Sodium current-induced calcium signals in isolated guinea-pig ventricular myocytes

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1. Na^+ current (I_{Na})-induced Ca^{2+} transients were studied in ventricular myocytes isolated from adult guinea-pig hearts. The fluorescent Ca^{2+} indicator fluo-3 or a mixture of fluo-3 and fura-red were used in conjunction with confocal microscopy to follow the intracellular Ca^{2+} concentration while membrane currents were measured simultaneously with the whole-cell configuration of the patch-clamp technique.

2. Ca^{2+} release from the sarcoplasmic reticulum (SR) could be triggered either by Ca^{2+} current (I_{Ca}) or Na^+ current (I_{Na}). Analysis of I_{Na}-induced Ca^{2+} signals at higher temporal resolution revealed a faster upstroke of these transients when compared with those triggered by I_{Ca}.

3. In the presence of 20 μM ryanodine to block SR Ca^{2+} release I_{Ca} elicited a verapamil-sensitive Ca^{2+} transient with a slow upstroke. I_{Na} also induced a residual Ca^{2+} transient that was insensitive to 10 μM verapamil and characterized by a rapid upstroke.

4. The existence of a residual Ca^{2+} transient in the absence of SR Ca^{2+} release and L-type I_{Ca} indicates that I_{Na} is indeed able to evoke an increase in $[Ca^{2+}]_i$ without uncontrolled activation of Ca^{2+} channels.

5. Substitution of extracellular Na^+ by Li^+ suppressed I_{Na}-induced Ca^{2+} transients, suggesting that the Ca^{2+} release and the residual Ca^{2+} transient can only be elicited by influx of Na^+ and not by Li^+. This result supports the notion that both the residual Ca^{2+} transient as well as the I_{Na}-induced Ca^{2+} release are mediated by the Na^+–Ca^{2+} exchange.

6. We conclude that during I_{Na} $[Na^+]$ increased underneath the cell membrane and activated the Ca^{2+} influx mode of the Na^+–Ca^{2+} exchange. The Na^+ current may serve as a safety factor in cardiac excitation–contraction coupling by accelerating the Ca^{2+} concentration changes responsible for signal transduction in the subsarcolemmal space.

In cardiac muscle rapid release of Ca^{2+} from the sarcoplasmic reticulum (SR) is the key event in the signal transduction process between electrical excitation of the surface membrane and mechanical activity of the cell. The release of Ca^{2+} itself is believed to be triggered by an influx of Ca^{2+} via voltage-gated Ca^{2+} channels, a mechanism known as Ca^{2+}-induced Ca^{2+} release (CICR) (Fabiato, 1985; for a review see Bers, 1991). Based on indirect experimental evidence it has recently been suggested that subcellular Ca^{2+} concentration gradients play an important role during the process of excitation–contraction (E–C) coupling (Lipp, Pott, Callewaert & Carmeliet, 1990; for a recent overview see Niggli & Lipp, 1993). Similar short-lived concentration gradients were also proposed to exist for Na^+ (Isenberg & Wendt-Galitelli, 1990; Leblanc & Hume, 1990; for a review see Carmeliet, 1992). This hypothesis is based on the observation that I_{Na} was able to trigger SR Ca^{2+} release in guinea-pig myocytes in the absence of I_{Ca}. This I_{Na}-induced Ca^{2+} release was explained by a mechanism involving the Na^+–Ca^{2+} exchange. During large I_{Na} (up to 100 nA, see Bers, 1991) the $[Na^+]$ may rise underneath the sarcolemma and activate the Ca^{2+}-influx mode of the Na^+–Ca^{2+} exchange. This hypothesis also predicted the existence of a residual Ca^{2+} transient resulting from the activation of the Na^+–Ca^{2+} exchange even in the absence of SR Ca^{2+} release. However, this residual signal has not yet been identified.

The proposed hypothesis involving Na^+–Ca^{2+} exchange-mediated SR Ca^{2+} release did not remain unquestioned, however. I_{Na}-induced Ca^{2+} release has also been explained by a loss of voltage control during the large I_{Na} (Sham, Cleemann & Morad, 1992). During loss of voltage control Ca^{2+} channels might be activated briefly and the resulting influx of Ca^{2+} ions would trigger the release of Ca^{2+} from the SR.

