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Sodium-Calcium Exchange in Heart: Membrane Currents and Changes in $[Ca^{2+}]_i$

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Recordings have been made of changes in intracellular calcium ion concentration ($[Ca^{2+}]_i$) that can be attributed to the operation of an electrogenic, voltage-dependent sodium-calcium (Na-Ca) exchanger in mammalian heart cells. Guinea pig ventricular myocytes under voltage clamp were perfused internally with fura-2, a fluorescent Ca^{2+} -indicator, and changes in $[Ca^{2+}]_i$ and membrane current that resulted from Na-Ca exchange were identified through the use of various organic channel blockers and impermeant ions. Depolarization of cells elicited slow increases in $[Ca^{2+}]_i$, with the maximum increase depending on internal $[Na^+]$, external $[Ca^{2+}]$, and membrane voltage. Repolarization was associated with net Ca^{2+} efflux and a decline in the inward current that developed instantaneously upon repolarization. The relation between $[Ca^{2+}]_i$ and current was linear, and the slope was made steeper by hyperpolarization.

THE EXISTENCE OF A SODIUM-CALCIUM (Na-Ca) exchanger has been established in many tissues (1), including intact heart tissue (2) and cardiac sarcolemmal vesicles (3). Experiments on single isolated heart cells from frog atria (4) and guinea pig ventricles (5) revealed currents that have been attributed to Na-Ca exchange. The role of Na-Ca exchange in the arrhythmogenic currents that accompany spontaneous fluctuations in intracellular calcium ion concentration ($[Ca^{2+}]_i$) (6, 7) has been shown (8), but $[Ca^{2+}]_i$ was not measured or controlled adequately in these studies. When recording the Ca^{2+} -activated fluorescence of fura-2 (9) in cardiac cells under voltage clamp (10), we observed that changes in fluorescence could be detected under conditions in which changes in $[Ca^{2+}]_i$ might be attributable to Na-Ca exchange. We now report (11) simultaneous measurements of changes in $[Ca^{2+}]_i$ and membrane current (4, 5), which we suggest are probably due to Na-Ca exchange.

The methods for voltage clamp preparation of single cells and for the recording

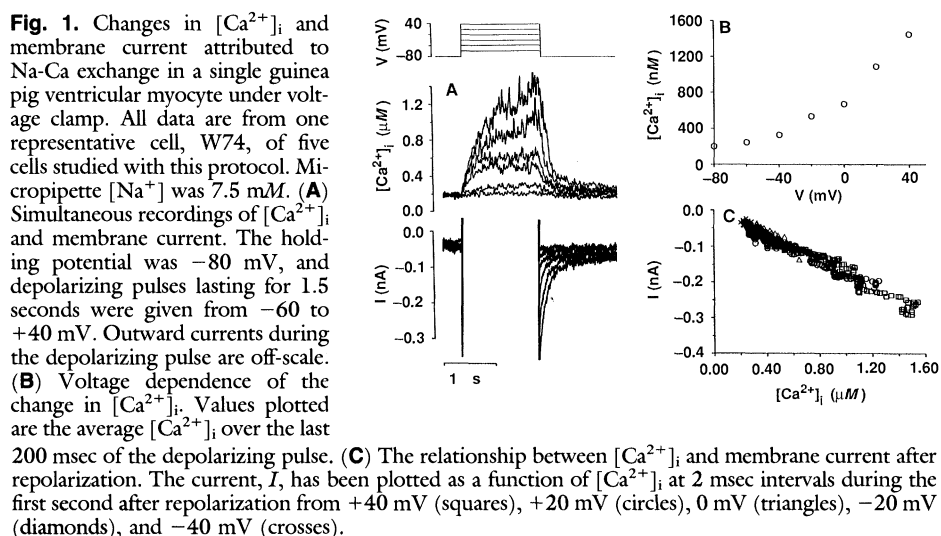
of fura-2 fluorescence were similar to those described (7, 10). The cells were internally perfused over 5 to 15 minutes with a micropipette electrode (1.8 to 3.5 MOhm) filled with 0.070 mM fura-2 (Molecular Probes), 130 mM CsCl, 2 mM $MgCl_2$, 2 mM adenosine 5'-triphosphate (sodium salt), 0 to 10 mM NaCl, and 10 mM Hepes (potassium or sodium salt) (pH 7.2). (The $[Na^+]$ in the

