Original Contributions

The Effect of Exchanger Inhibitory Peptide (XIP) on Sodium–Calcium Exchange Current in Guinea Pig Ventricular Cells

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We investigated the effect of exchanger inhibitory peptide (XIP) on Na–Ca exchange current (INa-Ca) in guinea pig ventricular cells. Cells were voltage-clamped with microelectrodes containing 20 mM Na⁺ and 14.0 mM EGTA ([Ca]o=100 nM). An outward putative exchange current was stimulated when extracellular Na⁺ was reduced from 144 mM to zero (Li⁺ replaced Na⁺). This outward current showed a significant dependence on extracellular Ca²⁺. When Na⁺ removal was delayed for up to 40 minutes (in the absence of extracellular K⁺ or the presence of 3.0 mM ouabain to block the Na⁺ pump), outward INa-Ca increased presumably because [Na]o increased. Time-dependent increases of outward current in the absence of K⁺ could be abolished by reapplication of K⁺, which presumably reactivates the Na⁺ pump and reduces intracellular Na⁺. This effect is blocked in the presence of 3.0 mM ouabain. The dependence of this current on extracellular Ca²⁺, its dependence on intracellular Na⁺, and activation by extracellular Na⁺ reduction, together with its resistance to ouabain all suggest that it is a Na–Ca exchange current. After dialyzing the cell with 10 μM XIP, outward INa-Ca was largely abolished. This indicates that XIP, which is a rather large molecule, can enter the cell via the microelectrode in sufficient quantities to inhibit exchange. Inward INa-Ca was blocked secondary to the blockade of outward INa-Ca. L-type Ca²⁺ current (ICa) was not measurably affected by XIP. It appears that XIP might be successfully used to separate the contribution of INa-Ca and ICa to excitation–contraction coupling and the regulation of contraction in isolated heart cells. (Circulation Research 1993;72:497–503)

KEY WORDS • Na–Ca exchange • exchanger inhibitory peptide • guinea pig ventricular cells • action potential • calcium current

Inhibitors of Na–Ca exchange already exist. These include various inorganic cations as well as derivatives of amiloride and bepridil.1 However these inhibitors lack specificity.2 In heart they inhibit both Na–Ca exchange and inward Ca²⁺ currents as well as other transport functions. This clearly limits their value in the study of cardiac excitation–contraction (E–C) coupling.

Recently the amino acid sequence of the cardiac Na–Ca exchanger has been determined.3 A polypeptide (XIP) has been synthesized based on a region of the exchanger that resembles a putative calmodulin binding site with possible autoinhibitory properties.4 When applied to sarcomemmal vesicles, XIP was a potent inhibitor of Na–Ca exchange and had no effect on sarcomemmal Ca²⁺ binding, Ca²⁺ permeability, the Na⁺,K⁺-ATPase and the sarcoplasmic reticulum (SR) Ca²⁺-ATPase.4 It did, however, appear to produce a large resting current when applied to the cell exterior.4

The effect of dialyzing intact cells with XIP has not been reported. Here we describe efforts to study the effect of this compound on guinea pig ventricular myocytes. We first attempted to determine whether this relatively large inhibitory molecule would enter cells through a micropipette in sufficient quantities to produce inhibitory effects on Na–Ca exchange. We were particularly interested in whether XIP exhibited any selectivity toward Na–Ca exchange when applied to the cell interior. For instance, could it be used to inhibit Na–Ca exchange without inhibiting Ca²⁺ currents? Clearly any substance that inhibits Na–Ca exchange without inhibiting Ca²⁺ currents will be of particular value in separating contributions of Na–Ca exchange and Ca²⁺ currents to E–C coupling and the regulation of contraction in heart. This issue has recently received much attention and discussion.5–7

Here we show that XIP inhibits outward Na–Ca exchange current (net reverse exchange corresponding to Ca²⁺ entry and Na⁺ exit). However, the compound does not affect ICa to any measurable extent. This suggests that the compound might be useful in the study of E–C coupling in the heart.

