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24. Samples were prepared as follows. CML-3b and CML-6: Five to ten drops of marrow diluted with phosphate-buffered saline (PBS) to prevent clotting were fixed in methanol/acetic acid and dropped on slides. CML-1, CML-2, CML-4, and CML-5: Peripheral blood or bone marrow, or both, was cultured in RPMI 1640 supplemented with 10% fetal bovine serum, an antibiotic mixture (gentamycin 500 μg/ml), and 1% L-glutamine for 24 hours. Cultures were synchronized according to J. J. Yunis and M. E. Chandler [*Prog. Clin Path* 7, 267 (1977)], and chromosome preparations followed L. M. Gibas and L. G. Jackson [*Karyogram* **11**, 91 (1985)]. CML-3a: Peripheral blood was centrifuged for 5 min at 1100 rpm, the buffy coat was pipetted off and diluted with same volume of PBS, spun down, fixed in methanol/acetic acid, and dropped on slides. Hybridization followed procedures described by D. Pinkel *et al* [*Proc Natl Acad Sci U.S.A.* **85**, 9138 (1988)], Trask *et al*. (*25*), and J. B. Lawrence, C. A. Villnave, and R. H. Singer [*Cell* **42**, 51 (1988)], with modifications. The *bcr* probe was nick-translated (Bethesda Research Laboratories Nick-Translation System) with digoxigenin-11–dUTP (deoxyuridine 5′-triphosphate) (Boehringer Mannheim Biochemicals) with an average incorporation of 25%. The *abl* probe was similarly nick-translated with biotin-11–dUTP (Enzo Diagnostics). Cells were thermally denatured at 72°C for 5 min, dehydrated in an ethanol series, air-dried, and placed at 37°C. A hybridization mixture (10 μl) containing each probe (2 ng/μl), 50% formamide/2× standard saline citrate (SSC), 10% dextran sulfate, and human genomic DNA (1 mg/ml, sonicated to 200 to 600 bp) was heated to 70°C for 5 min, incubated for 30 min at 37°C, placed on the warmed slides, covered with a 20 mm by 20 mm cover slip, sealed with rubber cement, and incubated overnight at 37°C. Slides were washed three times in 50% formamide/2× SSC for 20 min each at 42°C, twice in 2× SSC at 42°C for 20 min each, and rinsed at room temperature in 4× SSC. All subsequent steps were performed at room temperature. Slides were blocked in 100 μl of 4× SSC/1% bovine serum albumin (BSA) for 5 min under a cover slip. The biotinylated *abl* probe was detected by applying 100 μl of Texas red–avidin (Vector Laboratories Inc., 2 μg/ml in 4× SSC/1% BSA) for 45 min. The slides were washed twice for 5 min in 4× SSC/1% Triton X-100 (Sigma). The signal was amplified by applying biotinylated goat antibody to avidin {Vector Laboratories Inc., 5 μg/ml in PNM [0.1 M NaH_2PO_4/0.1 M Na_2HPO_4, pH 8 (PN) containing 5% nonfat dry milk and 0.02% sodium azide and centrifuged to remove solids]}, washed twice in PN for 5 min, followed by another layer of Texas red–avidin in PNM. The digoxigenin-labeled *bcr* probe was detected by incubation with sheep antibody to digoxigenin (obtained from D. Pepper, Boehringer Mannheim Biochemicals, Indianapolis, IN; 15.4 μg/ml in PNM) for 30 min, washed twice in PN for 5 min, followed by a rabbit-antibody to sheep conjugated with FITC (Organon Teknika-Cappel, 1:50 in PNM). After washing twice for 5 min in PN, the signal was amplified by applying a sheep antibody to rabbit immunoglobulin G (IgG) conjugated to FITC (Organon Teknika-Cappel, 1:50 in PNM). The slides were then rinsed in PN. Slides were mounted in 10 μl of fluorescence antifade solution [G. D Johnson and J. G. Nogueria, *J. Immunol. Methods* **43**, 349 (1981)] containing 4′,6-amidino-2-phenylindole (DAPI) at 1 μg/ml as a counterstain. The slides were examined with an FITC/Texas red double-band pass filter set (Omega Optical) on a Zeiss Axioskop.
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29. We used the method of S. Kohler *et al* [*Leukemia* **4**, 8 (1990)] for *bcr-abl* PCR on CML-2, CML-4, and CML-5. The oligonucleotide primers used were as follows: *abl*X3 antisense downstream 5′-TTT CTC CAG ACT GTT GAC TGG-3′; *abl*X2 sense upstream 5′-CCT TCA GCG GCC AGT AGC AT-3′; CML *bcr* upstream 5′-ACA GCA TTC CGC TGA CCA TC-3′; CML *abl* antisense detection 5′-TAT GCT TAG AGT GTT ATC TCC ACT-3′.
30. Method used for *bcr-abl* PCR by S. Hegewisch-Becker *et al*. [*J. Biol. Chem. Suppl* **13E**, 289 (1989)] on cases CML-3a, CML-3b, and CML-6. The oligonucleotide primers used were as follows: sense primer (upstream of *bcr*) 5′-AGG GTG CAC AGC CGC AAC GGC-3′; antisense primer (*abl*) 5′-GGC TTC ACT CAG ACC CTG AGG-3′; probe for the identification of *bcr3/abl2* junction sequence 5′-GAA GGG CTT TTG AAC TCT G-3′; probe for the identification of *bcr2/abl2* junction sequence 5′-GAA GGG CTT CTT CCT TAT-3′. Exon 3 of *bcr* is joined to *abl* exon 2 if a 314-bp fragment is amplified. Exon 2 of *bcr* is joined to *abl* exon 2 if a 239-bp fragment is amplified.
31. Southern blot on case CML-6 showed a rearranged Bgl II band with an OSI Transprobe-1 Kit (Oncogene Science catalog no. TP88).
32. We thank B. Trask for expertise in hybridization techniques, R. Seagraves and C. Dana Bangs for technical help, and R. Wooten for art and photographic assistance. D.C.T. is a recipient of a Centennial Fellowship from the Medical Research Council of Canada. C.A.W. is a James S. McDonnell Scholar in Molecular Medicine in Cancer Research. This work was performed under the auspices of the U.S Department of Energy by Lawrence Livermore National Laboratory under contract W-7405-ENG-48 with support from PHS grants CA 45919, CA49605, and CA44700.

