_I$) = 1.5 µM] and specific inhibitor of exchange activity (hence the name XIP for exchanger inhibitory peptide). XIP contains primarily basic and hydrophobic residues, is noncompetitive with both Na and Ca, and acts at the intracellular surface of the exchanger (15). Several residues of XIP are important in the inhibitory response (5, 25).

These experiments indicated that a peptide with the same sequence as the endogenous XIP region altered Na/Ca exchange activity but provided no evidence for a role for the endogenous XIP region itself. Subsequently, we performed mutational analysis of the endogenous XIP region (17). Strikingly, we found that all mutations altered a regulatory response of the exchanger known as Na-dependent inactivation (11). Na-dependent inactivation of the exchanger was first observed by Hilgemann (6), using the giant excised patch technique. When Na is applied to the intracellular surface of an excised membrane patch containing exchanger protein, an outward Na/Ca exchange current is activated if Ca is present in the patch pipette. The current rapidly peaks and then partially inactivates to a steady-state level over several seconds. We found that XIP region mutants had drastically altered inactivation kinetics and that, in some cases, inactivation was eliminated (17). The results strongly suggested that the endogenous XIP region is involved in the Na-dependent inactivation process.

The level of membrane phosphatidylinositol 4,5-bisphosphate (PIP$_2$) also exerts a strong influence on...
Na-dependent inactivation (8). An increase in membrane PIP2 stimulates exchange activity by eliminating inactivation. We hypothesized, therefore, that the XIP domain is a PIP2 binding site. The XIP region is modeled to be located in the exchanger near the membrane-lipid interface, providing accessibility to PIP2 (15). Binding of Na to transport sites at the cytoplasmic face of the exchanger, which initiates the inactivation process, could decrease the affinity of the exchanger for PIP2. In the simplest case, inactivation would correspond to the dissociation of PIP2 from the exchanger. Electrostatic interactions may play a major role: PIP2 is anionic, whereas the XIP region is cationic. A previous study has demonstrated binding of XIP peptide to phosphatidylserine and has proposed that XIP-phospholipid interactions regulate exchanger activity (23).

Here we begin to test this hypothesis. We examine the binding of XIP to PIP2 and test the effect of PIP2 on exchangers with mutations in the XIP region. The results confirm the plausibility of our hypothesis.

METHODS

125I-labeled XIP binding assays. Phospholipid vesicles [mixtures of phosphatidylcholine (PC) with either phosphatidylserine (PS) or PIP2] were prepared by sonication at 10 mg/ml in water until clarity was achieved. The vesicles were then applied to polyacrylamide (PEVDF) membranes using a Bio-Rad dot-blotting apparatus. The membrane was blocked with BSA (4%) in binding buffer (140 mM NaCl, 1 mM EGTA, 0.5 mM dithiothreitol, 0.5 mM NaN3, 0.05% Tween 20, 20 mM MOPS/Tris, pH 7.4). The filter was then incubated with 1 µM 125I-XIP (2 × 10^6 cpm/ml) in binding buffer containing 0.2% BSA in the presence or absence of other unlabeled peptides. The filter was washed, dried, and exposed to X-ray film.

125I-XIP was prepared by the chloramine-T method: Na125I (0.5 mCi; Amersham) was incubated with XIP (50 µg) and chloramine-T (10 µg) in 100 µl of 100 mM potassium phosphate buffer, pH 7.4, for 20 s, and the reaction was then stopped with 20 µl of Na2S2O5 (5 mg/ml). The reaction mixture was applied to a Sephadex G-10 column, and the 125I-XIP was eluted with the potassium phosphate buffer and stored at 4°C. Peptides were synthesized as described previously (5).

Mutants. Mutant Na/Ca exchangers were all constructed as part of an earlier study (17).