Materials and Methods

Guinea pig ventricular myocytes were obtained by enzymatic digestion using a method previously de-
scribed. Isolated myocytes were attached to the bottom of a cell bath with CR-laminin (Collaborative Research Inc., Bedford, Mass.) and viewed with an inverted phase-contrast microscope (Diaphot, Nikon, Tokyo). The cells were then superfused at 25°C with a modified Tyrode's solution containing (mM) NaCl 138, MgCl₂ 1.0, KCl 4.4, dextrose 11.0, CaCl₂ 2.7, and HEPES 12.0. The pH was adjusted to 7.4 with NaOH. This produced a total Na⁺ concentration of 144.4 mM.

Cells were voltage-clamped with single suction pipettes and a discontinuous voltage clamp circuit (Axoclamp-2A, Axon Instruments Inc., Foster City, Calif.). The suction pipettes were made from borosilicate capillary tubing (Corning 7052, 1.65 mm o.d., 1.2 mm i.d., A-M Systems, Everett, Wash.) and had resistances of 3–5 MΩ. During most of these experiments the cells were held at a potential of −40 mV and clamped at a frequency of 7–15 kHz. To measure outward exchange current the pipette contained (mM) NaCl 20, MgCl₂ 0.2, EGTA 14.0, MgATP 3.0, dextrose 5.5, and HEPES 10. Ca²⁺ (3.9 mM) was added as H₂CaEGTA. The solution pH was adjusted to 7.1 with CsOH. CsCl was added to give a final Cs⁺ concentration of 120 mM. The free Ca²⁺ was estimated to be 100 nM. When required, XIP was included in the pipette solution at a concentration of 10 μM. Ca²⁺ currents were measured in a superfusing solution identical to the above except that the Na⁺ was replaced with 136.6 mM tetraethylammonium chloride (TEA-Cl). The pH was adjusted to 7.1 with 5.2 mM CsOH. In a few experiments, Cs⁺ completely replaced Na⁺ (instead of TEA-Cl). This solution together with the following pipette solution was designed to pharmacologically isolate Ca²⁺ currents by removing contaminating K⁺ currents.

To activate outward exchange currents, voltage-clamped cells were superfused in a microstream containing (mM) NaCl 138.0, MgCl₂ 1.0, CaCl₂ 2.7, dextrose 11.0, and HEPES 12.0. The pH was adjusted to 7.4 with NaOH to give a final Na⁺ concentration of 144.4 mM. Outward exchange current was activated when the cell was abruptly immersed in an adjacent microstream of solution in which Li⁺ replaced Na⁺. These rapid solution changes were accomplished with a modified version of the switching device whose characteristics and design have previously been described. For these experiments the two adjacent microstreams simultaneously flowed from two square glass tubes (200 μm) separated by a 70-μm glass septum. For Ca²⁺ current measurement, the pipette contained (mM) MgCl₂ 0.2, EGTA 14.0, MgATP 3.5, and HEPES 10. The pH was adjusted to 7.1 with CsOH and the concentration of Cs⁺ was brought to 140 mM by adding CsCl. Inward L-type Ca²⁺ currents were activated by depolarizing the cell membrane from −40 mV to more positive values for 500 msec. Between measurements the cell was maintained at a holding potential of −80 to −90 mV. This prevented rundown of Ca²⁺ currents over lengthy trials.

Currents were digitized on line with a 12-bit A/D converter or stored on tape for future analysis. The digitized data was analyzed with a Compaq 386/20e computer and with both pCLAMP and AXOTAPE software (Axon). Statistical comparisons were made with a Student's t test and results are expressed as mean±SEM.

Results

Activation of Outward Exchange Current

We first investigated the effect of the putative inhibitor XIP on I₉₋₋. We found it convenient to study the effect of XIP on outward current, which corresponds to coupled Ca²⁺ entry and Na⁺ extrusion (net reverse exchange). We voltage-clamped myocytes and held their

