BIMODAL REGULATION OF Na^+–Ca^{2+} EXCHANGER BY β-ADRENERGIC PATHWAY IN SHARK VENTRICULAR MYOCYTES

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β-Adrenergic agonists are known to potentiate the force of cardiac contraction by enhancing Ca^{2+} current and Ca^{2+} release from the internal calcium store secondary to cAMP-dependent phosphorylation of the Ca^{2+} channel (Reuter, H., Nature 301:569-574, 1983; Callewaert et al., PNAS 85:2009-2013, 1988). Relaxant effect of catecholamines, on the other hand, is mediated by phosphorylation of phospholamban and troponin C resulting in stimulation of Ca^{2+} pump and decreased myofilament Ca^{2+} sensitivity (Sham et al., Am. J. Physiol. 261:H1344-H1349, 1991). Similar to the mammalian myocardium, the shark heart also exhibits some of the features associated with β-agonist response, namely enhancement of I_{Ca} and twitch force and suppression of maintained contracture tension (Maylie & Morad, Am. J. Physiol. 269:H1695-H1703, 1995; Maylie & Morad, MDIBL Bulletin 19:87, 1979). In light of previous reports showing little structural or functional evidence for Ca^{2+} release store in shark heart (Maylie et al., MDIBL Bulletin 19:84-87, 1979), the similarity of β-adrenergic response in shark and mammalian hearts is surprising. In shark heart with rudimentary SR, the Na^+-Ca^{2+} exchanger appears to serve both as a major Ca^{2+} influx and efflux pathway (Näbauer et al., J. Physiol. 457: 627-637, 1992). Here we report on the possible involvement of Na^+–Ca^{2+} exchanger in mediating the β-agonist tension relaxant effect. To probe the mechanism by which β-adrenergic agonists regulate the Na^+–Ca^{2+} exchanger activity we have compared the effects of β-agonist with that of a novel Na^+–Ca^{2+} exchanger blocker (KB-R7943).

Single ventricular cells were isolated from shark heart (*Squalus acanthias*) using previously described procedures (Maylie & Morad, Am. J. Physiol. 269:H1695-H1703, 1995). Briefly, male dogfish (2-7 kg), *Squalus acanthias*, were immobilized by complete spinal pithing. Hearts were removed and mounted in a Langendorff apparatus. The two major coronary vessels were cannulated and perfused with oxygenated Ca^{2+}-free elasmobranch solution containing (in mM) 270 NaCl, 4 KCl, 3 $MgCl_2$, 0.5 KH_2PO_4, 0.5 Na_2SO_4, 350 Urea, 10 HEPES, 0.5 glucose, pH 7.2 at 30° C for 10-15 min. The heart was then perfused for ~15 min with Ca^{2+}-free elasmobranch solution containing 1 mg ml^{-1} collagenase (type A, Boehringer Mannheim) and 0.2 mg ml^{-1} protease (type XIV, Sigma) at a rate of 6 ml min^{-1}. The heart was washed free of enzyme with 0.2 mM Ca^{2+}-containing elasmobranch solution for 10 min. The ventricle was then cut free of the cannula and gently agitated in 0.2 mM Ca^{2+}-containing elasmobranch solution. Dissociated myocytes were placed in a chamber on the stage of an inverted microscope and superfused with 2 mM Ca^{2+}-containing elasmobranch solution. Whole-cell currents were measured using 3-5 MΩ pipette attached to the input of a patch clamp amplifier (Dagan 8900). The pipette solution contained (in mM) 200 KCl, 60 NaCl, 300 Urea, 10 HEPES, 5 Mg-ATP, 10 TEA-Cl, 0.2 EGTA, pH 7.2 with KOH. To record Na^+–Ca^{2+} exchange current (I_{Na-Ca}) the external solutions were supplemented with 10 μM nifedipine to block Ca^{2+} channels and 0.1 mM $BaCl_2$ and 5 mM CsCl to block the inwardly rectifying K^+ channels. 5 mM Ni^{2+} was used in external solution to block I_{Na-Ca}. The experiments were carried out at room temperature and the data were collected, stored and analyzed on a personal computer using pCLAMP 5.5.1 (Axon Instruments) and ORIGIN software.

The ramp pulse protocol of Fig. 1*A*, preceded by short step depolarization to +60 mV to activate I_{Na-Ca}, was used to measure the voltage dependence of I_{Na-Ca} (Fig. 1*C*). The Ni^{2+} blockable I_{Na-Ca} (trace 1–trace 2) showed a reversal potential (E_{rev}) at −29.1 ± 5.7 mV (mean ± SEM, n=9), suggesting apparent E_{Ca} of ~72.5 mV ($[Ca^{2+}]_o$ = 2.0 mM) and an effective $[Ca^{2+}]_i$ of ~7.1 μM after a 40 ms depolarizing pulse to +60 mV ($[Na^+]_i$ = 60 mM; $[Na^+]_o$ = 270 mM). The density of the exchanger current at +60 mV was 5.57 ± 0.91 pA/pF (n=10).

