Knockout Mice for Pharmacological Screening Testing the Specificity of Na⁺-Ca²⁺ Exchange Inhibitors

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The role of the Na⁺-Ca²⁺ exchanger as a major determinant of cell Ca²⁺ is well defined in cardiac tissue, and there has been much effort to develop specific inhibitors of the exchanger. We use a novel system to test the specificity of two putative specific inhibitors, KB-R7943 and SEA0400. The drugs are applied to electrically stimulated heart tubes from control mouse embryos or embryos with the Na⁺-Ca²⁺ exchanger knocked out. We monitored effects of the drugs on Ca²⁺ transients. Both drugs depress the Ca²⁺ transients at low concentrations even in the absence of any Na⁺-Ca²⁺ exchanger. KB-R7943 and SEA0400 are not completely specific and should be used with caution as Na⁺-Ca²⁺ exchange inhibitors.

The Na⁺-Ca²⁺ exchanger (NCX) is the major Ca²⁺ efflux mechanism in cardiac muscle.¹,² Mediating cellular Ca²⁺ efflux is a role of special importance in cardiomyocytes because substantial transsarcolemmal Ca²⁺ fluxes occur with each contraction. The Na⁺-Ca²⁺ exchanger also plays a role in inotropic responses and is upregulated in many studies of hypertrophy and heart failure. The exact roles of the Na⁺-Ca²⁺ exchanger are difficult to study because of the multiplicity of Ca²⁺ flux pathways in cardiomyocytes and the lack of specific inhibitors.

In the present study, we investigate the specificity of two recently described Na⁺-Ca²⁺ exchange inhibitors. The first, KB-R7943,³,⁴ inhibits Na⁺-Ca²⁺ exchange activity with an IC₅₀ in the low micromolar range. The inhibitory mechanism of KB-R7943 has been investigated,³,⁵ and amino acids of the exchanger involved in binding KB-R7943 have been identified.⁶

In some studies,³,⁵,⁷ although not all,⁸–¹⁰ the action of KB-R7943 was more potent on the reverse mode (Ca²⁺ influx) than the forward mode (Ca²⁺ efflux) of exchange. Despite this inconsistency, KB-R7943 has been used as a specific inhibitor of reverse-mode Na⁺-Ca²⁺ exchange¹¹,¹² and has implied a role for reverse-mode exchange in arrhythmogenicity.⁷,¹³ Relative potent effects of KB-R7943 on other transporters and channels have been described. KB-R7943 binds to the norepinephrine transporter,¹⁴ blocks or binds to NMDA, K⁺, Na⁺, and Ca²⁺ channels,¹⁴,¹⁵ and blocks the nicotinic acetylcholine receptor.¹⁶

The second drug that we investigate is SEA0400,¹⁴,¹⁷ described as the most potent known inhibitor of Na⁺-Ca²⁺ exchange with an IC₅₀ of ≈20 nmol/L.¹⁴ SEA0400 reduced Ca²⁺-induced damage in cultured astrocytes¹⁴ and improved functional recovery after myocardial ischemia (T. Matsuda and A. Baba, unpublished observation, 2002), implicating a role for Na⁺-Ca²⁺ exchange in these processes. Moreover, SEA0400 had no effects on several ion channels and transporters and seems to have excellent specificity.

Four laboratories¹⁸–²¹ have found that knockout of the mouse Na⁺-Ca²⁺ exchanger, NCX1, is embryonic lethal by 11 days postcoitum (dpc). NCX1 is the only isoform of the exchanger in myocardium, and it might be expected that excitation-contraction coupling would fail in the absence of exchange activity. Koushik et al.¹⁹ however, found normal Ca²⁺ transients in heart tubes from 9.5-dpc NCX⁻/⁻ embryos. We reproduced this finding and used the NCX1 knockout model to identify the importance of the Na⁺-Ca²⁺ exchanger in the response of myocardium to ouabain.²¹

In this investigation, we use heart tubes from NCX⁻/⁻ mice to examine the specificity of KB-R7943 and SEA0400. Any effects of drugs on Ca²⁺ transients in NCX⁻/⁻ heart tubes must be due to modulation of something other than Na⁺-Ca²⁺ exchange.

Materials and Methods

Production of Na⁺-Ca²⁺ exchanger knockout mice has been described.²¹ All experiments were performed under approved institutional animal protocols. As before, the heart tubes from embryos at 9.5 dpc were loaded with fura 2, and Ca²⁺ transients were measured by fluorescence in a flow-through chamber on an inverted microscope. Heart tubes were paced at 1 Hz at 35°C.