In another interpretation it has been suggested that contaminating Ca^{2+} influx via Na^+ channels may trigger Ca^{2+} release from the SR (Johnson & Lemieux, 1991). This interpretation has been made unlikely by the observation that a Li^+ current carried by Na^+ channels was not able to

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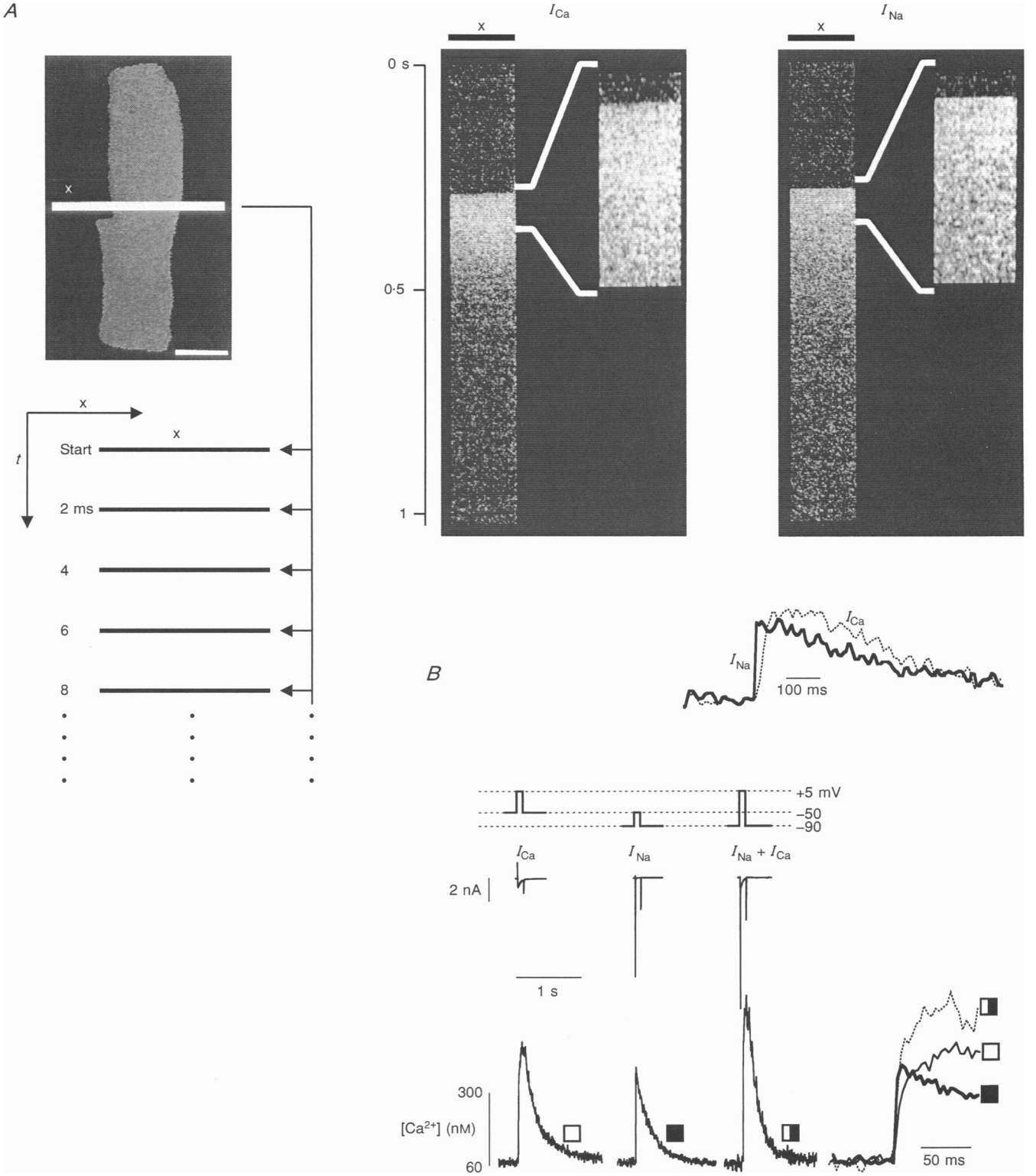

Figure 1. Ca^{2+} transients in cardiac myocytes induced by I_{Ca}, I_{Na} and a combination of I_{Ca} and I_{Na}
Unless mentioned otherwise, a pre-pulse protocol was used in order to provide a defined loading state of the SR. The cell was held at −90 mV and 10 conditioning pulses (duration 250 ms) to a membrane potential of +5 mV were applied at 0·5 Hz. Prior to the test pulse a resting period of 10 s was introduced. I_{Na} was elicited by depolarizing to −50 mV. I_{Ca} was activated by a voltage step to +5 mV after inactivating I_{Na} with a voltage ramp to −50 mV 3 s prior to evoking I_{Ca}. For experiments with I_{Na} and I_{Ca} the membrane potential was stepped from −90 mV to +5 mV. *A*, a single line (x) was repeatedly scanned with a temporal resolution of 2 ms. The lines were successively arranged in a top-to-bottom order to build up the line-scan images for both emission wavelengths. For ratio imaging the

induce Ca^{2+} release, suggesting that indeed the Na^+–Ca^{2+} exchange is involved in mediating the initial increase of $[Ca^{2+}]$ (Hume, Levesque & Leblanc, 1991; Lederer, Niggli & Hadley, 1991).