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Molecular Cloning and Functional Expression of the Cardiac Sarcolemmal Na^+-Ca^{2+} Exchanger

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The Na^+-Ca^{2+} exchanger of the cardiac sarcolemma can rapidly transport Ca^{2+} during excitation-contraction coupling. To begin molecular studies of this transporter, polyclonal antibodies were used to identify a complementary DNA (cDNA) clone encoding the Na^+-Ca^{2+} exchanger protein. The cDNA hybridizes with a 7-kilobase RNA on a Northern blot and has an open reading frame of 970 amino acids. Hydropathy analysis suggests that the protein has multiple transmembrane helices, and a small region of the sequence is similar to that of the Na^+- and K^+-dependent adenosine triphosphatase. Polyclonal antibodies to a synthetic peptide from the deduced amino acid sequence react with sarcolemmal proteins of 70, 120, and 160 kilodaltons on immunoblots. RNA, synthesized from the cDNA clone, induces expression of Na^+-Ca^{2+} exchange activity when injected into *Xenopus* oocytes.

THE Na^+-Ca^{2+} EXCHANGE TRANSporter of cardiac sarcolemma is a major pathway for transmembrane Ca^{2+} fluxes in cardiac myocytes (*1*). The exchanger uses the energy in the Na^+ gradient to move Ca^{2+} and is usually considered the dominant cellular Ca^{2+} efflux mechanism. The significance of Na^+-Ca^{2+} exchange in cardiac excitation-contraction coupling has recently attracted attention (*2*). Although both physiological and biochemical studies have defined the rate and magnitude of exchanger-mediated fluxes, molecular studies of the Na^+-Ca^{2+} exchanger have lagged because of the relatively low abundance and lability of the exchanger. We have previously correlated sarcolemmal Na^+-Ca^{2+} exchange activity with 70-, 120-, and 160-kD proteins (*3*). Here we report the molecular cloning, expression, deduced amino acid sequence, and apparent molecular size of the canine cardiac sarcolemmal Na^+-Ca^{2+} exchange protein.