Electrophysiology. Recordings of outward (reverse) Na/Ca exchange current were made in giant excised patches from Xenopus oocytes using the procedures and solutions described previously (7, 10, 19). All recordings were with CI-free solutions containing 20 mM Cs and 20 mM TEA on both membrane sides to block K and nonspecific currents. The extracellular (pipette) Ca concentration was 4 mM, and the cytoplasmic free Ca concentration was 1 µM with 10 mM EGTA as Ca buffer. The temperature was 32°C. PIP2 was incorporated into membrane patches by incubation of the excised patch with PIP2 liposomes as described previously (8, 12).

Materials. PIP2 antibody was from Perseptive Biosystems (Boston, MA). The reconstituted antibody solution was diluted 50-fold into cytoplasmic solutions. PC and PS were from Sigma and PIP2 was from American Radiolabeled Chemicals.

% PS

0 3 7 10 25 50

% PIP2

3 7 10

Fig. 1. Binding of 125I-labeled XIP to phosphatidylserine (PS)/phosphatidylcholine (PC) or phosphatidylinositol 4,5-bisphosphate (PIP2)/PC vesicles. Vesicles contained PS or PIP2 in the percentage shown, with the remainder of the phospholipid being PC. See METHODS for details. Data are representative of 3 experiments.

RESULTS

Binding of XIP to phospholipids. 125I-XIP was incubated with phospholipid vesicles immobilized on PVDF membranes. Phospholipids were PC mixed with different amounts of anionic phospholipids, either PS or PIP2. As shown in Fig. 1, the presence of substantial amounts of PS was necessary to detect binding of 125I-XIP as also seen in a previous report (23). In contrast, the binding of XIP to the vesicles was readily detected even at low PIP2 levels. No binding was observed in the absence of anionic phospholipid. Binding of XIP to PIP2 was equivalent in either a Na or K medium. The effects of Ca were not tested.

Thus XIP was capable of binding to vesicles containing PIP2. We next tested the specificity of this binding. 125I-XIP was incubated with vesicles containing 5% PIP2 in the presence or absence of excess unlabeled peptide. In the absence of competitor (Fig. 2 top left), strong binding of XIP was observed, as also seen in Fig. 1. A 50-fold excess of unlabeled XIP eliminated binding. However, six other peptides (labeled A–F in Fig. 2; see figure legend for peptide sequences), also representing different regions of the exchanger, were poor competitors of XIP binding. Some of these peptides, like XIP, were cationic. Thus the XIP peptide preferentially bound to PIP2 compared with the other basic peptides.

The specificity of XIP binding to PIP2 was further examined in experiments shown in Fig. 2 (bottom). The binding shown on the left ("None") again represents control binding of 125I-XIP to PIP2 in the absence of a competing peptide. Unlabeled XIP displaced most of the 125I-XIP. "Scrambled" XIP (sXIP), in which the amino acid sequence of XIP was randomly rearranged, was unable to displace 125I-XIP, whereas poly-L-lysine was a moderately successful competitor. Of interest were the effects of two peptides, F5E and K11Q, both analogs of XIP. In F5E, the phenylalanine at position 5 was replaced with a glutamate residue. In K11Q, the lysine at position 11 was exchanged for a glutamine residue. These same replacements were also present in mutants F223E and K229Q analyzed in our earlier functional study (17) and below. Although these peptides are quite similar, their effects on XIP/PIP2 interactions were remarkably different. F5E was an ineffective competitor, whereas K11Q was consistently a more...
pipette Ca was 4 mM. Under these conditions, the exchange current typically inactivates by 70 to 90% over 30 s, as shown. The 1 µM free cytoplasmic Ca used in these experiments is submaximal for secondary activation of the exchange current (9). After application of PIP2 for 2 min, the peak exchange current obtained on reapplication of cytoplasmic Na is increased only slightly, whereas the steady-state current is greatly increased. Thus the effect of PIP2 is largely due to an elimination of the Na-dependent inactivation process.

