Bimodal adrenergic regulation of Na^+-Ca^{2+} exchanger in Ca^{2+}-buffered native ventricular myocytes from the spiny dogfish shark (Squalus acanthias)

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The Na^+-Ca^{2+} exchanger (NCX) is the dominant Ca^{2+}-efflux mechanism in mammalian heart. In non-mammalian vertebrate hearts (shark, frog), where intracellular Ca^{2+}-release pools are vestigial, NCX appears to also contribute significantly to Ca^{2+}-influx and has unique β-adrenergic regulation. We have reported that while in frog NCX is uniformly suppressed by adrenergic regulation, in shark there is differential regulation of Ca^{2+}-efflux and influx pathways ('bimodal regulation'[4]). Specifically, we found that outward current, I_{NaCa}, generated by NCX in the Ca^{2+}-influx mode was strongly suppressed compared to inward I_{NaCa}. These findings were obtained in native voltage-clamped freshly dissociated shark ventricular cardiomyocytes dialyzed with an internal solution containing minimal Ca^{2+}-buffers (0.2 mM EGTA). Consequently, during voltage-clamp pulses lasting 30-200 ms and exposure to adrenergic agents (5 μM isoproterenol), we observed considerable changes (~30 mV) in the reversal potential of I_{NaCa} ($E_{NaCa}= 3E_{Na} - 2E_{Ca}$) indicative of changes in $[Ca^{2+}]_i$. These effects were carefully considered in our data analysis, but they do call for additional experiments where $[Ca^{2+}]_i$ is either highly buffered or measured directly.

Here we pursued this strategy and obtained evidence that 5 μM epinephrine, while suppressing Ca^{2+}-influx via NCX in voltage-clamped Ca^{2+}-buffered shark cardiomyocytes, in some cells may actually enhances Ca^{2+}-efflux. Fluorometric measurements of $[Ca^{2+}]_i$ in non-dialyzed cells depolarized with K^+-rich solutions produced less clear-cut results on adrenergic regulation, but provide guidelines for future efforts in this direction. In a companion article in this volume of the MDIBL Bulletin we report the completion of the sequencing of shark NCX and the functional expression of the entire molecule and a truncated mutant in mammalian cells where preliminary evidence was obtained for adrenergic regulation.

Hearts were excised from dogfish sharks following institutional and national guidelines. Single ventricular cardiomyocytes were dispersed by enzymatic digestion as previously described (2,4) and used within 2-8 hours.

In one set of experiments we measured the effect of 5 μM epinephrine on I_{NaCa} in single voltage-clamped cells dialyzed with an internal solution containing: 200 mM KCl, 50 mM NaCl, 300 mM urea, 10 mM Hepes (titrated to pH 7.2 with KOH), 5 mM Na_2ATP, 5 mM $MgCl_2$, 10 mM TEA, 10 mM EGTA, and 6 mM $CaCl_2$ yielding an estimated $[Ca^{2+}]_i$ of 200 nM[2]. K^+ currents were suppressed by omission of K^+ from the external solution during voltage clamp procedures (270 mM NaCl, 350 mM urea, 10 mM Hepes (titrated to pH 7.4 with NaOH), 2 mM $MgCl_2$, 0.5 mM NaH_2PO_4, 0.5 mM Na_2SO_4, and 2 mM $CaCl_2$) and by inclusion of TEA in the internal solution. Na^+-current was suppressed by using a relatively depolarized holding potential (-60 mV).

Figure 1 illustrates how a ramp-clamp protocol (panel A, top) was repeated at 10 sec intervals, before and after the exposure to a saturating concentration of epinephrine (5 μM, panel B) and how repeated exposures to 5 mM Ni^{2+} were used to separate I_{NaCa} from residual current components (Cl^-, leak, *etc.*). The graph in the lower part of panel B shows 4 current traces recorded immediately before (a), during (b and c) and after (d) rapid application of 5 mM Ni^{2+}. From these traces we obtained I_{NaCa} as the Ni^{2+}-sensitive membrane current ((a-b-c+d)/2). By relating the current measured at different times to simultaneous values of the membrane potential during the ramp-clamp, we constructed the

current-voltage relation shown as "Control" in panel C. The same procedure was used to measure current voltage relations 2½ min (e f g h in panel B, Epi_1 in panel C) and 4 min (i j k l in panel B, Epi_2 in panel C) after application of epinephrine. The current voltage-relations in panel C show that I_{NaCa} was significantly reduced by epinephrine at + 60 mV, while I_{NaCa} from -120 to -60 mV and E_{NaCa} (-58 → -54 mV) were essentially unchanged. Panel B shows the time course of the membrane currents at the indicated potentials measured at 10 sec intervals. The dip in the "Control" current-voltage relation around 0 mV (panel C) is thought to represent residual Ca^{2+}-current (I_{Ca}), in part because it disappeared in later experiments including 5 µM nifedipine. The quality of recordings was assessed from the stability of a) currents at all potentials prior to application of epinephrine and b) the Ni^{2+}-insensitive currents throughout the experiment.