We used a polyclonal antibody against a partially purified preparation of the exchanger (*3*) to screen an amplified λgt11 expression library (*4*). A 3.2-kb clone, designated A4, was isolated from the library for further study. Northern blot analysis of polyadenylated [poly(A)$^+$] RNA from dog heart tissue probed with DNA from A4 indicated that the complete exchanger transcript is 7 kb (*5*). Sequence analysis and our inability to express exchange activity in oocytes with RNA synthesized from the A4 clone indicated that this clone did not contain the entire coding region for the exchanger.

To obtain the 5′ end of the coding region of the exchanger clone, we constructed a

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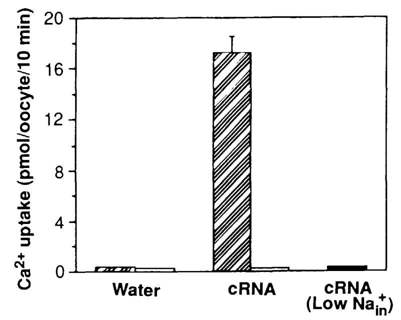

Fig. 1. Expression of Na^+-Ca^{2+} exchange activity in *Xenopus laevis* oocytes. Oocytes were dissected, injected, and assayed as described (*8*). The oocyte intracellular Na^+ concentration was elevated by treatment with nystatin in Barth's solution (*20*). Na^+ gradient–dependent uptake of $^{45}Ca^{2+}$ (hatched bars) was measured by placing the oocytes into a K^+ medium [90 mM KCl, 10 μM $CaCl_2$ (6.25 μCi of $^{45}CaCl_2$ per milliliter), 250 μM $MgCl_2$]. The background, Na^+ gradient–independent $^{45}Ca^{2+}$ uptake (white bars) was determined by placing the oocytes into a medium identical to the K^+ medium, but with Na^+ substituted for K^+. A control to show that the induced Ca^{2+} uptake required internal Na^+ (Na^+_{in}) is shown in the right column (black bar) where intracellular Na^+ was not elevated by incubation with nystatin (*8*), and the oocytes were placed into K^+ medium for the Ca^{2+} uptake assay. Oocytes were injected with 50 nl of water or synthetic RNA (cRNA, 50 ng) 6 days prior to the Na^+-Ca^{2+} transport assay. n = 10. Where omitted, SEM bars are less than 8% of Ca^{2+} uptake.

canine heart oligo(dT)-primed cDNA library and screened the unamplified library with DNA probes from the A4 clone (*6*). Several long cDNA clones (about 6 kb) were isolated. By restriction endonuclease analysis, we found that these clones had long 3′ extensions (>2 kb) relative to the A4 clone, although none had poly$(A)^+$ tails. One of these clones, TB11, also had a 5′ extension of 80 base pairs and was used for further analysis.

To confirm that the cDNA clone encoded for the Na^+-Ca^{2+} exchanger, we synthesized RNA (*7*) from TB11 and injected the RNA into *Xenopus laevis* oocytes. Six days later, we measured Na^+ gradient–dependent Ca^{2+} uptake (*8*). The induction of Na^+ gradient–dependent Ca^{2+} uptake into the oocytes is shown in Fig. 1. The expressed Na^+-Ca^{2+} exchange activity required internal Na^+ and the absence of external Na^+, as expected. The magnitude of Ca^{2+} uptake was at least one order of magnitude greater than that observed with oocytes injected with poly$(A)^+$ RNA from cardiac tissue (*8*). An even greater amount of induced uptake might have been expected from the cRNA, but may not have occurred for a variety of reasons. For example, the cRNA does not have a poly$(A)^+$ tail, which may affect stability of the RNA or the efficiency of its translation (*9*). Also, maximal exchange activity may require the presence of associated regulatory proteins. The induction of Na^+-Ca^{2+} exchange activity demonstrates that a single gene product can mediate exchange.