The aim of the current paper was to examine further the kinetic properties of the I_{Na}-induced Ca^{2+} release and to reveal a key element predicted by the Na^+–Ca^{2+} exchange hypothesis – a residual I_{Na}-induced Ca^{2+} transient in the absence of CICR. This residual Ca^{2+} transient represents the missing link between I_{Na} and Ca^{2+} release from the SR.

METHODS

Cell preparation and experimental solutions

Guinea-pigs were killed by cervical dislocation after stunning. Single ventricular myocytes were dissociated from the heart using an enzymatic method as described recently (Lipp & Niggli, 1993*a*). The extracellular solution contained (mM): NaCl, 145; CsCl, 2; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 1; Hepes/NaOH, 10; pH 7·4. In order to block L-type Ca^{2+} channels 10 μM verapamil was used. SR Ca^{2+} release was inhibited by pre-incubation of the myocytes in a solution containing 20 μM ryanodine (Penick, Lyndhurst, NJ, USA) for at least 30 min. For Na^+ substitution experiments the external solution contained an equimolar concentration of LiCl. Solutions were applied by a rapid ($t < 200$ ms) perfusion system described recently (Lipp, Pott, Callewaert & Carmeliet, 1992). The solution for the internal dialysis of the myocytes contained (mM): caesium aspartate, 120; NaCl, 5; Mg-ATP, 5; $MgCl_2$, 1; Hepes/CsOH, 10; pH 7·2; fluo-3$(NH_4)_5$, 0·033; fura-red$(NH_4)_4$, 0·066 (or fluo-3$(NH_4)_5$ only, 0·1) (Molecular Probes, Eugene, MO, USA).

Current recordings

The membrane current was recorded in the whole-cell configuration of the patch-clamp technique. A detailed description of the set-up for current recordings has been described elsewhere (Lipp & Niggli, 1993*a*).

Fluorescence recording and image analysis

The set-up for ratiometric confocal fluorescence measurements has been described recently (Lipp & Niggli, 1993*a*). Briefly, the experimental set-up was based on a Bio-Rad MRC-600 confocal microscope (Bio-Rad, Wetzikon, Switzerland) equipped with an argon-ion laser (available wavelengths 488 and 514 nm). For excitation of fluo-3 alone the 488 nm line of the laser was used, while the 514 nm line excited the mixture of indicators in the dual emission mode. The fluorescence was measured at 540 nm (± 15 nm, fluo-3) and above 600 nm (fura-red). In order to obtain a high temporal resolution (either 6 or 2 ms), the line-scan mode of the confocal set-up was used (for a detailed description of the line-scan mode see Lipp & Niggli, 1993*a*). Image analysis was performed on an Apple Macintosh IIcx (Apple, Walisellen, Switzerland) with image-processing software (NIH-Image). Line-scan ratio images were produced with pixel-by-pixel division of the fluo-3 and fura-red image and scaling of the resulting ratios into the available 256 gray-scale levels. For the line plots each line of the line-scan image was averaged. In experiments with fluo-3 as the only indicator, we used a normalization procedure to obtain 'pseudo-ratios' (i.e. the actual fluorescence level was normalized by the resting level: F/F_{rest}).

RESULTS

I_{Na}-induced Ca^{2+} transients rise faster than I_{Ca}-activated signals

In the first set of experiments the existence and kinetic characteristics of I_{Na}-induced Ca^{2+} release were examined and compared to I_{Ca}-induced Ca^{2+} transients. In order to maintain a defined Ca^{2+} loading state of the SR throughout the experiment a pre-pulse protocol was applied prior to each test pulse (for details see legend of Fig. 1). Figure 1*A* illustrates that I_{Ca}, as well as I_{Na}, was able to trigger Ca^{2+} release from the SR, as has been shown previously (Leblanc & Hume, 1990). The simultaneous increase of $[Ca^{2+}]_i$ in the entire scanned line indicates that Ca^{2+} release was homogeneous throughout the cell, a property that characterized both Ca^{2+} transients. Closer inspection of the Ca^{2+} transients revealed kinetic differences in the rising phase of the Ca^{2+} signals as illustrated in the line plots in the lower part of panel *A*.