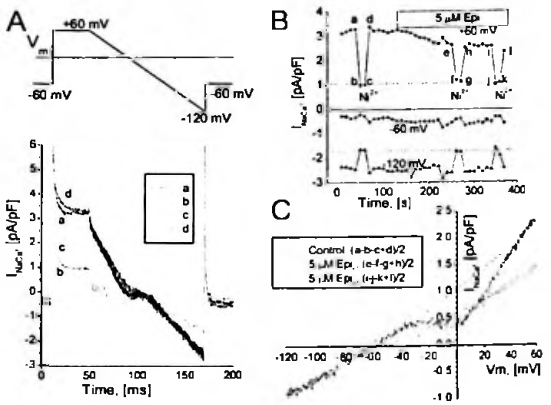


Fig. 1. Epinephrine primarily suppresses NCX in the Ca^{2+}-influx mode. A: Ramp clamp protocol (top) used to measure membrane current before (a) during (b, c) and after (d) application of 5 mM Ni^{2+}. B: Membrane current at +60, -60 (holding potential) and -120 mV recorded at 10s intervals throughout an experiment with 3 brief exposures to 5 mM Ni^{2+} and long lasting exposure to 5 µM epinephrine. C: Current-voltage relations for the Ni^{2+}-sensitive component of the membrane current measured once before ("Control") and twice after application of epinephrine (Epi_1, Epi_2). (Experiment 08/26b)

Figure 2 summarizes results from 7 cells. On average epinephrine did not change I_{NaCa} at -120 ($I_{Epi}/I_{Control}$ = 1.04±0.19 (SEM), n=7). In marked contrast I_{NaCa} at +60 which was nearly cut in half ($I_{Epi}/I_{Control}$ = 0.60±0.04, n=7). E_{NaCa} was not significantly altered (-52±5 → -51±4 mV, n= 9). The enhancement of I_{NaCa} at -120, observed in 2 out of 7 cells, has intriguing physiological implications and might be more prevalent in experiments, were it not for the rundown observed for many current components, including I_{NaCa}.

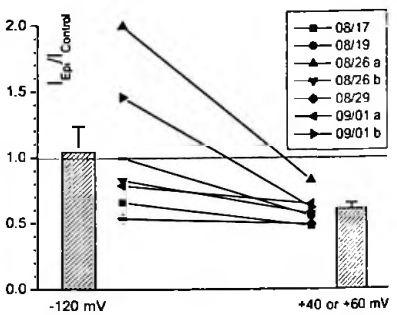


Figure 2. Differential effect of epinephrine on inward and outward I_{NaCa} (measured respectively at -120 mV and at +60 or +40 mV). The histograms show average values of relative current ($I_{Epi}/I_{Control}$). 7 experiments are represented by different symbols and connecting lines.

Figure 3 illustrates one of the experiments where epinephrine clearly enhanced inward I_{NaCa}, but also increased outward I_{NaCa}, thereby lessening the tendency for outward rectification (panel A). The relative change of I_{NaCa} at different potentials (panel B) was found to vary smoothly with the membrane potential (V_m), except near E_{NaCa} where it was poorly defined, and could be approximated by Boltzmann factor (exp{-0.12*FV_m/RT}) with effective charge of 0.12 . This behavior may be interpreted in terms of the sequential model[1], frequently used to describe I_{NaCa} (Fig. 3A, inset). The "graph-method" gives the solution:

$$I_{NaCa} = \frac{\text{[graph]} - \text{[graph]}}{\sum \text{[graph]} + \text{[graph]} + \text{[graph]} + \cdots} \qquad \text{(Eq. 1)}$$

The two terms in the nominator (each the product of 6 rate constants) represent respectively the Ca^{2+}-influx and -efflux modes. Of the 36 terms in the denominator (each the product of 5 rate constants) the significant ones are those defined by the most densely populated states and the highest activation energies. If the denominator has only one significant term, the solution is the difference between two rate constants:

$$I_{NaCa} \cong \text{[graph]} - \text{[graph]} \qquad \text{(Eq. 2)}$$

(each open arrows represent division by an elemental rate constant) that are determined by going around the loop in different direction from the predominant state (lowest energy) to the most difficult transition (highest energy). The observed effect of epinephrine can therefore be seen as modulating the charge movements associated with the NCX molecule by additional 0.12 units moving within the electric field of the membrane.