The identity of the polypeptide or polypeptides involved in Na^+-Ca^{2+} exchange in sarcolemmal membranes has been uncertain (*3*). To resolve this question, we raised an antibody (*10*) against a peptide derived from the deduced amino acid sequence of the exchanger (amino acids 680 to 694) (Fig. 2A). As determined by enzyme-linked immunosorbent analysis (ELISA), the antiserum contains an antibody that reacts with the peptide but does not bind to the native exchanger. However, the antiserum does specifically react with denatured sarcolemmal proteins on immunoblots (Fig. 3). The reaction with sarcolemmal proteins (Fig. 3, lanes d and e) is similar to the reaction of the previously described (*3*) antibody to the exchanger (Fig. 3, lane a), which was used to isolate A4. Both antibodies react with exchanger polypeptides of 160, 120, and 70 kD. The reaction of the antiserum with sarcolemmal proteins can be blocked by peptide (Fig. 3, lane c), but not by keyhole limpet hemocyanin (Fig. 3, lane e), which was coupled to the peptide for antibody production. This verifies our previous correlation of Na^+-Ca^{2+} exchange activity with proteins of these apparent molecular weights (*3*). Although the origin of the multiple protein bands is unclear, there are indications that the 70-kD peptide arises from proteolysis of the 120-kD protein (*3*). Differential processing may also account for the multiple protein bands.

The deduced amino acid sequence of the exchanger (Fig. 2A) was obtained from the combined nucleotide sequence of the A4 clone and the 5′ end of the TB11 clone. The first ATG is 26 base pairs from the 5′ end of TB11 in an open reading frame. The nucleotide sequence around this potential start site, CAACATGC, is similar to the Kozak consensus initiation site (*11*). The open reading frame continues for 2910 bases to a TAA stop codon and encodes a protein of 970 amino acids with a molecular size of 108

A

```
                  1      ⇓                            *
  1  MLQLRLLPTF SMGCHLLAVV ALLFSHVDLI SAETEMEGEG NETGECTGSY
                                       2
 51  YCKKGVILPI WEPQDPSFGD KIARATVYFV AMVYMFLGVS IIADRFMSSI
                                  *         *         3
101  EVITSQEKEI TIKKPNGETT KTTVRIWNET VSNLTLMALG SSAPEILLSV
        *                         4
151  IEVCGHNFTA GDLGPSTIVG SAAFNMFIII ALCVYVVPDG ETRKIKHLRV
                  5                            6
201  FFVTAAWSIF AYTWLYIILS VISPGVVEVW EGLLTFFFFP ICVVFAWVAD
251  RRLLFYKYVY KRYRAGKQRG MIIEHEGDRP SSKTEIEMDG KVVNSHVDNF
301  LDGALVLEVD ERDQDDEEAR REMARILKEL KQKHPEKEIE QLIELANYQV
                                               ◆
351  LSQQQKSRAF YRIQATRLMT GAGNILKRHA ADQARKAVSM HEVNTEVAEN
401  DPVSKIFFEQ GTYQCLENCG TVALTIIRRG GDLTNTVFVD FRTEDGTANA
451  GSDYEFTEGT VVFKPGETQK EIRVGIIDDD IFEEDENFLV HLSNVKVSSE
501  ASEDGILEAN HVSALACLGS PSTATVTIFD DDHAGIFTFE EPVTHVSESI
551  GIMEVKVLRT SGARGNVIVP YKTIEGTARG GGEDFEDTCG ELEFQNDEIV
                    *
601  KTISVKVIDD EEYEKNKTFF LEIGEPRLVE MSEKKALLLN ELGGFTITGK
651  YLYGQPVFRK VHAREHPIPS TVITIAEEYD DKQPLTSKEE EERRIAEMGR
701  PILGEHTKLE VIIEESYEFK STVDKLIKKT NLALVVGTNS WREQFIEAIT
                                     7
751  VSAGEDDDDD ECGEEKLPSC FDYVMHFLTV FWKVLFAFVP PTEYWNGWAC
             8                          9
801  FIVSILMIGI LTAFIGDLAS HFGCTIGLKD SVTAVVFVAL GTSVPDTFAS
                    *                  10
851  KVAATQDQYA DASIGNVTGS NAVNVFLGIG VAWSIAAIYH AANGEQFKVS
                        11                               12
901  PGTLAFSVTL FTIFAFINVG VLLYRRRPEI GGELGGPRTA KLLTSCLFVL
951  LWLLYIFFSS LEAYCHIKGF
```

B

	212																					234
NaCaX	Y	T	W	L	Y	I	I	L	S	V	I	S	P	G	V	V	E	V	W	E	G	L	L
NaK	Y	T	W	L	E	A	V	I	F	L	I	G	I	I	V	A	N	V	P	E	G	L	L
	308																					330