Figure 3B shows the typical responses of the Δ229–237 exchanger mutant to the same protocols. In this mutant, nine amino acids of the endogenous XIP region are eliminated. The records are from the same oocyte batch from which the wild-type recordings in Fig. 3A were obtained. Under control conditions, the outward exchange current shows almost no inactivation on application of cytoplasmic Na (left) as seen previously (17). The outward current still requires the presence of regulatory Ca on the cytoplasmic side in the micromolar concentration range (not shown). Application of PIP2 (30 µM) has almost no effect on the current, although more current can still be elicited by increasing cytoplasmic Ca. Thus, under these conditions, disruption of the Na-dependent inactivation process appears to be associated with a loss of the response to PIP2.

Figure 4 shows the responses of two other mutants (K229Q and F223E) to PIP2. These records were also obtained in the presence of 1 µM free cytoplasmic Ca. The K229Q mutation in the XIP domain results in loss of Na-dependent inactivation (Fig. 4A) (17), similar to that seen with the deletion mutation (Fig. 3). With the point mutation, however, a large component of exchange current is insensitive to removal of cytoplasmic Ca (see response to 0 Ca). Application of PIP2 (30 µM) stimulates the exchange current by only ~5%.

The data on the effects of PIP2 on Na-dependent inactivation are consistent with the interpretation that the inactivation process reflects a reduced affinity of the exchanger for PIP2. Thus inactivation may reflect the dissociation of PIP2 from the exchanger. In this context, we would predict that an exchanger with rapid inactivation would have a reduced affinity for PIP2. As shown in Fig. 4B and as described previously (17), the F223E mutation results in a much more pronounced and rapid Na-dependent inactivation. As also shown, PIP2 has almost no effect on the exchange current of the F223E mutant. In some experiments, a somewhat more pronounced effect was observed. The largest effect was obtained in a patch with only 75% inactivation and, in this case, the stimulatory effect of the PIP2 was still only 20% of the peak exchange current obtained on applying Na (not shown). Similar results for mutant F223E were also obtained in excised patches from BHK

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**Fig. 2. Specificity of binding of 125I-XIP to PIP2/PC vesicles.** Vesicles contained 5% PIP2. Top: competition of 125I-XIP binding to PIP2-containing vesicles with other basic peptides. Binding of 125I-XIP to vesicles was performed in the absence or presence of a 50-fold excess (50 µM) of unlabeled XIP or other peptides. Sequences of peptides are as follows: XIP; RRLFYKYYVRYRAGKORG; peptide A: QEKEITKKPNGGETT; peptide B: KYYVRYRAGKORGMIIEHE; peptide C: EERDDEEARERMARILKE; peptide D: EERARERMARILKEKQKHPE; peptide E: MARILKEKQKHPEKEIEQL; peptide F: ITGYLYGQVFRKVHAREH. Bottom: competition of 125I-XIP binding to PIP2PC vesicles by XIP analogs and by poly-L-lysine. Conditions as above. See text for details on the peptides. The sequence of sXIP (“scrambled” XIP) is YQLRGFRGRKHYAVRYKYLK. Data are representative of 3 (top) or 4 (bottom) experiments.

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**Fig. 3. Effects of PIP2 on Na/Ca exchange currents in giant inside-out membrane patches from Xenopus oocytes expressing the wild-type (NCX1.1) and Δ229–237 exchangers.** Pipette contains 4 mM Ca, cytoplasmic solution contains 1 µM free Ca, and 90 mM Na is substituted for 90 mM Cs on the cytoplasmic side to activate outward exchange current. A: wild-type Na/Ca exchanger. Typical outward current transients obtained before (left) and after (right) application of PIP2 (30 µM). B: Δ229–237 exchanger. Current transients before (left) and after (right) application of PIP2 (30 µM). Similar data were obtained in 2 additional experiments. Na+, intracellular Na.
Thus both the loss of Na-dependent inactivation (as in mutant K229Q) and the enhancement of Na-dependent inactivation (as in mutant F223E), as a result of mutations in the XIP domain, can result in a loss of the stimulatory effect of PIP2. Because the loss of inactivation by mutation K229Q results in a phenotype that is functionally similar to the PIP2-stimulated wild-type exchanger, it is important to distinguish clearly between these two different functional states. This is accomplished with an antibody to PIP2 that reverses the effects of endogenous PIP2 (8) in excised membrane patches. Figure 5 shows the typical response of wild-type and K229Q exchangers to PIP2 antibody. To mimic the loss of inactivation for the wild-type exchanger, the current was first stimulated with 3 mM ATP, which increases membrane PIP2 (8), before obtaining the record shown in Fig. 5A. As expected, the exchange current shows greatly reduced inactivation on application of cytoplasmic Na, similar to the function of the K229Q mutant. As shown in Fig. 5, however, the ATP-stimulated wild-type exchanger is strongly inhibited by application of PIP2 antibody, whereas the K229Q exchanger is not. Thus the K229Q mutation appears both to activate exchange current by removing inactivation and to make the exchanger independent of membrane PIP2.