Ca^{2+} transients triggered by I_{Na} alone or by a combination of I_{Na} and I_{Ca} exhibited a substantially faster increase of $[Ca^{2+}]_i$ than those triggered by I_{Ca} in the absence of I_{Na} (Fig. 1*B*). In addition, the I_{Ca}-induced Ca^{2+} transient increased during the entire duration of I_{Ca}, a property that was also found in the Ca^{2+} transients triggered by a combination of I_{Na} and I_{Ca}. Quite in contrast, the rising phase of the I_{Na}-induced Ca^{2+} transient was extremely short (< 6 ms). In addition, comparison of the Ca^{2+} transients in panel *B* suggests that I_{Na} also increased the amplitude of the signal.

It has been suggested recently that the activation of Ca^{2+} release by I_{Na} may be due to a loss of the voltage control during the large I_{Na} (Sham *et al.* 1992). This would activate Ca^{2+} channels and thereby trigger Ca^{2+} release from the SR. We tested this hypothesis by applying 10 μM verapamil, an L-type Ca^{2+} channel blocker (for review see Bers, 1991). Figure 2 shows that the I_{Ca}-triggered Ca^{2+} release was sensitive to verapamil while the I_{Na}-activated Ca^{2+} transient was not affected. This result indicates that uncontrolled

fluo-3 line-scan image was divided pixel-by-pixel by the fura-red image. The resulting ratio images for I_{Ca} (left) and I_{Na} (right) show a homogeneous Ca^{2+} release throughout the entire scanned line (see also the insets for a higher temporal resolution). Scale bar: 20 μm. The line plots of the Ca^{2+} transients at the bottom of panel *A* illustrate the time course of the ratios and reveal kinetic differences between the two transients. *B*, the three membrane currents elicited by the appropriate pulse protocol and the corresponding Ca^{2+} transients are shown after applying an *in vitro* calibration. Characteristic kinetic differences were found when the upstroke velocity of the individual transients was analysed.