Fig. 2. Amino acid sequence of the Na^+-Ca^{2+} exchanger and similarity to the Na^+,K^+-ATPase. (**A**) Deduced amino acid sequence of the exchanger. The entire A4 clone and the 5′ end of TB11 were sequenced in both directions with the dideoxynucleotide chain termination method (*6, 21*). Double-stranded templates were sequenced as subcloned restriction fragments or unidirectionally deleted clones (Promega Erase-a-Base System). In some cases, oligonucleotides were synthesized for use as specific primers. Potential membrane-spanning segments are double underlined and labeled with numbers according to the model. The first amino acid encoded in clone A4 is indicated with an arrow. Potential N-linked glycosylation sites of the type NXS/T are indicated with asterisks and the potential phosphorylation site with a diamond. Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. The exchanger nucleotide and amino acid sequence data are available through GenBank, accession number M36119. (**B**) Sequence similarity between the exchanger (NaCaX) and the Na^+,K^+-ATPase (NaK) (*18*). Protein data bases were searched by the FastA program of University of Wisconsin Genetics Computer Group (*14*). Identical amino acids are enclosed by boxes.

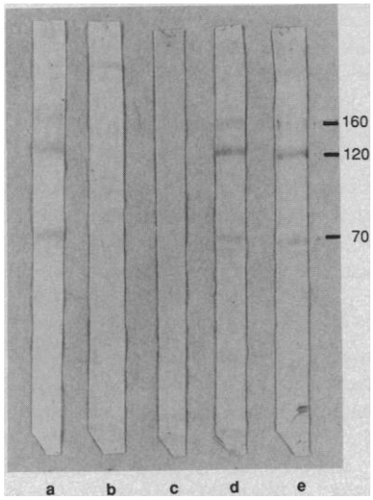

Fig. 3. Immunoblot analysis of sarcolemmal proteins with antibodies to the synthetic peptide. SDS–polyacrylamide gel electrophoresis, blotting, and antibody reactions with horseradish peroxidase and diaminobenzidine were performed as described (*3*). Each lane contained about 10 μg of canine cardiac sarcolemmal protein. Nitrocellulose strips were reacted with (lane a) a previously described (3) polyclonal antibody to the exchanger proteins (1:3000 dilution); (lane b) pre-immune serum from the rabbit used to produce the antibodies to the peptide (1:200 dilution); and (lanes c, d, and e) antiserum (1:200 dilution) to the synthetic peptide. Lane c also contained free peptide (0.7 mg/ml) to compete with the antibody, and lane 3 contained keyhole limpet hemocyanin (0.2 mg/ml) in addition to antibody.

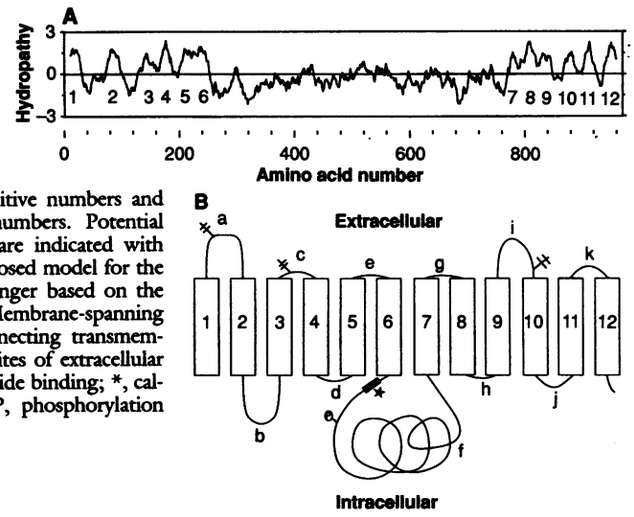

Fig. 4. Hydropathy plot and model for the Na^+-Ca^{2+} exchanger. (**A**) Hydropathy plot for the exchanger determined by the method of Kyte and Doolittle (*22*) with a window of 20 amino acids. On the ordinate, hydrophobicity is indicated by positive numbers and hydrophilicity by negative numbers. Potential membrane-spanning regions are indicated with numbers 1 to 12. (**B**) A proposed model for the sarcolemmal Na^+-Ca^{2+} exchanger based on the hydropathy plot. 1 to 12, Membrane-spanning segments; a to k, loops connecting transmembrane segments. ‡, Possible sites of extracellular asparagine-linked oligosaccharide binding; *, calmodulin binding site; and P, phosphorylation site.

kD. The native exchanger protein has a maximal apparent molecular size of about 160 kD (Fig. 3). The reason for this discrepancy in unknown, though the exchanger may be heavily glycosylated. There are six potential sites for N-linked glycosylation of the exchanger (Fig. 2A).