**DISCUSSION**

The cardiac Na/Ca exchanger undergoes an inactivation process. This is most easily seen during measurements of outward exchange currents using the giant excised patch technique (6, 11). After induction of exchange current by the application of internal Na, exchange activity decays to a new steady-state level over a period of several seconds (e.g., Fig. 3A). We have previously suggested that a portion of the exchanger protein known as the endogenous XIP region is involved in the inactivation process (17). The XIP region is 20 amino acids in length and forms the aminoterminal region of a large intracellular loop of the exchanger. The XIP region begins at amino acid position 219 and has the sequence RRLFYKYYKRYRAGKQRG. Mutations of the XIP region (17) either eliminate inactivation (e.g., Δ229–237 and K229Q) or increase the rate of inactivation (e.g., F223E). Perhaps movement of the XIP region following exchanger activation places the protein into an inactivated state.

Inactivation can be removed by interventions that increase the membrane level of PIP2. Experimentally, this can be achieved by application of PIP2 (Fig. 3) or ATP (Fig. 5) to an excised patch. Other anionic phospholipids can also activate the exchanger (2, 24), although not with the potency of PIP2 (8, 16). The exchanger remains highly sensitive to the presence of anionic lipids even after reconstitution into liposomes, implying that other proteins are not involved. Thus PIP2 very likely exerts its effect by directly interacting with the exchanger protein. We hypothesize that the anionic PIP2 interacted with the cationic XIP region. We find (Fig. 1) that a 20-amino acid peptide with the same sequence as the XIP region binds avidly to PIP2.

**Fig. 4.** Representative outward exchange currents for mutants K229Q and F223E. Same conditions as Fig. 3. A: K229Q shows almost no inactivation, and regulatory Ca and PIP2 have only small effects. B: F223E shows greatly enhanced inactivation, and PIP2 has only a very small stimulatory effect. Similar data were obtained in 2 additional experiments.

**Fig. 5.** Effects of PIP2 antibody on wild-type and K229Q exchange currents. A: Wild-type exchanger. Exchange current was first stimulated by application of 2 mM ATP for 2 min prior to the recording. The current shows almost no inactivation, and application of the PIP2 antibody results in an 80% inhibition of current over 2 min. B: K229Q exchanger. Outward exchange current shows almost no inactivation on applying cytoplasmic Na, and application of the PIP2 antibody has no effect. Almost identical data were obtained in a second experiment.
There was clearly some specificity in the XIP/PIP2 interaction. Unlabeled XIP peptide was able to displace \(^{125}\)I-XIP from PIP2, whereas other cationic peptides could not (Fig. 2, top). Also, scrambled XIP and poly-L-lysine were ineffective competitors of \(^{125}\)I-XIP. Modest changes in the XIP peptide sequence altered PIP2 binding. The FSE and K11Q peptides (Fig. 2, bottom) had decreased and increased interactions with PIP2, respectively. We conclude that there is a specific binding interaction between the XIP peptide and PIP2. We do not have direct evidence that the endogenous XIP region of the intact exchanger protein interacts with membrane PIP2, but the data are strongly suggestive that this interaction may also occur.