FIGURE 1. Panel A: Na-Ca exchange current (I₉₋₋) activated by removal and subsequent reapplication of external Na⁺. The cell was voltage clamped and held at −40 mV and perfused with a solution containing 144 mM Na⁺ and 0 mM K⁺ (to inhibit the Na⁺ pump). The micropipette contained both 20 mM Na⁺ and 14.0 mM EGTA which buffered free Ca²⁺ to 100 nM. After allowing the cell to accumulate Na⁺ for 20 minutes, external Na⁺ was reduced for 5 seconds. This activated a large transient outward I₉₋₋. Reapplication of Na⁺ activated a transient-inward exchange current. Panel B: The superimposed exchange currents were recorded at 5-minute intervals after impaling the cell. These currents exhibited a progressive increase in magnitude which presumably parallels the accumulation of intracellular Na⁺. Holding potential, −40 mV.
membranes at −40 mV with pipettes containing 14.0 mM EGTA and a sufficient amount of added Ca\(^{2+}\) to ensure that free Ca\(^{2+}\) was buffered at 100 nM in free solution. However, this solution could not buffer intracellular Ca\(^{2+}\) indefinitely in the face of a large Ca\(^{2+}\) influx when used to dialyze the cell interior. The pipettes also contained 20 mM Na\(^+\). An outward putative exchange current was activated when extracellular Na\(^+\) was rapidly reduced (Figure 1A). Replication of Na\(^+\) suppressed this outward current and, as shown in the example in Figure 1A, activated a transient inward current. We briefly activated outward current every 5 minutes for up to 50 minutes, after which impalements were invariably lost. At first, outward current was small but increased significantly during every 5-minute period that it was activated (Figure 1B). We also noted that upon reperfusing the cell with 144 mM Na\(^+\), not only did the outward exchange current disappear, but a net transient inward current was stimulated. This increased in a way that tended to parallel the increase in outward current (Figure 1B). This behavior is expected if EGTA failed to buffer incoming Ca\(^{2+}\) in the vicinity of the exchanger. The resulting rise of free Ca\(^{2+}\) would result in forward exchange activity upon Na\(^+\) application.

Rapid reduction of extracellular Na\(^+\) should activate outward exchange current. However, these experiments were conducted with K\(^+\)-free solutions, which we assumed would largely block electrogenic Na\(^+\) pumping.\(^\text{12}\) We explored the possibility that the application of zero Na\(^+\) replaced with 144 mM Li\(^+\) might re activate electrogenic Na\(^+\) pumping.\(^\text{13-15}\) Thus the outward current measured in the absence of Na\(^+\) might have been partially or completely attributable to Na\(^+\) pump current. The following results, however, argue against this. First, if external Ca\(^{2+}\) is reduced to 10\(^{-7}\) M, outward current that is activated by Na\(^+\) removal is dramatically reduced when Ca\(^{2+}\) is removed. Thus approximately 75% of the outward current depends on external Ca\(^{2+}\) (Figure 2). This result was observed in two cells and is clearly a property attributable to Na-Ca exchange current. With intracellular Ca\(^{2+}\) buffered to approximately 10\(^{-7}\) M and the cell held at −40 mV (external [Ca\(^{2+}\)] 10\(^{-7}\) M), there is still a significant inward Ca\(^{2+}\) gradient.

With zero external Na\(^+\), the driving force for outward exchange remains infinite. It therefore remains possible that some or all of the remaining current is due to Na-Ca exchange. On the other hand, we cannot exclude the possibility that the remaining current is due to the Na\(^+\) pump.

We further tested the possibility that outward currents were largely due to electrogenic Na\(^+\) pumping.

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**Figure 2.** Outward Na-Ca exchange current. Tracing A: Rapid Na\(^+\) removal activates outward current in the presence of 2.7 mM Ca\(^{2+}\). Tracing B: If the Ca\(^{2+}\) concentration in the superfusing solution is 10\(^{-7}\) M, Na\(^+\) removal activates an outward current whose magnitude is approximately fourfold less than that activated in the presence of 2.7 mM Ca\(^{2+}\).

**Figure 3.** Outward exchange current was activated at intervals for up to 12 minutes. ●, Increase in outward current measured in a cell exposed to zero K\(^+\) to block the Na\(^+\) pump; △, outward current activated in a cell exposed to both zero K\(^+\) and 3.0 mM ouabain.

**Figure 4.** Panel A: In the absence of K\(^+\) (assumed to block the Na\(^+\) pump), outward exchange current was activated as intervals continually increased. After approximately 12 minutes, 4.4 mM K\(^+\) was applied and outward currents began to decline and reached negligible values within a few minutes of K\(^+\) application. Panel B: Ouabain (3.0 mM) blocks this decline in outward current that occurs when K\(^+\) is applied.
Outward currents were repeatedly activated in the manner and with the solutions described above. In the presence of 3.0 mM ouabain, outward current could be activated by reducing Na⁺ (Figure 3). Moreover, when K⁺ was absent, the presence or absence of ouabain had little effect on the time-dependent outward current that was activated by Na⁺ removal. With this concentration of ouabain, the observed outward current is unlikely to be due to electrogenic Na⁺ pumping.