The heart tubes were from embryos that are NCX⁻/⁻, NCX⁺/+ , or NCX⁺/+ . However, in several series of experiments, the hemizygous knockouts and wild-type embryos had identical properties. Therefore, we combined data obtained with NCX⁻/⁻ and NCX⁻/+ heart tubes into one group representing NCX1-containing heart tubes and refer to this group as NCX⁻/⁻.

KB-R7943 and SEA0400 were kind gifts from Kanebo and Taisho Pharmaceutical, respectively.

Results

Stock solutions of KB-R7943 and SEA0400 were in DMSO, and we tested whether DMSO by itself altered Ca²⁺ transients. Figure 1 shows that 0.1% DMSO had no effects on Ca²⁺ transients in heart tubes from NCX⁻/⁻ embryos. Figure 1 also demonstrates the excellent stability of the system. No
Changes were observed in Ca\(^{2+}\) transients over the 10-minute period in DMSO.

Typical effects of KB-R7943 (5 \(\mu\)mol/L, 5 minutes) on Ca\(^{2+}\) transients in embryonic heart tubes are shown in Figure 2A and are summarized in Figure 2B. In NCX\(^{+}\) heart tubes, KB-R7943 had substantial effects on diastolic Ca\(^{2+}\) and relaxation time. However, even in the absence of the Na\(^{+}\)-Ca\(^{2+}\) exchanger, KB-R7943 significantly reduced Ca\(^{2+}\) by 34\(^\pm\)8%. Continued exposure to KB-R7943 further reduced the Ca\(^{2+}\) transients. After 10 minutes of exposure to KB-R7943 (Figure 2C), Ca\(^{2+}\) was largely eliminated for both the NCX\(^{+}\) and NCX\(^{-}\)/NCX\(^{-}\) heart tubes.

Effects of SEA0400 (0.1 \(\mu\)mol/L) on the Ca\(^{2+}\) transients of embryonic heart tubes are shown in Figure 3A and are summarized in Figure 3B. The effects are similar to those obtained with KB-R7943, although achieved with much lower concentrations. Both drugs elevated diastolic Ca\(^{2+}\) and reduced Ca\(^{2+}\) in NCX\(^{+}\) heart tubes and reduced Ca\(^{2+}\) in NCX\(^{-}\)/NCX\(^{-}\) heart tubes.

The voltage of the field stimulation needed to be increased to maintain Ca\(^{2+}\) transients after application of both drugs. This occurred with both NCX\(^{+}\) and NCX\(^{-}\)/NCX\(^{-}\) heart tubes. The voltage typically would need to be increased from 30 to 40 or 50 V after 3 minutes of drug exposure.

**Discussion**

The usefulness in physiological settings of the Na\(^{+}\)-Ca\(^{2+}\) exchange inhibitors KB-R7943 and SEA0400 requires specificity. We compared the effects of the drugs on Ca\(^{2+}\) transients in embryonic heart tubes of control and Na\(^{+}\)-Ca\(^{2+}\) exchanger knockout mice. If a drug affects the Ca\(^{2+}\) transient in the absence of exchanger, it is certainly not a specific inhibitor of the exchanger. We have recently used this approach to demonstrate that the presence of the exchanger is required to elicit a response to ouabain or to removal of external Na\(^{+}\).\(^{21}\)
KB-R7943 and SEA0400 both depress Ca\(^{2+}\) transients even in the absence of the Na\(^+-\)Ca\(^{2+}\) exchanger. This depress effect clearly occurs by a mechanism not involving Na\(^+-\)Ca\(^{2+}\) exchange. KB-R7943 has already been noted to have multiple other effects,\(^{14,16}\) but SEA0400 had seemed promising as a specific exchange inhibitor.\(^{14,17}\)

The differences between the effects of the drugs on myocardium containing or lacking the exchanger is informative. Both drugs elevate diastolic Ca\(^{2+}\) only in the presence of the exchanger. Diastolic Ca\(^{2+}\) in NCX\(^{-/-}\) hearts was unaffected. The difference is likely related to a direct inhibitory effect of the drugs on the exchanger. Acute inhibition of the exchanger apparently blocks a component of Ca\(^{2+}\) efflux and the Ca\(^{2+}\) transient never fully relaxes. The fact that both drugs required the field stimulation to be increased for maintenance of Ca\(^{2+}\) transients suggests that ion channels involved in tissue excitation are being affected.

In summary, KB-R7943 and SEA0400 are not specific inhibitors of the Na\(^+-\)Ca\(^{2+}\) exchanger. Although we observe a lack of specificity, we have not determined the other actions of these drugs on embryonic heart tubes. KB-R7943 and SEA0400 may have uses in Na\(^+-\)Ca\(^{2+}\) exchange research but should be used with caution.

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References


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