The cardiac exchanger is regulated by adenosine triphosphate (ATP), although phosphorylation has not been implicated (*12*). The most likely phosphorylation site, RKAVS (amino acids 385 to 389), could be a substrate for either a calmodulin-dependent kinase or an adenosine 3′,5′-monophosphate (cAMP)–dependent kinase (*13*).

Comparison of the sequence of the Na^+-Ca^{2+} exchanger with other proteins in the available databases indicates that the exchanger contains almost no sequence similarity to any other protein. However, a region of 23 amino acids with 48% identity to the α subunit of the Na^+- and K^+-dependent adenosine triphosphatase (Na^+,K^+-ATPase) received the highest similarity score (*14*) (Fig. 2B). This region may perform a similar structural or functional role in the two proteins.

A hydropathy map for the exchanger protein is shown in Fig. 4A. The protein can be divided into three regions: a hydrophobic NH_2-terminal portion containing six potential membrane-spanning segments, a long hydrophilic region, and a hydrophobic COOH-terminal portion containing six potential membrane-spanning segments. Division into long hydrophobic and hydrophilic regions appears to be a common feature of ATP-dependent cation pumps (*15*) and ion exchangers (*16*). The overall similarity of the hydropathy plots of the Na^+-Ca^{2+} exchanger and the ATP-dependent cation pumps (*15*) is notable. Some of the proposed transmembrane segments have polar residues at regular intervals of three to four amino acids. For example, segments three and five both have six hydroxyl-containing or acidic residues, all of which would be on the same surface of an amphipathic helix. In conjunction with other amphipathic helices, these surfaces may form portions of ion translocation pathways. The first hydrophobic segment could be a cleavable NH_2-terminal signal sequence. The consensus cleavage site would be between residues 32 and 33 (*17*).

By analogy to models of other transporters (*15, 16*), we place the long hydrophilic region (520 amino acids, residues 250 to 769, loop f) on the cytoplasmic side of the membrane and propose a secondary structure model (Fig. 4B). The region of similarity between the Na^+,K^+-ATPase and the Na^+-Ca^{2+} exchanger (Fig. 3, lane b) occurs at homologous positions in the two proteins (*18*). The region of similarity is in transmembrane segments immediately NH_2-terminal to a long cytoplasmic loop. By this model, three of the potential N-linked glycosylation sites are on the extracellular surface, and the potential phosphorylation site is on the intracellular surface. The other three potential glycosylation sites are in putative cytoplasmic loops b and f.

Overall, the exchanger is a very acidic protein, with 137 acidic and only 108 basic residues (including histidines). Most of the disparity between numbers of acidic and basic residues is in cytoplasmic loop f, which contains 103 (20%) acidic and only 75 (14%) basic amino acids. The acidic amino acids are spread throughout cytoplasmic loop f, although there are regions very enriched in acidic residues (for example, positions 755 to 765) that may be involved in cation binding. In contrast, the basic residues in cytoplasmic loop f tend to be clustered primarily at the NH_2-terminal and COOH-terminal ends of the loop. The clustering of basic residues may also have some functional significance. For example, the basic region at residues 251 to 270 is a calmodulin binding domain (*19*). The model for the exchanger should provide a basis for future structure and function studies of the exchanger.