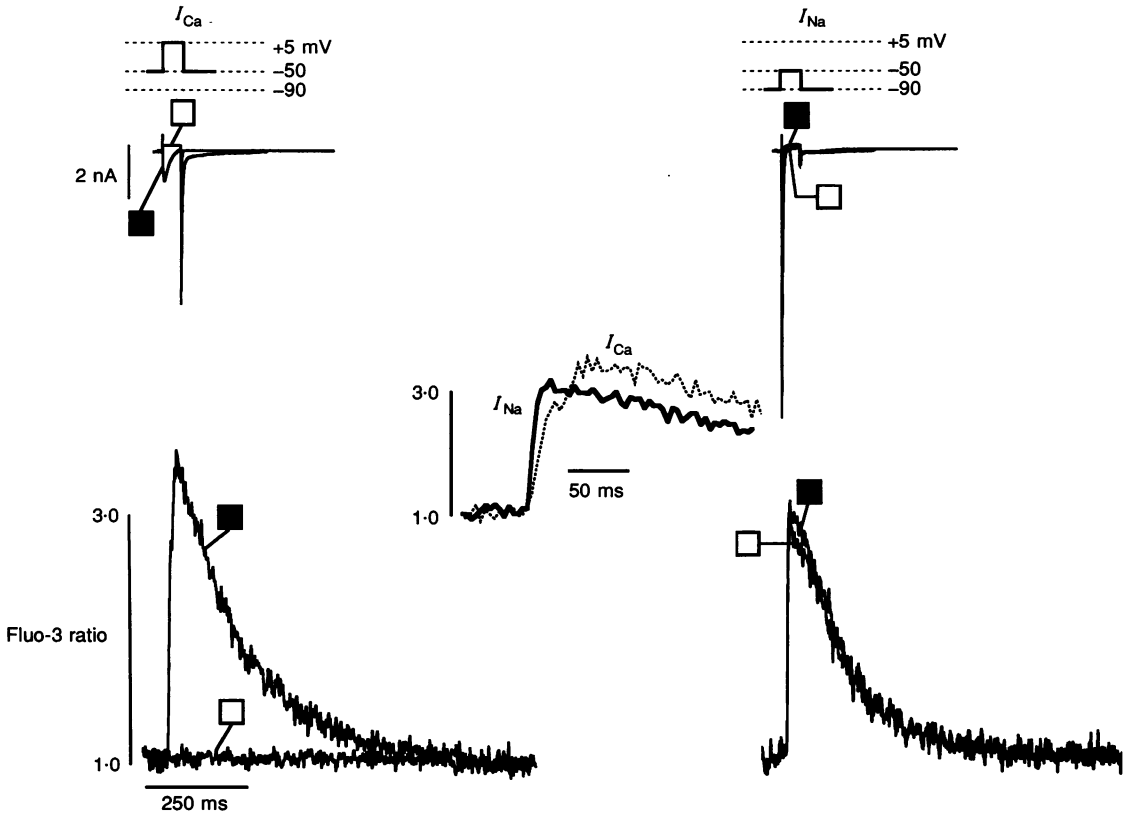

Figure 2. I_{Na}-induced Ca^{2+} release is verapamil insensitive
The fluo-3 ratios for the line plots were obtained by normalizing the fluo-3 signal with the resting fluorescence (F/F_{rest}). In all experiments with verapamil a different pre-pulse protocol was applied. The cells were held at −50 mV and depolarizing pulses (duration 500 ms) were applied at 0·75 Hz to a membrane potential of +60 mV to load the SR with Ca^{2+} via activation of the Na^+–Ca^{2+} exchange in the Ca^{2+}-influx mode. I_{Ca} was activated by depolarizing to +5 mV. For I_{Na} the membrane potential was stepped to −90 mV 50 ms prior to the test pulse (to −50 mV). The inset illustrates the differences in upstroke velocity at higher temporal resolution. After the control experiments (■) the application of 10 μM verapamil (□) effectively blocked I_{Ca} and I_{Ca}-induced Ca^{2+} release, while the I_{Na}-induced Ca^{2+} transient was not affected.

spurious opening of Ca^{2+} channels during the activation of I_{Na} was negligible in our experiments. Therefore, I_{Na} itself served as the trigger for Ca^{2+} release from the SR, either directly or indirectly.

I_{Na} produces residual Ca^{2+} transients in the presence of ryanodine

In order to examine the increase of $[Ca^{2+}]_i$ generated by I_{Na} or I_{Ca} alone, the SR Ca^{2+} release function was inhibited by pre-incubation of the cells in a solution containing 20 μM ryanodine. Figure 3*A* illustrates that Ca^{2+} influx through Ca^{2+} channels still produced a substantial Ca^{2+} transient. As predicted by the Na^+–Ca^{2+} exchange hypothesis, I_{Na} also resulted in an increase of $[Ca^{2+}]_i$ although much smaller than the transient induced by I_{Ca} (Fig. 2, right panel). Moreover, the signal induced by I_{Ca} was completely abolished by 10 μM verapamil while the transient caused by I_{Na} was not affected at all (Fig. 3*B*). This result rules out a significant uncontrolled activation of Ca^{2+} channels due to a loss of voltage control during I_{Na}.

Li^+ current does not produce Ca^{2+} transients

Nevertheless, the activation of T-type Ca^{2+} channels (Hille, 1992) could still result in a small increase of $[Ca^{2+}]_i$ since T-type Ca^{2+} channels are not blocked by verapamil. To examine a putative contribution of T-type Ca^{2+} channels to the increase of $[Ca^{2+}]_i$ during I_{Na} we compared the ability of I_{Na} to induce Ca^{2+} release when it was carried by Na^+ or Li^+. Since Li^+ is able to substitute for Na^+ as the charge carrier in the Na^+ channel with almost equal permeability (Hille, 1992), the Li^+ current (I_{Li}) resulted in a similar loss of voltage control, but Li^+ would not substitute for Na^+ in the Na^+–Ca^{2+} exchange mechanism (Fig. 4).

The solution change from Na^+ to Li^+ was performed 2 s before the test pulse was applied. This procedure minimized the effect of Na^+ substitution on resting $[Ca^{2+}]_i$ (see inset in Fig. 4). It is obvious from Fig. 4 that I_{Li} (□) was not able to trigger Ca^{2+} release from the SR. The Ca^{2+} transient visible in the presence of extracellular Na^+ was completely suppressed and no residual transient was elicited. This