micropipette is indicated.) The cells were superfused at room temperature (23°C) with a physiological saline solution containing 2.5 mM $CaCl_2$, 135 mM NaCl, 10 mM CsCl, 1 mM $MgCl_2$, 0.33 mM NaH_2PO_4 , 10 mM dextrose, 10 mM Hepes, 10 mM tetraethylammonium (TEA), 10 μM verapamil, and 10 μM ryanodine; pH was 7.3 with the addition of NaOH. Interfering potassium (K^+) currents were blocked by substituting impermeant ions (Cs^+ and TEA). Verapamil and ryanodine (12) blocked the interfering changes in $[Ca^{2+}]_i$ that result from entry of Ca^{2+} through surface membrane Ca^{2+} channels (13) or from release of Ca^{2+} from the sarcoplasmic reticulum (SR), respectively.

From a holding potential of -80 mV, depolarization for 1.5 seconds was associated with a slow increase in $[Ca^{2+}]_i$ (Fig. 1). As the membrane was depolarized more, the change in $[Ca^{2+}]_i$ became larger and faster. The $[Ca^{2+}]_i$ at the end of the depolarizing pulse increased monotonically with clamp-pulse potential (Fig. 1B). In Ca^{2+} -free solution, these changes did not occur. This is evidence for a voltage-dependent process that controls $[Ca^{2+}]_i$ in cells in which Ca^{2+} entry through channels and Ca^{2+} release from SR are blocked.

Repolarization to -80 mV was associated with the instantaneous development of a large inward current and the initiation of the decline in $[Ca^{2+}]_i$. Throughout the decline in $[Ca^{2+}]_i$, the current was directly proportional to $[Ca^{2+}]_i$ (Fig. 1C). At -80 mV, all ordered pairs ($[Ca^{2+}]_i$, current) fall on the same line independent of time or previous history of depolarization.

In the working scheme of electrogenic Na-Ca exchange (14), which we will use here, the exchange current (Eq. 3) is proportional to the difference between Ca^{2+} influx and efflux, where each unidirectional



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flux (Eqs. 1 and 2) is proportional to the product of a voltage-dependent rate constant and the concentration of the exchanger (E) bound at both inner (i) and outer (o) ion-binding sites to either three Na^+ or one Ca^{2+} (15).

$$\text{Ca}^{2+} \text{ efflux (forward mode)} = k \times \exp[-VF(1-r)/RT] \times [\text{ENa}_3\text{Ca}^1] \quad (1)$$

$$\text{Ca}^{2+} \text{ influx (reverse mode)} = k \times \exp(VFr/RT) \times [\text{ENa}_3\text{Ca}^0] \quad (2)$$

$$I_{\text{Na-Ca}} = k_{\text{Na-Ca}} \times (\text{Ca}^{2+} \text{ influx} - \text{Ca}^{2+} \text{ efflux}) \quad (3)$$

where k is the rate constant of exchanger translocation at $V = 0$; r is the partition parameter, according to rate theory; V is the membrane potential; R is the gas constant; T is the absolute temperature (in degrees Kelvin); F is the Faraday constant; and $k_{\text{Na-Ca}}$ is a proportionality factor comprised of the rate constants for ion binding. According to this scheme for Na-Ca exchange, changes in Na-Ca exchange current at constant membrane potential and internal and external $[\text{Na}^+]$ can only be produced by one of the partial reactions of Na-Ca exchange:

Na^+_{o} -dependent Ca^{2+} efflux, the forward mode (Eq. 1). Since $[\text{Ca}^{2+}]_{\text{o}}$ is constant and $[\text{Na}^+]_{\text{i}}$ is assumed to be constant, there can be no changes in exchange current resulting from the other partial reaction, Na^+_{i} -dependent Ca^{2+} influx, the reverse mode (Eq. 2). Thus, the $[\text{Ca}^{2+}]_{\text{i}}$ -dependent current accompanying Ca^{2+} efflux (Fig. 1C) may be attributable to the forward mode of Na-Ca exchange.