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Voltage-Independent Calcium Release in Heart Muscle

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The Ca^{2+} that activates contraction in heart muscle is regulated as in skeletal muscle by processes that depend on voltage and intracellular Ca^{2+} and involve a positive feedback system. How the initial electrical signal is amplified in heart muscle has remained controversial, however. Analogous protein structures from skeletal muscle and heart muscle have been identified physiologically and sequenced; these include the Ca^{2+} channel of the sarcolemma and the Ca^{2+} release channel of the sarcoplasmic reticulum. Although the parallels found in cardiac and skeletal muscles have provoked valuable experiments in both tissues, separation of the effects of voltage and intracellular Ca^{2+} on sarcoplasmic reticulum Ca^{2+} release in heart muscle has been imperfect. With the use of caged Ca^{2+} and flash photolysis in voltage-clamped heart myocytes, effects of membrane potential in heart muscle cells on Ca^{2+} release from intracellular stores have been studied. Unlike the response in skeletal muscle, voltage across the sarcolemma of heart muscle does not affect the release of Ca^{2+} from the sarcoplasmic reticulum, suggesting that other regulatory processes are needed to control Ca^{2+}-induced Ca^{2+} release.

THE HYPOTHESIS THAT THE EFFECTS of a small amount of trigger Ca^{2+} could be amplified to provide the elevated intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) needed to activate contraction was first proposed to explain excitation-contraction (EC) coupling in skeletal muscle (*1*), although this concept of "Ca^{2+}-induced Ca^{2+} release" (CICR) is now broadly applied to excitable and nonexcitable cells (*2*–*7*). Even though CICR has a limited role in EC coupling of skeletal muscle (*8*), CICR may modulate EC coupling in skeletal muscle under specific conditions (*9*). CICR has been demonstrated in skinned heart muscle cells (*2*) and may be responsible for amplifying the effects of the Ca^{2+} influx through L-type Ca^{2+} channels in intact heart cells. Experiments in intact isolated heart muscle cells also show that their CICR can be directly activated by a photochemically produced step increase in $[Ca^{2+}]_i$ (*10*). However, it is not known if sarcolemmal membrane potential can influence CICR or directly modulate EC coupling in heart, although several reports (*11, 12*) provide support for CICR in heart muscle: when the Ca^{2+} current (I_{Ca}) is reduced by depolarizations to the electrochemical potential of Ca^{2+} (E_{Ca}), there is no Ca^{2+} release. This is in clear contrast to findings in skeletal muscle where similar depolarizations do not result in reduction in the voltage-activated $[Ca^{2+}]_i$ transient (*13*).

One difficulty with the concept of CICR arises from the potentially large positive feedback (gain) inherent in this process. Because the CICR produces its own trigger signal as the output signal, this system would not release Ca^{2+} in a graded way, unless its gain were low. However, the experimentally observed gain of this positive feedback system was variable depending on the conditions, suggesting that the gain may be regulated (*14*). Furthermore, in heart muscle, $[Ca^{2+}]_i$ transients can be abbreviated by early repolarization (*11*), a finding not expected from CICR with a high gain. Examination of I_{Ca} showed that the flux of Ca^{2+} itself was important in triggering CICR, since replacing outside Ca^{2+}, $Ca^{2+}{}_o$, with other ions that can permeate the Ca^{2+} channels prevented CICR, as did other procedures that blocked Ca^{2+} flux through the Ca^{2+} channels (*15*). From these findings, it was concluded that the voltage dependence of Ca^{2+} release was mediated entirely by Ca^{2+} flux through Ca^{2+} channels and thus by the voltage dependence of these channels. However, additional voltage-dependent mechanisms may be involved. The voltage-dependent sodium current (I_{Na}) and the intracellular Na^+ concentration $[(Na^+]_i)$–dependent and voltage-dependent Na^+-Ca^{2+} exchanger can trigger CICR (*16*), again raising the question whether voltage itself can influence the gain of CICR in heart muscle. In order to address this point experimentally, it is necessary to activate CICR and control voltage independently. We have achieved this by activating CICR using photorelease of caged Ca^{2+} (*17*) while independently controlling membrane potential by using a patch-clamp method in the whole-cell mode.

In the absence of photorelease of Ca^{2+}, depolarization of the sarcolemmal membrane activated contraction in the normal manner (Fig. 1A), and repolarization of the cell membrane produced a small aftercontraction that reflects the I_{Ca} tail current. A twitch contraction was triggered at a holding potential of −40 mV in the same cell by photorelease of Ca^{2+} with a 230–W-s discharge through the flashlamp (Fig. 1B). The photochemically induced contraction, although similar to the contraction induced by depolarization, exhibited some expected

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