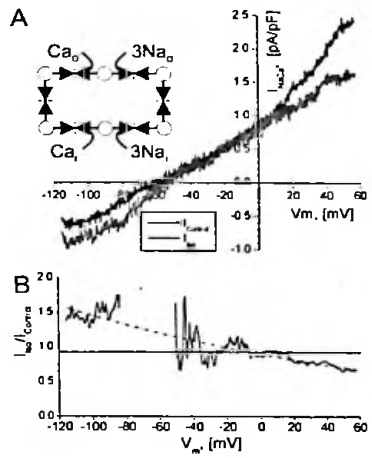


Figure 3. Enhancement of inward I_{NaCa} by epinephrine. A: Current-voltage relations and sequential model (inset). B: Relative change in I_{NaCa} (Experiment 09/01b)

In a second set of experiments the membrane was clamped at a fixed holding potential (-60 mV) while the extracellular Na^+ concentration, $[Na^+]_o$, was either increased or nearly eliminated for 2 sec by rapid superfusion. Figure 4 A shows that these manipulations were effective in activating respectively inward and outward I_{NaCa}. When $[Na^+]_o$ was returned from 10 mM to the control value, we found (in 3 out of 6 cells) very large transient inward currents (>6 pA/pF) suggesting that the Ca^{2+}-influx during a 2 sec pulse of low $[Na^+]_o$ may be sufficient to overpower the added Ca^{2+}-buffers and raise $[Ca^{2+}]_i$. Panel B shows an experiment where epinephrine enhanced inward current activated by elevated $[Na^+]_o$. Additional experiments will be required to detect how this effect compares to the effect of epinephrine on I_{NaCa} activated by low $[Na^+]_o$.

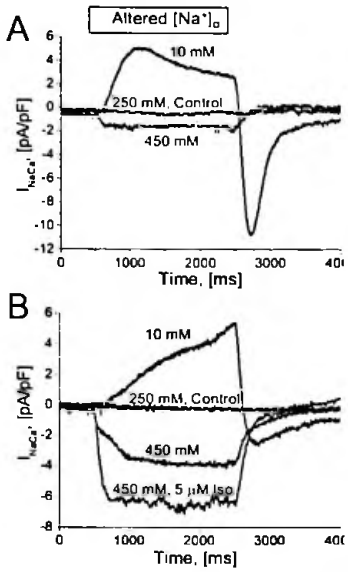
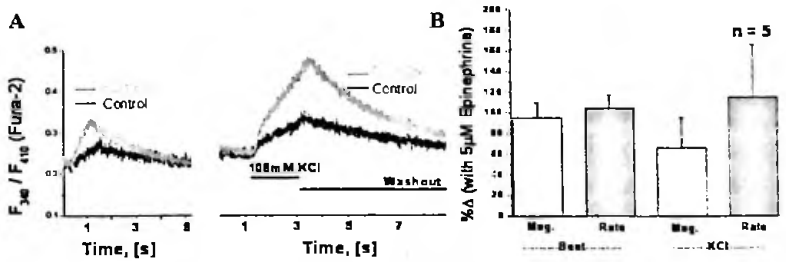


Figure 4. Activation I_{NaCa} by changing $[Na^+]_o$. A: Results from an experiment where the outward I_{NaCa} activated by reducing $[Na^+]_o$ (250 → 10 mM, Cs^+ substitution) was significantly larger than the inward I_{NaCa} activated low by increased $[Na^+]_o$ (250 → 450 mM, urea replacement). B: Effect of Epinephrine on inward I_{NaCa} activated by high $[Na^+]_o$.

Figure 5. Effect of epinephrine on $[Ca^{2+}]$ measured with Fura-2AM epi-fluorescence during field–stimulated action potentials and KCl-depolarications. A: Ratio-metric measurements of Ca^{2+}-dependent fluorescence (Fura-2, F_{340}/F_{410}) during beats and KCl-exposure in the absence and presence of 5 µM epinephrine. B: Epinephrine-induced increase in percent of the magnitude (Mag.) and rate of decay if Ca-transients.