A fundamental feature of a Na-Ca exchanger, regardless of the specific details of its operation, is that an elevation of $[\text{Na}^+]_{\text{i}}$ will promote the reverse mode, exchange of Na^+_{i} for $\text{Ca}^{2+}_{\text{o}}$ (1). To increase $[\text{Na}^+]_{\text{i}}$, we exposed cells to the Na-ionophore monensin (4) at a concentration of $30 \mu\text{M}$ for 15 minutes. The changes in $[\text{Ca}^{2+}]_{\text{i}}$ elicited by depolarization in the presence of monensin were increased, particularly at the more positive membrane potentials (Fig. 2B). The relationship between $[\text{Ca}^{2+}]_{\text{i}}$ and current was not changed (Fig. 2C).

These experiments provide evidence that the process controlling $[\text{Ca}^{2+}]_{\text{i}}$ is both voltage-dependent and electrogenic, which is of interest to determine the molecular mechanism of the exchanger (16). According to

our scheme, the efflux of Ca^{2+} produces an inward (negative) current, which will be favored energetically by hyperpolarization and, thus, be voltage-dependent (17). We have examined this issue quantitatively in experiments in which the voltage clamp protocol was designed to reveal the effect of membrane potential on the relationship between $[\text{Ca}^{2+}]_{\text{i}}$ and current during Ca^{2+} efflux (Fig. 3A). The slope of the linear relation between $[\text{Ca}^{2+}]_{\text{i}}$ and current varied with membrane voltage (Figs. 3, B and C).

The possibility that a significant fraction of the current that is closely related to $[\text{Ca}^{2+}]_{\text{i}}$ is not produced by Na-Ca exchange but is $[\text{Ca}^{2+}]_{\text{i}}$ -activated background current (5) is unlikely for the following reasons: (i) In 14 of 16 experiments, the current activated by depolarizing pulses was essentially flat or decreased with time (Fig. 3A), as expected for a Na-Ca exchange current; only in two experiments did the current increase (Fig. 2A), as might be expected for Ca^{2+} -activated K^+ (18) or nonspecific cation current (19). (ii) Currents through single Ca^{2+} -activated channels in cultured atrial cells (8) are large enough that they would have been detected as noise on the macroscopic current record. (iii) Neither the voltage dependence of the relationship between $[\text{Ca}^{2+}]_{\text{i}}$ and current (Figs. 3, B and C) nor the absence of an effect of Na^+ loading on this relationship (Fig. 2C) is consistent with a Ca^{2+} -activated, ohmic, nonspecific cation current reversing between -30 mV and 0 mV (5, 19). (iv) The existence of Ca^{2+} -activated nonspecific cation channels (19) in adult guinea pig ventricular heart has never been proved. We conclude that under our experimental conditions, a majority of the current is carried by Na-Ca exchange and that Na-Ca exchange is electrogenic and voltage-dependent.

Experimentally, the relationship between $[\text{Ca}^{2+}]_{\text{i}}$ and changes in membrane current was approximately linear over the range of $[\text{Ca}^{2+}]_{\text{i}}$ examined (100 to 1000 nM). An approximately linear relationship would be observed if $[\text{Ca}^{2+}]_{\text{i}}$ was lower than the dissociation constant (K_D) for the internal Ca^{2+} -binding site; in cardiac sarcolemmal vesicle preparations values of K_D range from 15 to $22 \mu\text{M}$ (3).