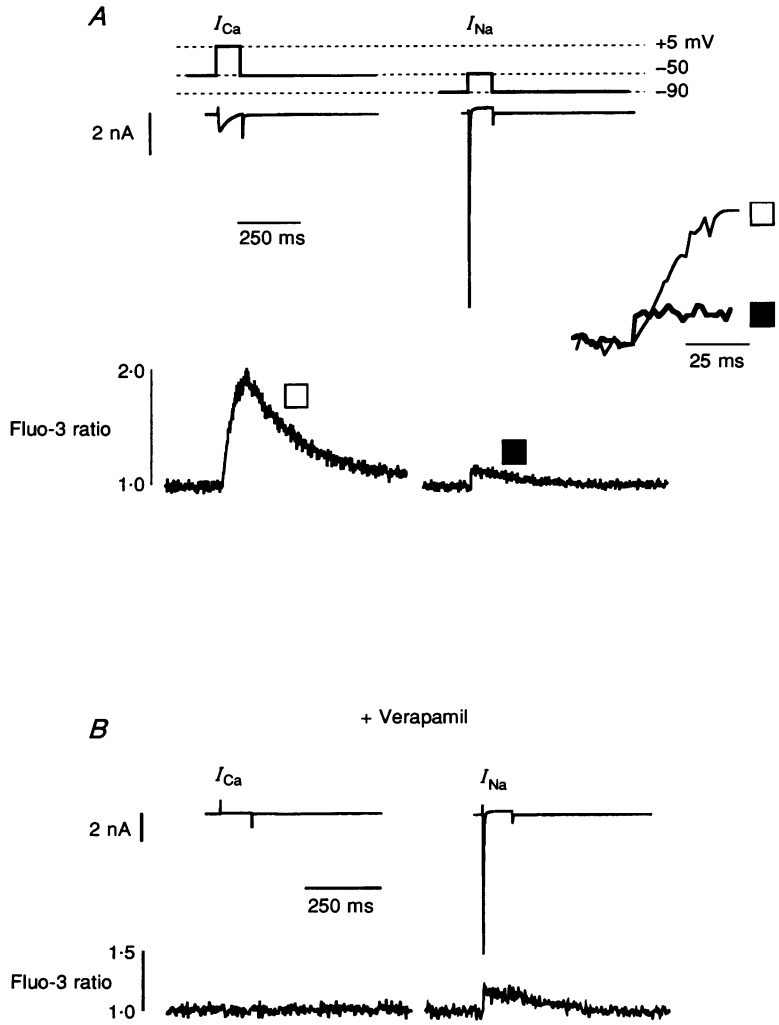

Figure 3. Effect of verapamil on Ca^{2+} transients in the absence of SR release function
A, the left traces show membrane current and fluo-3 ratio traces measured in a myocyte due to activation of I_{Ca}. The cell had been treated with 20 μM ryanodine for at least 30 min. Due to the absence of SR Ca^{2+} release function the ratio transient exhibited a slower upstroke when compared with signals recorded in the absence of ryanodine. I_{Na} also resulted in a small transient despite the presence of ryanodine. Nevertheless, the kinetic differences observed in the presence of SR Ca^{2+} release function were preserved as indicated in the inset. *B*, while application of 10 μM verapamil effectively blocked I_{Ca} and the corresponding Ca^{2+} transient, I_{Na} and the residual Ca^{2+} transient remained unaffected.

result indicates that Na_o^+ was required for both: to trigger Ca^{2+} release or to induce a residual Ca^{2+} transient.

DISCUSSION

For several years CICR from the SR of cardiac myocytes was postulated to be exclusively triggered by Ca^{2+} influx through Ca^{2+} channels in the cell membrane (Sham *et al.* 1992; for a review see Bers, 1991). However, I_{Ca} activated during the action potential is not the only pathway for Ca^{2+} influx. Besides the established role of the Na^+–Ca^{2+} exchange during later phases of the intracellular Ca^{2+} transient, a recent report provided indirect evidence for an additional role of the Na^+–Ca^{2+} exchange during early events in the E–C coupling process (Leblanc & Hume, 1990).

The authors reported I_{Na}-induced Ca^{2+} release in cardiac myocytes that was dependent on the presence of extracellular Ca^{2+} and sensitive to ryanodine. This observation suggests that I_{Na} (i.e. Na^+ influx) results in an increase of $[Ca^{2+}]_i$ via the Na^+–Ca^{2+} exchange subsequently triggering Ca^{2+} release from the SR. Although some evidence for the participation of the Na^+–Ca^{2+} exchange in this phenomenon was presented (see also Lederer, Niggli & Hadley, 1990; Hume *et al.* 1991), the predicted link between influx of Na^+ during I_{Na} and release of Ca^{2+} from the SR has not been identified, i.e.