During the entire course of these experiments, the cell was only briefly exposed to zero Na⁺. Since experiments were conducted either in the absence of K⁺ or in the absence of K⁺ and presence of ouabain, we expected progressive Na⁺ accumulation due to Na⁺ pump blockade. Outward exchange current is known to be linearly dependent on [Na⁺]. If the Na⁺ pump is blocked but the influx of Na⁺ continues to occur across the cell membrane and from the pipette (dialysis), we expect Na⁺ to accumulate to some extent. We therefore suggest that progressive increases in outward exchange current could be explained by continual intracellular Na⁺ accumulation. To test this idea we activated putative exchange current with Na⁺-free solutions after cell attachment. Between activation periods the cell was maintained in a Na⁺-containing solution deficient in K⁺. Superfusion with zero K⁺ for 12 minutes, during successive reductions of Na⁺, caused a progressive increase in outward current (Figure 4A). A similar result was obtained in another cell treated with 3.0 mM ouabain (Figure 4B). Application of 4.4 mM K⁺ reduced outward current as shown in Figure 4A. This effect is completely blocked in the cell treated by 3.0 mM ouabain (Figure 4B). It seems clear that progressive increases in outward current occur when the Na⁺ pump is blocked, whether by K⁺ deprivation or the presence of ouabain. If the outward current was due largely to Na⁺ pumping, it should have been blocked in the presence of ouabain rather than continuing to increase as observed. On the contrary, it is when the pump is reactivated that outward current disappears. From this we conclude that outward current is dependent on intracellular Na⁺ and increases because intracellular Na⁺ increases. When the Na⁺ pump is reactivated by K⁺ intracellular Na⁺ is reduced and outward current diminishes. Taken together with the observed Ca²⁺ dependence of this current and the conclusion that most of the current is unlikely to reflect electrogenic Na⁺ pumping, we conclude that this current is outward Na-Ca exchange current. We also suggest that the progressive increase in this current is due to continual Na⁺ accumulation as a result of Na⁺ pump blockade. This process is doubtless slowed by the buffering effect of the electrode. This appears to be the same current that Kimura et al observed in the absence of external Na⁺ (Li⁺ replacement) but in the presence of external Ca²⁺. Transient behavior is characteristic of outward Na-Ca exchange current measured in whole cells. It appears that, as outward current and hence Ca²⁺ entry increases, the

![Figure 5](http://circres.ahajournals.org/)

**Figure 5.** Panel A: Effect of XIP on normalized exchange currents. The progressive increase in the amplitude of reverse exchange current observed in control cells (●). With 10 µM XIP in the pipette, this increase in the amplitude of exchange current was abolished (∗). Current magnitudes were normalized by expressing the magnitude of net outward current at any time relative to the magnitude of outward current immediately after cell attachment. Thus after 40 minutes putative exchange current had increased approximately 7.5-fold. The normalized current amplitude in the control and experimental cells was significantly different less than 20 minutes after impalement (p≤0.01). Bars, ±1 SEM. Panel B: The upper panel exhibits an outward current measured initially and a much larger current measured after 30 minutes. The effect of XIP on outward current is displayed in the lower panel. The larger current was measured soon after impalement and the smaller current was measured 30 minutes after impalement with an XIP-containing pipette. This result (which is one of those contributing to the result in panel A) suggests that some of the initial current (measured immediately after attachment) was the result of Na-Ca exchange.
The foregoing results suggest a straightforward way of testing the hypothesis that the peptide XIP might effectively inhibit reverse Na-Ca exchange. After introducing XIP into the cell via the suction pipette we observed inhibition of the progressive increase in outward current that accompanies Na⁺ accumulation (Figure 5A). Presumably the presence of forward exchange depends on the initial entry of Ca²⁺ during reverse exchange. Therefore, inhibition of outward current will also result in inhibition of Na⁺-dependent inward current. It is clear (Figure 5B) that this expectation is fulfilled. After an initial modest increase the outward exchange is significantly less than its maximum value measured in the absence of XIP (Figure 1B). In fact, differences in the amplitude of outward current in the XIP-treated and -untreated cells were significant in less than 20 minutes (p≤0.01) (Figure 5A). The small initial increase in outward current may be explained if we assume that the rate at which Na⁺ accumulated somewhat exceeded the rate at which inhibitory quantities of XIP entered the cell. Presumably the activating effect of the former modestly exceeds the inhibitory effect of the latter.