Fig. 1 also shows the effect of 5 μM isoproterenol (ISO) on I_{Na-Ca}. The application of ISO suppressed the magnitude of outward I_{Na-Ca} at steady-state by 20 % and accelerated its inactivation (Fig. 1*A* and *B*). In the absence or presence of ISO, 5 mM Ni^{2+} rapidly and reversibly suppressed the exchanger current to the same extent. The slow kinetics of appearance of ISO-induced effect on I_{Na-Ca} is consistent with the activation of a second messenger-signaling pathway. To examine whether this effect was mediated by the binding of the hormone to the β-adrenoceptor and the activation of the adenylate-cyclase dependent signaling pathway, adenylate-cyclase was directly activated by forskolin. 10 μM forskolin mimicked the ISO-suppressive effect on I_{Na-Ca} (data not shown), which was accompanied by −24.8 ± 1.8 mV (n=4) shift in its E_{rev}. These findings suggest that substantial reduction of Ca^{2+} influx and $[Ca^{2+}]_i$ by ~2.6 fold.

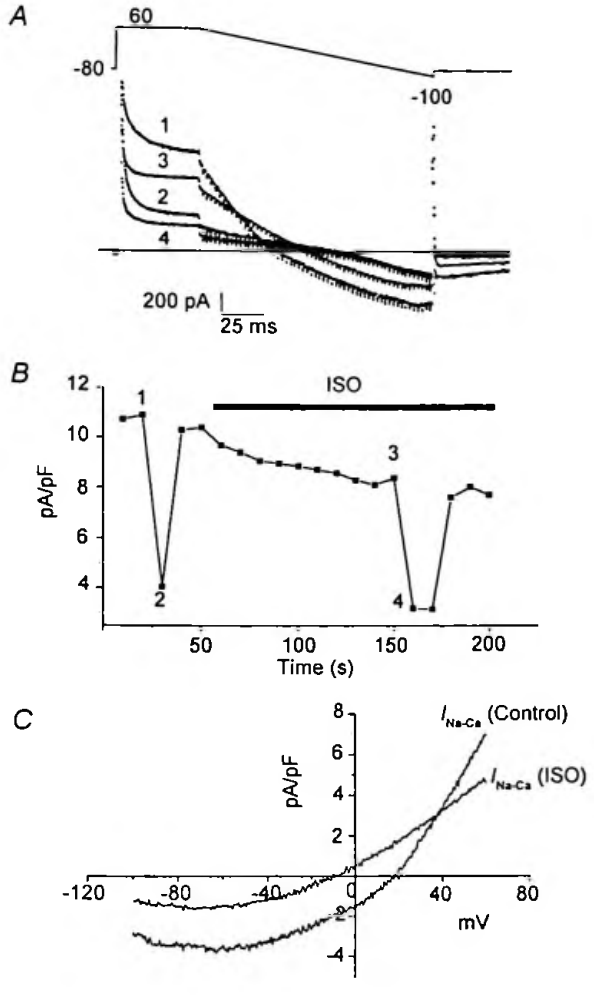
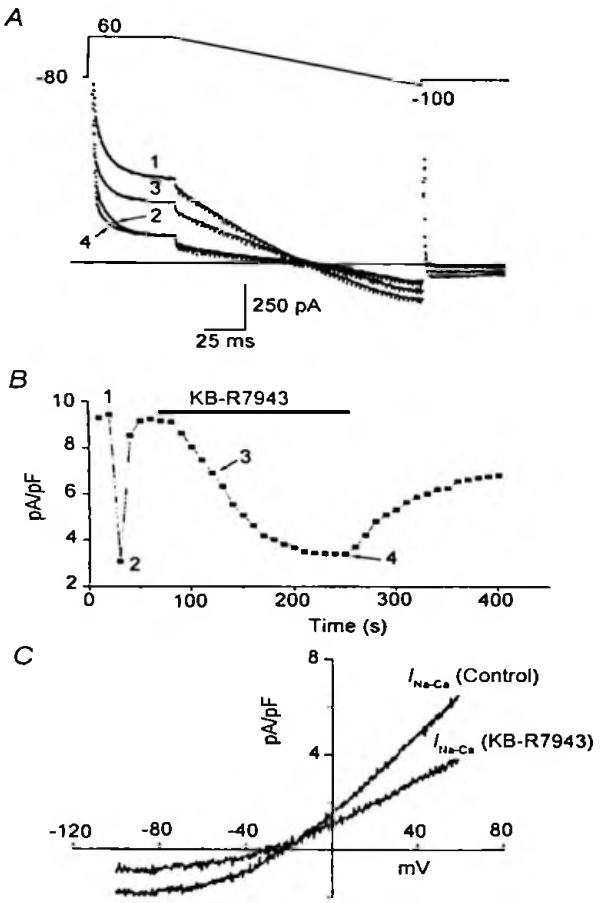