In a third set of experiments, ratiometric epi-fluorescent measurements with Fura-2AM were performed to measure the effects of epinephrine on Ca_i-transients in non-dialyzed cardiomyocytes during field-stimulated action potentials (beats) and 2 sec KCl-depolarizations (Fig. 5A). Epinephrine enhanced the magnitude and rate of the Ca_i-transients by about 100% (Fig. 5). At the onset of field-stimulated or KCl-induced depolarization we observed an initial rapid increase in $[Ca^{2+}]_i$ that is thought to depend directly on I_{Ca}. This was followed by a more gradual rise that may represent both I_{Ca} and Ca^{2+}-influx *via* NCX. On the other hand, the rate of decay (slope) of the Ca^{2+} transients following repolarization, thought to reliably reflect Ca^{2+}-efflux via the exchanger, was significantly enhanced. The observed increases in the rates of decay (shaded columns in panel B) are consistent therefore with enhancement of the Ca^{2+}-efflux mode of NCX by epinephrine.

Using Fluo-3AM-loaded non-dialyzed cardiomyocytes and total internal reflectance fluorescence (TIRF) microscopy, we performed additional measurements of Ca^{2+}-transients evoked by KCl-depolarizations (Fig. 6). These Ca^{2+} transient (panel A) were recorded from fluorescence within areas of cell/glass adhesion (panel B) that remained well defined during contraction (panels C and D). In these experiments we also observed an initial I_{Ca}-mediated rise in $[Ca^{2+}]_i$, but the following slowly rising phase was suppressed by epinephrine (n=3) consistent with suppression of the Ca^{2+}-influx mode of NCX, but the decay, follow washout of KCl, was not noticeably enhanced (panel A).

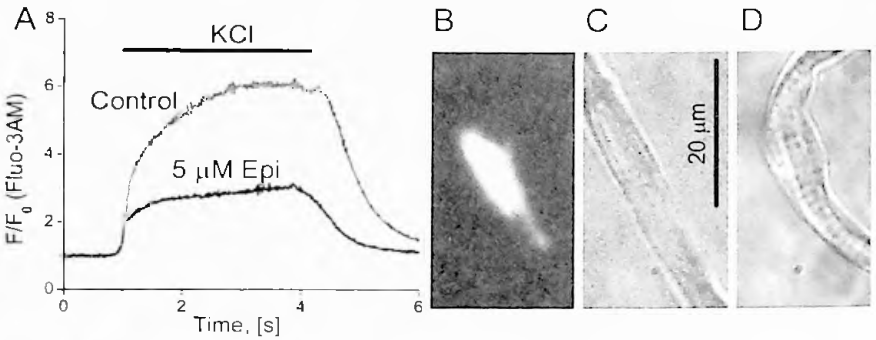


Figure 6. Effect of epinephrine on $[Ca^{2+}]$ measured with Fluo-3 TIRF microscopy during KCl-depolarizations. KCl- evoked Ca_i-transients were measured in the absence (Control) and presence of 5 μM epinephrine. A: Fluorescence from a stable area of adhesion. B and C: Bright field images of central portion of ventricular cardiomyocyte in the absence (B) and presence of flow/contraction.

The two sets of measurements of $[Ca^{2+}]_i$ demonstrate two strategies for high-speed recordings that give consistent results with respect to the initial rapid rise of the Ca_i-transients, but conflicting results with respect to the following slowly rising phase. This discrepancy may partly be related to differences in technique (Iso- vs. hypertonic KCl-solutions, possibly saturation of Ca^{2+} indicator dye, photo- and electrical damage, degree of rundown of I_{Ca} and I_{NaCa}, absence or presence of pacing, etc.) and partly to the variability observed also in the voltage-clamp experiments (Fig. 2).

The present experiments with voltage-clamped cardiomyocytes provide additional support for bimodal adrenergic regulation of shark NCX (Figs. 1, 2, 3). The experiments were conducted in cells with Ca^{2+}-buffered solutions to eliminate or strongly reduce changes in I_{NaCa} that may occur secondarily to changes in $[Ca^{2+}]_i$ which would both alter the driving force for I_{NaCa} ($V_m - E_{NaCa}$) and affect the intracellular regulatory Ca^{2+}-binding sites[3]. The epinephrine-induced enhancement of inward I_{NaCa} observed in some cells (Figs. 2, 3, 4) may represent one of mechanism by which shark cardiomyocytes achieve rapid and complete relaxation when adrenergic stimulation increases the heart rate and shortens the diastolic interval. The finding that epinephrine is more effective in suppressing I_{NaCa} at more positive potentials implies that the outward rectification, typical of vertebrate NCXs, is lessened, possibly due to change in charge movements generated by the NCX molecules during translocations of Na^+ and Ca^{2+}. This explanation may be evaluated by identification of the most densely populated states and the rate limiting transitions of NCX in the absence and presence of epinephrine and raises the question how phosphorylation, supposedly at an intracellular site, might affect movements of charges in regions of the NCX molecule that are exposed to the electric field of the membrane. Supported by RO1 HL 16152 and R21 EB 003474.

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