We conclude from these results that sufficient XIP entered the cells under the circumstances of this experiment to significantly inhibit reverse Na-Ca exchange.

**Effect of XIP on L-Type Ca²⁺ Current**

To separate the contributions of Iₓ and Iₓ₋ₐ to E-C coupling it is necessary to demonstrate that XIP does not inhibit L-type Ca²⁺ currents. We measured pharmacologically isolated Ca²⁺ currents (Figure 6). Cs⁺ completely replaced K⁺ in the micropipette, which also contained EGTA at a concentration of 14.0 mM. Free Ca²⁺ was effectively zero. In a few experiments external Na⁺ was replaced with Cs⁺. In the majority of experiments the superfusing solution contained TEA-Cl and small quantities of Cs⁺. Cells were clamped at −40 mV and then depolarized to +10 mV to activate Ca²⁺ currents. Currents were activated as soon as possible after establishing a clamp and then every 5 minutes for as much as 30 minutes (Figure 6A). Identical experiments were conducted with pipettes containing XIP (Figure 6B). It is clear from Figure 6C that XIP did not have an inhibitory effect on Ca²⁺ currents. We also constructed current–voltage relations in the presence and absence of XIP (Figure 7). Normalized averaged results from five XIP-treated cells and three untreated cells showed no difference (Figure 7). From this we conclude that XIP exerts no effect on the voltage dependence of Ca²⁺ current.

**Discussion**

Our main results indicate that 1) when XIP is included in the micropipette at a concentration of 10 μM, sufficient quantities dialyze the cell to produce considerable inhibition of the outward exchange current within 30 minutes. Significant inhibition of the current is measurable in less than 15–20 minutes. Thus the compound is capable of inhibiting net reverse exchange, which corresponds to coupled Ca²⁺ entry and Na⁺ extrusion from the cell. 2) XIP was not capable of blocking pharmacologically isolated L-type Ca²⁺ currents.

A relatively specific inhibitor of Na-Ca exchange would clearly be of particular value when studying the contribution of exchange to E-C coupling and the regulation of contraction in heart. After analyzing the amino acid sequence of the exchange, Li et al synthesized XIP. Its structure was based on a region of the exchanger with possible autoinhibitory properties resembling a putative calmodulin binding site. These authors showed that XIP is a potent inhibitor of Na-Ca exchange when present at the intracellular sarcolemmal surface. However the mechanism by which the XIP inhibits exchange is not yet understood.
We have concluded that the outward current that we measured upon Na"+ removal consists primarily of net outward exchange current. In these experiments Na"+ was replaced with equimolar Li"+. For the entire duration of the experiment the cells were perfused with solutions deficient in K"+ to block the Na"+ pump. It is possible that sudden application of Li"+ (Na"+ removal) might reactivate the Na"+ pump.13-15 Moreover, as intracellular Na"+ accumulates, the pump current would become larger every time Li"+ replaced Na"+. However, several lines of evidence suggest that most of this outward current is not a Na"+ pump current. In the first case these currents can be elicited in the presence of 3.0 mM ouabain. It should be emphasized that in some cases ouabain was applied in the absence of K"+, which should increase its effectiveness. Moreover, outward current characteristics are not observably altered by ouabain. These outward currents exhibit a clear dependence on external Ca"²⁺ and are greatly suppressed (about fivefold) when extracellular Ca"²⁺ is 10⁻⁷ M. The Na-Ca exchange, unlike the Na"+ pump, would be expected to show a striking dependence on external Ca"²⁺. The time-dependent increase in outward current is eliminated by K"+ application (Figure 4A). This effect can be explained if one assumes that reactivation of the Na"+ pump reduces intracellular Na"+ and hence outward exchange.