Figure 1. The effect of isoproterenol (ISO) on I_{Na-Ca} in shark ventricular myocyte. *A*, pulse protocol and corresponding currents obtained in control (1), after 5 mM Ni^{2+} (2), in 5 μM ISO (3) and in 5 mM Ni^{2+} and ISO (4). *B*, time course of changes of the steady-state outward current density at +60 mV in response to pulse shown in *A*, delivered at a frequency of 0.1 Hz. *C*, voltage dependencies of I_{Na-Ca} in control (1–2) and ISO (3–4) were constructed from ramp records after subtraction of Ni^{2+}-resistant current shown in *A*.

We compared the effect of ISO with that of KB-R7943 (Iwamoto et al., J. Biol. Chem. 271:22391-22397, 1996). The application of 10 μM KB-R7943 produced gradual suppression of I_{Na-Ca} magnitude, reaching the steady state suppressive effect in 2 min after the application of the drug (Fig. 2*B*). Although the maximal effect of 10 μM KB-R7943 was almost the same as that of application of 5 mM Ni^{2+} the inactivation kinetics of I_{Na-Ca} in the presence of KB-R7943



appeared to be faster than those found in Ni^{2+} (Fig. 2*A*). To compare the effect of KB-R7943 on the E_{rev} of I_{Na-Ca} with that in the presence of ISO, we selected values that had similar level of inhibition (~20 %) (point 3 of Fig. 2*B*) to that in ISO (Fig. 1*B*). KB-R7943-induced inhibition of I_{Na-Ca} was accompanied by an −8 mV shift of I_{Na-Ca} reversal potential (Fig. 2*C*), suggesting a reduction of $[Ca^{2+}]_i$ by ~1.2 fold. Although the level of outward I_{Na-Ca} block by KB-R7943 was almost the same as that induced by ISO, the magnitude of the decrease in the $[Ca^{2+}]_i$ was markedly larger in the presence of ISO. This finding strongly implies that ISO may have additional effects on the exchanger.

Figure 2. The effect of KB-R7943 on I_{Na-Ca}. *A*, ramp voltage-clamp protocol and corresponding original currents obtained in control (1), after 5 mM Ni^{2+} (2), in 10 μM KB-R7943 (3) and in 5 mM Ni^{2+} and KB-R7943 (4). *B*, time course of changes of the steady-state outward current density at +60 mV in response to voltage-clamp pulses shown in *A*, delivered at a frequency of 0.1 Hz. *C*, Current density-voltage relations in control and KB-R7943 were constructed from ramp records after subtraction of Ni^{2+}-resistant current.

To quantitate the efficacy of the β-adrenergic regulatory effect on the Ca^{2+} influx or efflux mode of the exchanger, we used an "envelope" pulse protocol where both the inward and outward component of the exchanger-transported currents could be independently assessed. Fig. 3 illustrates the effect of ISO on the envelope of exchanger-activated currents. In the presence of ISO both inward and outward currents appeared to be reduced (Fig. 3 *A* and *B*). The decrease of Ca^{2+} influx via the Na^+–Ca^{2+} exchanger by ISO may by itself produce a reduction of Ca^{2+} efflux during repolarization. Surprisingly, however, the efflux of Ca^{2+} on the exchanger appeared to be enhanced when corrected for the reduced Ca^{2+} influx. This is reflected in larger ratio of the inward to outward I_{Na-Ca} ($I_{inward}/I_{outward}$) in the presence of ISO compared to control solutions (Fig. 3*C*), suggesting more efficient Ca^{2+} extrusion by the exchanger in the presence of ISO. Consistent with this finding, ISO increased the ratio of charge (Q) carried by Na^+–Ca^{2+} exchanger during the inward tail currents to that during outward current by about 3-4 fold (Fig. 3*D*). We compared the effect of ISO on the ratio I_{Na-Ca} ($I_{inward}/I_{outward}$) with that recorded in the presence of KB-R7943. 10 μM KB-R7943, although suppressing both the inward and outward I_{Na-Ca}, similar to the effect of ISO, it did not alter the ratio of the inward to outward current (data not shown). These findings suggest that the larger shift in E_{rev} in the presence of ISO (Fig. 1*C*) results from enhancement of Ca^{2+} efflux via Na^+–Ca^{2+} exchanger. Thus ISO appears to

differentially regulate the Ca^{2+} efflux and Ca^{2+} influx modes of the exchanger. In frog heart a similar PKA-dependent phosphorylation of Na^+–Ca^{2+} exchanger was found in response to β-adrenergic agonists (Fan et al., PNAS 93:5527-5532, 1996). However, the analysis of Fig. 3 of Fan et al., (1996) does not show a similar bimodal regulation of the exchanger, suggesting that bimodal regulation of the exchanger may be unique to the shark heart.