We next examined the effects of PIP2 on the function of Na/Ca exchangers with mutations in the XIP region. The mutations had all been previously characterized to alter Na-dependent inactivation (17). Two of the mutations (∆229–237 and K229Q) eliminated the inactivation process. These mutants were also no longer affected by PIP2. The result is consistent with the hypothesis that the XIP region is the site of interaction with PIP2. In the simplest model, when the XIP region is bound to PIP2, the exchanger is activated. Binding of Na to transport sites induces a conformational change, which favors the dissociation of XIP from PIP2. The XIP peptide altered to match the sequence of K229Q (i.e., peptide K11Q) appeared to bind more strongly to PIP2 than did the XIP peptide itself. Thus the XIP region of K229Q may interact with the membrane bilayer so strongly that little inactivation ever occurs.

Although dissociation of the XIP region from PIP2 or other anionic phospholipids may favor the inactive state of the Na/Ca exchanger, the situation is likely to be more complicated. The cationic XIP region, once dissociated from PIP2, likely finds some other site on the exchanger to which to bind, and this binding may induce the inactive state. This unknown site may also be the site to which exogenous XIP peptide binds to inhibit exchange activity (4, 15). Thus a mutation of the XIP region can affect both binding to PIP2 and to some other site on the exchanger. The final phenotype may reflect effects at both sites. For example, in mutant ∆229–237, the XIP region may be incapable of binding to either PIP2 or to its alternative binding site on the exchanger protein. Likewise, exogenous XIP peptide may have two sites of action: binding to PIP2 and to a specific site on the exchanger protein itself. A model in which the XIP domain of the exchanger can interact with two different sites has also been proposed by Shannon et al. (23).

For mutant K229Q, we have proposed (above) that the endogenous XIP region binds tightly to PIP2, preventing inactivation. If the mechanism of inhibition by exogenous XIP peptide is to displace PIP2 from the XIP region of the protein, then we would expect the potency of XIP to be diminished for this mutant. However, XIP sensitivity is not remarkably different for the wild-type and K229Q exchangers (S. Matsuoka, unpublished observations). This would imply that a site of action of XIP peptide is on the protein itself, and effects of XIP peptide are not due solely to an interaction with phospholipids. In a previous study (5), the peptide K11Q was moderately less potent than XIP itself as an inhibitor of exchange activity. This would suggest that peptide K11Q binds less strongly to the protein than does XIP peptide. We have not been able to devise unequivocal experiments to confidently define the relative inhibitory effects of XIP due to direct inhibition of the exchanger vs. indirect inhibition due to binding to PIP2.

In exchanger mutant F223E, the rate of inactivation is markedly accelerated. In this case, the interaction of the endogenous XIP region with membrane lipids may be tenuous. Initiation of exchange activity may induce a rapid dissociation and a concomitant fast inactivation. Consistent with this interpretation, peptide F5E binds poorly to PIP2. Also, we had previously found that peptide F5E inhibits exchange activity much less potently than XIP peptide (5). This would be expected if peptide F5E inefficiently displaces the endogenous XIP region of the exchanger from anionic phospholipids and/or if peptide F5E binds weakly to the exchanger protein itself.

Notably, PIP2-sensitive inward rectifier K channels, such as the KATP and ROMK channels (8, 12), are inhibited by relatively low concentrations of XIP (10 μM) (Hilgemann, unpublished observations). These observations are consistent with the XIP domain of the Na/Ca exchanger being a PIP2 binding site. Thus exogenous XIP would be expected to competitively displace PIP2 from PIP2 binding sites on other proteins.

For simplicity, we have presented an interpretation in which the XIP region associates and dissociates from PIP2 to regulate activity. Although we have evidence that binding does occur, there is no direct evidence that reversible association/dissociation governs activity. A plausible alternative, for example, is that the XIP region is always membrane bound, although different lipid-bound conformations exist. Furthermore, mutations of the XIP region also modulate regulation of the exchanger by Ca through interactions that are not yet understood (17). In any case, our results may form the framework for further investigation. Modulation of Na-dependent inactivation may represent a physiologically relevant mechanism for regulation of Na/Ca exchange activity.

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