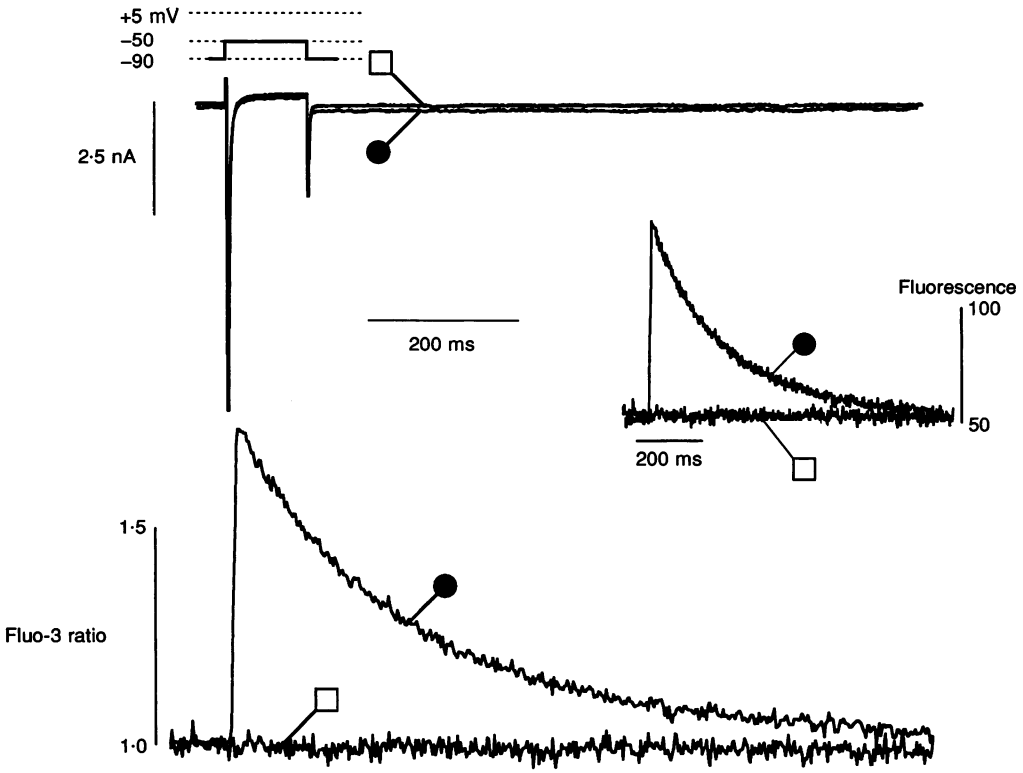

Figure 4. Substitution of Na^+ by Li^+ suppressed I_{Na}-induced Ca^{2+} release
Membrane current (upper traces) and fluo-3 ratio (lower traces) are shown. In this experiment extracellular Na^+ was replaced with Li^+ 2 s before the test pulse. Traces marked with filled symbols were recorded in the presence of extracellular Na^+, while those marked with open symbols were measured with Li^+ in the extracellular solution. Obviously, I_{Li} was not able to trigger Ca^{2+} release from the SR or to cause a residual Ca^{2+} transient. The inset illustrates the two original fluo-3 fluorescence traces recorded in the presence and absence of extracellular Na^+. Comparing absolute fluorescence levels prior to the test pulse indicates that resting $[Ca^{2+}]_i$ did not increase substantially.

the residual I_{Na}-induced Ca^{2+} transient in the absence of SR Ca^{2+} release function.

I_{Na}-induced Ca^{2+} transients rise faster than I_{Ca}-induced transients

Due to fundamental differences in the kinetics of I_{Na} and I_{Ca} one might expect kinetic differences in the Ca^{2+} transients elicited by I_{Na} and I_{Ca}, respectively. However, in a study where Ca^{2+} transients were measured with the fluorescent indicator indo-1 no kinetic differences could be resolved (Leblanc & Hume, 1990). In the present paper we show that I_{Na}-induced Ca^{2+} signals were characterized by a much faster onset. It is likely that we were able to resolve these kinetic differences because we used the fast Ca^{2+} indicator fluo-3 (Eberhard & Erne, 1989) with the line-scan mode of the confocal microscope.

Ca^{2+} release from the SR is believed to be terminated as soon as I_{Ca} is discontinued (Cannell, Berlin & Lederer, 1987; Näbauer, Callewaert, Cleemann & Morad, 1989). Our findings with different kinetics for I_{Na}- and I_{Ca}-induced Ca^{2+} release support this notion. Since I_{Na} is very brief, only a short pulse of Ca^{2+} influx was possible and Ca^{2+} release terminated rapidly. However, the trigger signal for Ca^{2+} release mediated by I_{Ca} lasted as long as the voltage-clamp pulse (100–150 ms) and therefore Ca^{2+} influx, and most probably also Ca^{2+} release, was maintained over a longer period of time.