Ca^{2+} and Na^+ compete for binding to a site on the exchanger in cardiac sarcolemmal vesicles (3). Inside intact cardiac cells, however, this competition was not evident since elevation of internal Na^+ did not change the linear relationship between $[\text{Ca}^{2+}]_{\text{i}}$ and the current produced by the forward mode (Eq. 1; Fig. 2C). This can be explained if the $[\text{Na}^+]_{\text{i}}$ was well below the K_D at this site. In this case, elevation of $[\text{Na}^+]_{\text{i}}$ would have increased the probability of an exchanger to operate in the reverse mode (Eq. 2). Since

Fig. 2. The effects of loading a cell with Na^+ on changes in $[\text{Ca}^{2+}]_{\text{i}}$ and currents attributed to Na-Ca exchange. (A) Simultaneous recordings of $[\text{Ca}^{2+}]_{\text{i}}$ and membrane current during depolarization to $+30 \text{ mV}$ over 1.5 seconds in the absence and presence (arrow) of monensin (cell T55; micropipette $[\text{Na}^+]_{\text{i}}$, 4 mM). (B) Dependence of $[\text{Ca}^{2+}]_{\text{i}}$, measured at the end of the 1.5-second depolarizing pulse, on pulse voltage in the absence (circles) and presence (triangles) of monensin (cell T55). (C) Relationship between $[\text{Ca}^{2+}]_{\text{i}}$ and membrane current after repolarization to -80 mV in the absence (circles) and presence (squares) of monensin (cell T67; micropipette $[\text{Na}^+]_{\text{i}}$, 4 mM). The data were fit by linear regression. The effect of monensin was studied in seven cells with this or a similar protocol.

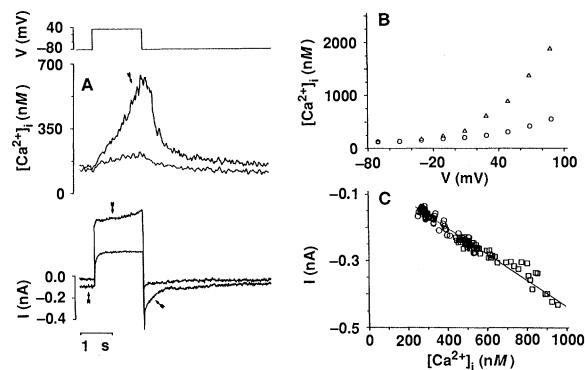
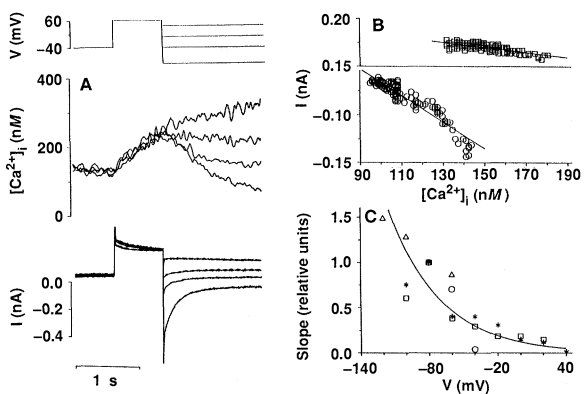


Fig. 3. Voltage dependence of Ca^{2+} efflux and membrane current. (A) Depolarizing pulses to $+60 \text{ mV}$ were followed by repolarization to various voltages (cell H62; micropipette $[\text{Na}^+]_{\text{i}}$, 4 mM). (B) Plots of current as a function of $[\text{Ca}^{2+}]_{\text{i}}$ after repolarization to 0 mV (upper) and to -100 mV (lower). The slopes of the two lines, fit by regression analysis, are -260 nA per mM and -1380 nA per mM , respectively (cell H71; micropipette $[\text{Na}^+]_{\text{i}}$, 4 mM). (C) Normalized slopes from four cells plotted as a function of voltage (slopes were normalized in each cell by dividing the slope at each voltage by that at -80 mV). The equation of the best fitting exponential function, obtained by linear regression after logarithmic transformation of the data, is: $\text{slope} = \exp[-VF(1-r)/RT]$ (14), with the partition parameter, r , equal to 0.45 (90% confidence interval for r ; $0.31 < r < 0.59$).



reverse mode is favored by depolarization (Eq. 2), this would have resulted as observed, in a much larger Ca^{2+} influx (unidirectional) during depolarizing pulses.