Some outward current does remain when Na"+ is removed in the presence of 10⁻⁷ M Ca"²⁺. We do not know the origin of this current. Some of it might be attributable to a Na"+ background current similar to that measured in the rabbit sinoatrial node.21 If this current was normally inward, presumably Na"+ removal would suppress or completely reverse it. This would result in the appearance of an outward current. The other possibility is that this remaining outward current is Na"+ pump current. It seems likely that such a current, if present, is a relatively small fraction of the total outward current (Figure 2).

To summarize, the increase in outward current during Na"+ pump blockade indicates that it is dependent on intracellular Na"+. The current's dependence on both internal and external Na"+, its dependence on external Ca"²⁺ and its resistance to ouabain all suggest that it is largely a Na-Ca exchange current. It is however probable that this current is contaminated to some extent by other currents.

Net reverse Na-Ca exchange can be largely inhibited if 10 μM XIP is included in the dialyzing pipette solution. It is well known that increases in intracellular Na"+ increase reverse exchange.16,22,23 As we have explained, when Na"+ accumulates, larger exchange currents can be activated. The time course with which these exchange currents enlarge is difficult to follow. In only one of five control cells was it possible to obtain data points beyond 20 minutes. With intracellular Ca"²⁺ buffered to 100 nM, the membrane held at −40 mV and extracellular Na"+ at a concentration of 144 mM, we determined that the exchange will reverse when intracellular Na"+ reaches 9 mM.24 With 20 mM Na"+ in the pipette it therefore seems likely that reverse Na-Ca exchange occurred for nearly the entire course of the experiment. Presumably the capacity of EGTA to buffer this incoming Ca"²⁺ is exceeded with resulting irreversible contracture and cell death. In cells treated with 10 μM XIP, data points were measured in all four cells for as long as 35–60 minutes after impalement. If XIP prevents the continual accumulation of intracellular Ca"²⁺ by reverse exchange secondary to Na"+ pump inhibition during the entire course of the experiment, excessive Ca"²⁺ entry and cell death will be prevented. We assume that under these circumstances any passive
Ca\(^{2+}\) leak is compensated by the sarcolemmal Ca\(^{2+}\) pump. There is an initial small increase in reverse Na-Ca exchange in the XIP-treated cells that peaks within approximately 15 minutes (Figure 5A). We assume that initially the rate with which Na\(^{+}\) accumulates in the cell exceeds the rate at which the inhibitory effect of XIP is exerted. Apparently sufficient XIP enters the cell to exert maximum inhibition after approximately 30 minutes. It is difficult to give a precise estimate of the fractional inhibition of exchange by XIP because a background Na\(^{+}\) current apparently exists in certain heart cells.\(^25\) The outward exchange is activated in this study by removing external Na\(^{+}\). If this process removes a small inward background current or reduces a non-specific current, a small outward current will appear in the record. It is impossible at this stage to distinguish a current of this nature from a small exchange current that escaped inhibition. However, the results in Figure 2B suggest that at least 90% of the exchange can be blocked by XIP and it is very likely that all of it is inhibited.

We failed to demonstrate any inhibition of L-type Ca\(^{2+}\) currents by XIP. These currents were recorded in the presence of K\(^+\) channel blockers. We also used 14.0 mM EGTA in the pipettes to eliminate as much as possible Ca\(^{2+}\)-dependent inactivation and to preserve Ca\(^{2+}\) currents for as long as possible. Apparently the voltage dependence of Ca\(^{2+}\) current is not affected by XIP.

It is known that if XIP is applied to the cell exterior it induces the presence of large membrane currents.\(^4\) Not much is known about this effect, but it does suggest that, when XIP is applied to the cell exterior, its effects may lack specificity. However, when applied to the cell interior XIP appears to be a relatively specific inhibitor of the Na-Ca exchange though other effects may yet be described. It is fairly straightforward to demonstrate significant inhibition of reverse exchange. As far as E-C coupling is concerned, it is important that XIP appears not to inhibit the Ca\(^{2+}\) current. Thus it should prove useful in separating the contributions of reverse exchange and the Ca\(^{2+}\) current to the mechanism of SR Ca\(^{2+}\) release and also to the regulation of contraction.

### References

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