I_{Na} caused a residual Ca^{2+} transient in the absence of SR Ca^{2+} release function

The hypothesis of the Na^+–Ca^{2+} exchange providing the trigger signal for Ca^{2+} release also predicted the existence of a small increase in $[Ca^{2+}]_i$ due to I_{Na}. We were able to reveal the predicted residual Ca^{2+} transient produced by I_{Na} in the presence of ryanodine. The kinetic characteristics were identical to the SR Ca^{2+} release signal in the absence of ryanodine, and the Ca^{2+} transient was insensitive to verapamil. Therefore, this transient represents the missing link in the signal-transduction mechanism responsible for triggering Ca^{2+} release due to the flow of I_{Na}. The identification of this missing link provides additional support for the view that the Na^+–Ca^{2+} exchange is involved in I_{Na}-induced Ca^{2+} release, possibly mediated by spatial and functional coupling of the two mechanisms via preferential access to a space underneath the membrane (Lederer *et al.* 1990; for a recent review see Niggli & Lipp, 1993). It should

be pointed out again that the pre-pulse protocol was applied in order to provide a high SR load. Most probably the high level of SR loading caused a high positive feedback of the amplification in the Ca^{2+} release mechanism. Therefore the small Ca^{2+} influx signal caused by I_{Na} was able to trigger a large intracellular Ca^{2+} transient.

I_{Na}-induced Ca^{2+} transients are independent from L- and T-type Ca^{2+} current

During the huge I_{Na} in cardiac myocytes a loss of voltage control is inevitable in the whole-cell configuration of the patch-clamp technique. Ca^{2+} release during I_{Na} as well as during I_{Li} has been reported in the absence of inhibitors for I_{Ca} (Sham *et al.* 1992) suggesting that a significant loss of voltage control during I_{Na} might indeed activate Ca^{2+} channels in *their* experiments. However, our observation of substantial kinetic differences in the I_{Na}- and I_{Ca}-induced Ca^{2+} transients indicates that voltage escape cannot be the only explanation for I_{Na}-induced Ca^{2+} signals. This view is supported by the effects of the Ca^{2+} channel blocker verapamil, which clearly affected only I_{Ca}-induced Ca^{2+} release but had virtually no effect on I_{Na}-triggered release.

Additional evidence against a spurious activation of Ca^{2+} channels but also against a contamination of I_{Na} with Ca^{2+} (Johnson & Lemieux, 1991) could be derived from the Li^+ substitution experiments. Li^+ ions in the extracellular solution can substitute for Na^+ ions in carrying current via Na^+ channels (Hille, 1992) and thus generated a similar loss of voltage control. However, Li^+ is not able to substitute for Na^+ in the Na^+–Ca^{2+} exchange cycle. Therefore, the absence of Ca^{2+} release during application of Li^+ demonstrates that (i) the voltage step itself was not capable of inducing Ca^{2+} release; (ii) spurious activation of L- or T-type Ca^{2+} channels due to a loss of voltage control during I_{Li} and thus also during I_{Na} was not sufficient to trigger Ca^{2+} release or to induce a residual Ca^{2+} transient; (iii) Ca^{2+} contamination with the Na^+ influx was insignificant (Fig. 4); and (iv) direct activation of the Ca^{2+} influx mode of the Na^+–Ca^{2+} exchange by the voltage step was negligible. Because the probability of SR Ca^{2+} release also seems to depend on the Ca^{2+} loading state of the SR (Leblanc & Hume, 1990) and Ca^{2+} release may even occur spontaneously (Lipp & Niggli, 1993*b*), the Li^+ effect on the residual Ca^{2+} transient is an even more direct test for the role of the Na^+–Ca^{2+} exchange activation. Since the Na^+–Ca^{2+} exchange is highly specific for Na^+ over Li^+, the absence of a residual transient when Li^+ is the charge carrier represents clear evidence for the importance of a rise in $[Na^+]_i$ and the Na^+–Ca^{2+} exchange in the generation of I_{Na}-induced Ca^{2+} signals.

Physiological role of I_{Na}-induced Ca^{2+} release

The existence of significant concentration gradients during cardiac E–C coupling has been reviewed recently for Ca^{2+} (Niggli & Lipp, 1993) and Na^+ (Carmeliet, 1992). While the important role of I_{Ca} during cardiac E–C coupling is unquestioned, a possible direct role of I_{Na} in this process is still disputed. In this paper we show that I_{Na} has an accelerating effect and also increases the amplitude of the Ca^{2+} transient. During the upstroke of the action potential I_{Na} rapidly activates Ca^{2+} release from the SR and thus may serve as a safety factor for cardiac E–C coupling. While I_{Na} provides a rapid and close functional coupling between cell membrane and SR Ca^{2+} release channels, I_{Ca} ensures sustained release and serves as a source for refilling the intracellular stores.

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