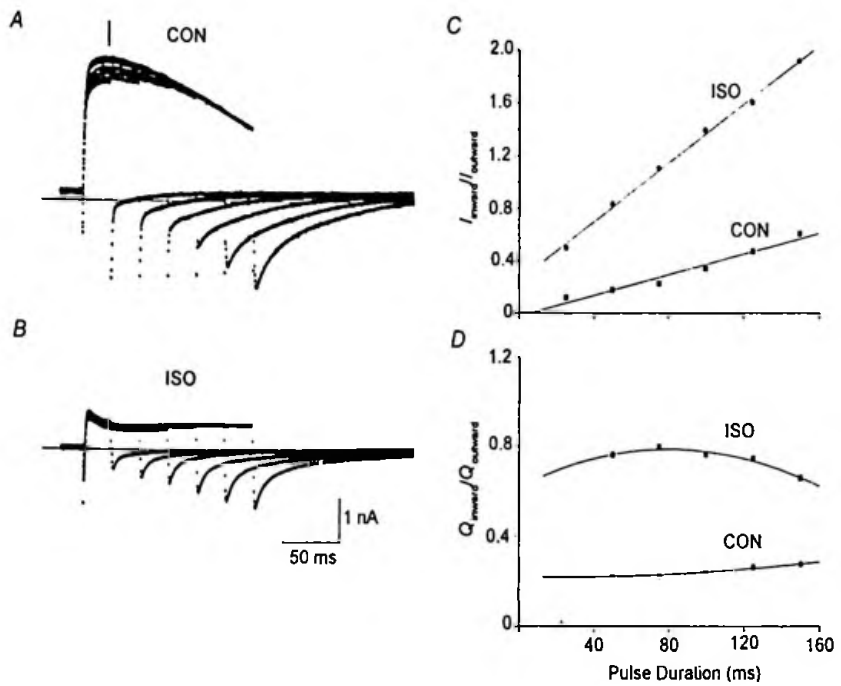


Figure 3. The effect of ISO on the ratio of the inward to outward I_{Na-Ca}. *A* and *B*, superimposed traces of I_{Na-Ca} in control and 5 μM ISO obtained by subtraction of 5 mM Ni^{2+}-resistant component, respectively. Voltage-clamp pulses from –80 to +60 mV were applied in increasing duration from 25 to 150 ms in 25-ms increments. *C*, the relationship between the pulse duration and the ratio of the maximum amplitude of tail current (I_{inward}) to the outward current ($I_{outward}$) measured at the time point marked as a vertical dot-line (in panel A) in control (CON) and ISO. *D*, the relation between the pulse duration and the ratio of the charge (Q) carried via Na^+–Ca^{2+} exchanger during the tail current to that during the depolarizing pulse in CON and ISO.

In quantifying the Ca^{2+} influx and efflux modes of the exchanger, we found that as the duration of the depolarizing pulse gradually increased the ratio of I_{inward} to $I_{outward}$ in control as well as ISO-treated myocytes also increased (Fig. 3*C*). A possible explanation for this observation is that, the higher $[Ca^{2+}]_i$ achieved during the longer depolarizations may further facilitate Ca^{2+} efflux by Na^+–Ca^{2+} exchanger. In the presence of ISO, the slope of the curve, (reflecting the efficacy of Ca^{2+} efflux via the exchanger, Fig. 3*C*) and the ratio ($I_{inward}/I_{outward}$ or $Q_{inward}/Q_{outward}$) were larger than those in control. A possible mechanism for the enhancement of Ca^{2+} efflux by ISO even in the lower $[Ca^{2+}]_i$ is that ISO-induced phosphorylation of the Na^+–Ca^{2+} exchanger increases the affinity of the exchanger for its ionic substrates.

Thus, it appears that ISO not only suppresses the influx of the Ca^{2+} on the exchanger during the action potential to reduce the Ca^{2+} load of the myocyte, but also enhances the Ca^{2+} extrusion mode of the exchanger to facilitate its relaxation. Bimodal regulation of the exchanger by PKA-mediated phosphorylation in parallel with enhancement of Ca^{2+} channel current allows for the shift in the weight of Ca^{2+} influx from a slow, exchanger-mediated, to a faster, Ca^{2+} channel-mediated pathway. This β-adrenergic-induced shift in the mode of Ca^{2+}-influx and the bimodal regulation of the exchanger, in hearts lacking significant SR and Ca^{2+} ATPase, may have evolved to meet the evolutionary demands for faster contraction and accelerated relaxation in animals subjected to fight, fright and flight conditions. (Supported by NIH #16152)