The working scheme (14) predicts that the slope of the linear relationship between $[\text{Ca}^{2+}]_i$ and current will depend exponentially on voltage as a result of translocation of one positive charge with each ion exchange reaction. The exponential equation that best fits the data (Fig. 3C) indicates a partition parameter r of 0.45, which is similar to the value of 0.35 obtained by measuring putative Na-Ca exchange currents in these cells (5).

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Structure of a Psoralen Cross-Linked DNA in Solution by Nuclear Magnetic Resonance

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One- and two-dimensional nuclear magnetic resonance (NMR) methods were used to determine a three-dimensional model of an eight-base-pair DNA fragment (d-GGGTACCC) cross-linked with psoralen in solution. Two-dimensional nuclear Overhauser effect experiments were used to assign the spectrum and estimate distances for 171 proton pairs in the cross-linked DNA. The NMR-derived model shows a 53° bend into the major groove that occurs primarily at the site of drug addition and a 56° unwinding that spans the eight-base-pair duplex.

PSORALENS (FIG. 1A) ARE A CLASS OF furocoumarins (1) that are used for treatment of psoriasis and other skin disorders (2), and have also been used as probes of nucleic acid structure and function because they covalently cross-link nucleic acids between opposing strands of duplex regions of DNA (3). A three-step mechanism of psoralen cross-linking with DNA has been proposed (4). The planar psoralen first intercalates into a double helical region, and ultraviolet (UV, 320 to 400 nm) irradiation initially induces a single cyclobutane addition with a pyrimidine base (Fig. 1B). Photochemistry can occur on either the furan (f) or pyrone (p) side. The furan-side monoadduct still absorbs in this wavelength region, so that a second photoaddition can occur with the pyrone side to form the cross-link (Fig. 1C). Thymine is the preferred site for monoadduct formation (5), and psoralen cross-linking occurs at 5'-TpA-3' sites in DNA (6, 7). Psoralen addition products are primarily in the cis-syn conformation (5, 8), as confirmed by the x-ray structure of a 8-methoxypsoralen monoadduct [8MOP-thymine (9)] and by the NMR analysis of the thymine-psoralen-thymine diadduct (6). Physical models based on the monoadduct structure led to the proposal that psoralen cross-links cause a sharp kink in the double helical DNA structure and unwinding of the duplex (10, 11).

We have used one- and two-dimensional nuclear magnetic resonance (2D NMR) methods, including through-space nuclear Overhauser effect spectroscopy (NOESY), to analyze the solution structure of a self-

complementary DNA octanucleotide d-GGGTACCC that has a 5'-TpA site cross-linked with psoralen. Distance information was obtained by peak integration of NOESY cross peaks. Both model-based refinements and distance geometry methods were then used with the NMR data to generate a solution structure of cross-linked DNA.

A stepwise monomer-addition phosphoramidite method was used to synthesize d-

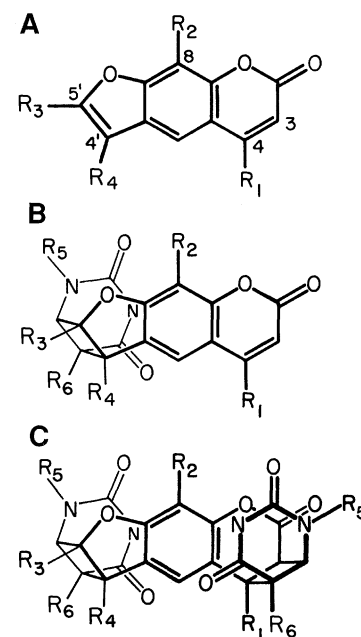


Fig. 1. (A) Generalized formula for psoralen; in AMT, R1, R2, and R3 are methyl groups and R4 is CH_2NH_2 ; in 8-methoxypsoralen (8MOP), R1, R3, and R4 are hydrogens and R2 is OCH_3 . (B) One of the observed psoralen-thymine monoadduct structures in cis-syn conformation; R5 is deoxyribose sugar and R6 is methyl group of thymine. (C) Cross-link of psoralen with thymine; each adduct has the cis-syn configuration.

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