Rapid Report

Intracellular Ca^{2+} release contributes to automaticity in cat atrial pacemaker cells

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1. The cellular mechanisms governing cardiac atrial pacemaker activity are not clear. In the present study we used perforated patch voltage clamp and confocal fluorescence microscopy to study the contribution of intracellular Ca^{2+} release to automaticity of pacemaker cells isolated from cat right atrium.

2. In spontaneously beating pacemaker cells, an increase in subsarcolemmal intracellular Ca^{2+} concentration occurred concomitantly with the last third of diastolic depolarization due to local release of Ca^{2+} from the sarcoplasmic reticulum (SR), i.e. Ca^{2+} sparks. Nickel (Ni^{2+}; 25–50 μM), a blocker of low voltage-activated T-type Ca^{2+} current ($I_{Ca,T}$), decreased diastolic depolarization, prolonged pacemaker cycle length and suppressed diastolic Ca^{2+} release.

3. Voltage clamp analysis indicated that the diastolic Ca^{2+} release was voltage dependent and triggered at about −60 mV. Ni^{2+} suppressed low voltage-activated Ca^{2+} release. Moreover, low voltage-activated Ca^{2+} release was paralleled by a slow inward current presumably due to stimulation of Na^{+}–Ca^{2+} exchange ($I_{Na\text{-}Ca}$). Low voltage-activated Ca^{2+} release was found in both sino-atrial node and latent atrial pacemaker cells but not in working atrial myocytes.

4. These findings suggest that low voltage-activated $I_{Ca,T}$ triggers subsarcolemmal Ca^{2+} sparks, which in turn stimulate $I_{Na\text{-}Ca}$ to depolarize the pacemaker potential to threshold. This novel mechanism indicates a pivotal role for $I_{Ca,T}$ and subsarcolemmal intracellular Ca^{2+} release in normal atrial pacemaker activity and may contribute to the development of ectopic atrial arrhythmias.

Electrical excitation of the mammalian heart originates from specialized pacemaker cells located in specific regions of the right atrium. The right atrium contains both primary (sino-atrial (SA) node) and secondary (latent) pacemaker cells. Latent atrial pacemakers are specialized cells localized in specific regions of the inferior right atrium (Rubenstein *et al.* 1987). SA node pacemaker cells maintain the normal heartbeat whereas latent atrial pacemakers contribute to various types of atrial arrhythmias. In general, the electrical activity of cardiac pacemakers is thought to depend exclusively on ion channel currents within the plasma membrane, including: (i) the hyperpolarization-activated inward current, (ii) time-dependent decay of K^{+} conductance, (iii) inward T-type and L-type Ca^{2+} currents, (iv) inward leak currents, and (v) the lack of background K^{+} conductance (Noma *et al.* 1984; Hagiwara *et al.* 1988; DiFrancesco, 1991; Wu *et al.* 1991; Campbell *et al.* 1992; Zhou & Lipsius, 1992, 1993, 1994). In particular, low voltage-activated T-type Ca^{2+} current ($I_{Ca,T}$) is thought to contribute to the last third or late phase of diastolic depolarization in both SA node and latent atrial pacemakers (Hagiwara *et al.* 1988; Zhou & Lipsius, 1994). However, inhibition of intracellular Ca^{2+} release from the sarcoplasmic reticulum (SR) by ryanodine depresses diastolic depolarization in both SA node (Lipsius, 1989; Rigg & Terrar, 1996; Li *et al.* 1997) and latent atrial (Rubenstein & Lipsius, 1989; Zhou & Lipsius, 1993) pacemakers, indicating that intracellular Ca^{2+} release also plays a prominent role in bringing the late pacemaker potential to threshold. Diastolic Ca^{2+} release is thought to depolarize the pacemaker potential by stimulating inward Na^{+}–Ca^{2+} exchange current ($I_{Na\text{-}Ca}$) (Zhou & Lipsius, 1993). However, a clear demonstration of diastolic Ca^{2+} release in pacemaker cells has not yet been provided and the mechanisms responsible for initiating diastolic Ca^{2+} release in these cells are not understood. In the present study, we report that atrial pacemaker cells exhibit a local release of subsarcolemmal intracellular Ca^{2+}, i.e. Ca^{2+} sparks (Cheng *et al.* 1993), during late diastole, which is triggered by low voltage-activated $I_{Ca,T}$. This novel pacemaker mechanism

contributes to normal pacemaker function and may underlie abnormal atrial pacemaker activities promoted by alterations in intracellular Ca^{2+} release.

METHODS

Adult cats of either sex were anaesthetized with sodium pentobarbital (70 mg kg^{-1} I.P.). After bilateral thoracotomy, the heart was rapidly excised and placed on a Langendorff perfusion apparatus for cell isolation, as described previously (Wang & Lipsius, 1996). Pacemaker cells are distinguished from atrial muscle cells by their morphology (smaller size, lack of striations, tapered ends), pacemaker activity and the presence of hyperpolarization-activated inward current (Wu *et al.* 1991; Zhou & Lipsius, 1992). Latent pacemaker cells were isolated from a specific region of the inferior right atrium at its junction with the inferior vena cava, and SA node pacemaker cells were isolated from the superior right atrium at its junction with the superior vena cava (Rubenstein *et al.* 1987). Isolated cells were plated on glass coverslips and transferred onto the stage of an inverted microscope (Axiovert 100, Carl Zeiss) attached to a confocal scanner unit (LSM 410, Carl Zeiss). The Ca^{2+} indicator fluo-3 (Molecular Probes) was introduced into the myoplasm by exposure of the cells to the membrane-permeant acetoxymethyl ester form of the dye (fluo-3 AM, 5 μM for 30 min at room temperature). During experiments, cells were continuously superfused with Tyrode solution containing (mM): NaCl, 137; KCl, 5·4; $MgCl_2$, 1·0; $CaCl_2$, 2·0; Hepes, 5; glucose, 11; titrated with NaOH to a pH of 7·4. All experiments were performed at 33 ± 2 °C. Membrane voltage and currents were recorded with an Axopatch 200A amplifier (Axon Instruments) using a nystatin (150 μg ml^{-1})-perforated patch (Horn & Marty, 1988) whole-cell recording method (Hamill *et al.* 1981). The internal pipette solution contained (mM): potassium glutamate, 100; KCl, 40; $MgCl_2$, 1·0; Na_2-ATP, 4; EGTA, 0·5; Hepes, 5; titrated with KOH to a pH of 7·2. Fluo-3 fluorescence was excited using an argon-ion laser at 488 nm and simultaneously recorded at wavelengths $\geqslant 515$ nm. Confocal linescan images were acquired at a rate of 250 Hz. $[Ca^{2+}]_i$ images were calculated using the formula:

$$[Ca^{2+}]_i = K_d R/((K_d/[Ca^{2+}]_{rest}) - R + 1)$$

(Cannell *et al.* 1994), where R is the normalized fluorescence (F/F_{rest}) and $K_d = 1{\cdot}1$ μM. A trigger signal generated by the voltage clamp protocol was recorded simultaneously by the confocal imaging system to allow synchronization of electrophysiological and Ca^{2+} measurements. For further details of methods see Hüser *et al.* (1996) and Blatter *et al.* (1997).

The animal procedures used in this study were in accordance with the guidelines of the Animal Care and Use Committee of Loyola University Medical Center.

RESULTS

Figure 1 illustrates pacemaker action potentials (*A*) and spatially resolved changes in intracellular Ca^{2+} concentration (*B*) recorded from a spontaneously beating latent atrial pacemaker cell. The subsarcolemmal Ca^{2+} concentration ($[Ca^{2+}]_{ss}$) is known to regulate plasma membrane conductances, including $I_{Na\text{-}Ca}$. Therefore, changes in $[Ca^{2+}]_{ss}$ were visualized by positioning the repetitively scanned line (250 Hz) used to obtain the linescan image parallel to the longitudinal axis of the cell and close to the sarcolemmal membrane (Fig. 1*D*, inset). Each pacemaker action potential coincided with a rapid rise in $[Ca^{2+}]_{ss}$ due to Ca^{2+} release from the SR triggered by Ca^{2+} entry through voltage-gated Ca^{2+} channels. Ca^{2+} release occurred almost simultaneously throughout the subsarcolemmal space. No change in $[Ca^{2+}]_{ss}$ was observed immediately following repolarization. However, $[Ca^{2+}]_{ss}$ gradually increased during the late phase of the pacemaker depolarization prior to the action potential, resulting in a small diastolic 'Ca^{2+} pedestal' in recordings averaged over the subsarcolemmal space (Fig. 1*B*). Recordings of $[Ca^{2+}]_{ss}$ from a restricted region (~1 μm) close to an active release site (Fig. 1*C*) revealed that the Ca^{2+} pedestal was caused by the summation of individual local Ca^{2+} release events, i.e. Ca^{2+} sparks. The surface plot (Fig. 1*D*) demonstrates the absence of Ca^{2+} sparks during early diastolic depolarization immediately following the prior pacemaker action potential, and a gradual increase in Ca^{2+} spark frequency during the late pacemaker depolarization. In this cell, the diastolic Ca^{2+} pedestal became detectable with the occurrence of the first subsarcolemmal Ca^{2+} spark at a membrane potential of −57 mV. Similar results were obtained in seven pacemaker cells.

To determine whether the mechanism responsible for triggering diastolic Ca^{2+} release depended on membrane depolarization or whether it was a voltage-independent mechanism inherent to the SR, we studied Ca^{2+} release in pacemaker cells under voltage clamp conditions. The pacemaker action potential was approximated by a ramp–step voltage protocol (Fig. 2*A–C*, left panels) which followed nine pre-conditioning pulses to ensure steady-state loading of SR Ca^{2+}. The $[Ca^{2+}]_{ss}$ transient elicited by L-type Ca^{2+} current ($I_{Ca,L}$) was preceded by a smaller Ca^{2+} release triggered during the ramp depolarization at a potential of −57 mV. This low voltage-activated Ca^{2+} release (LVCR) seen under voltage clamp conditions mimicked the diastolic Ca^{2+} pedestal recorded from spontaneously beating pacemaker cells, and successfully reproduced the pattern of changes in $[Ca^{2+}]_{ss}$. Like the diastolic Ca^{2+} pedestal, LVCR resulted from the summation of individual subsarcolemmal Ca^{2+} sparks. As shown in Fig. 2*A–C* (right panels), when the ramp depolarization was omitted and membrane voltage was held constant (−70 mV) prior to the voltage step, diastolic Ca^{2+} release was not observed. These findings clearly rule out the possibility that a spontaneous time-dependent mechanism is responsible for the increase in frequency of Ca^{2+} sparks during late diastole. Rather, Ca^{2+} release during the ramp and therefore during the late phase of diastolic depolarization is dependent on membrane depolarization. The voltage threshold of −57 mV excludes activation of L-type Ca^{2+} channels and subsequent Ca^{2+}-induced Ca^{2+} release (CICR) as the underlying mechanism. It is compatible, however, with CICR induced by Ca^{2+} entry through low voltage-activated T-type Ca^{2+} channels (Zhou & January, 1998; Sipido *et al.* 1998). Similar results were obtained in four pacemaker cells.

The results shown in Fig. 3 support the idea that $I_{Ca,T}$ triggers diastolic Ca^{2+} release. Micromolar concentrations of

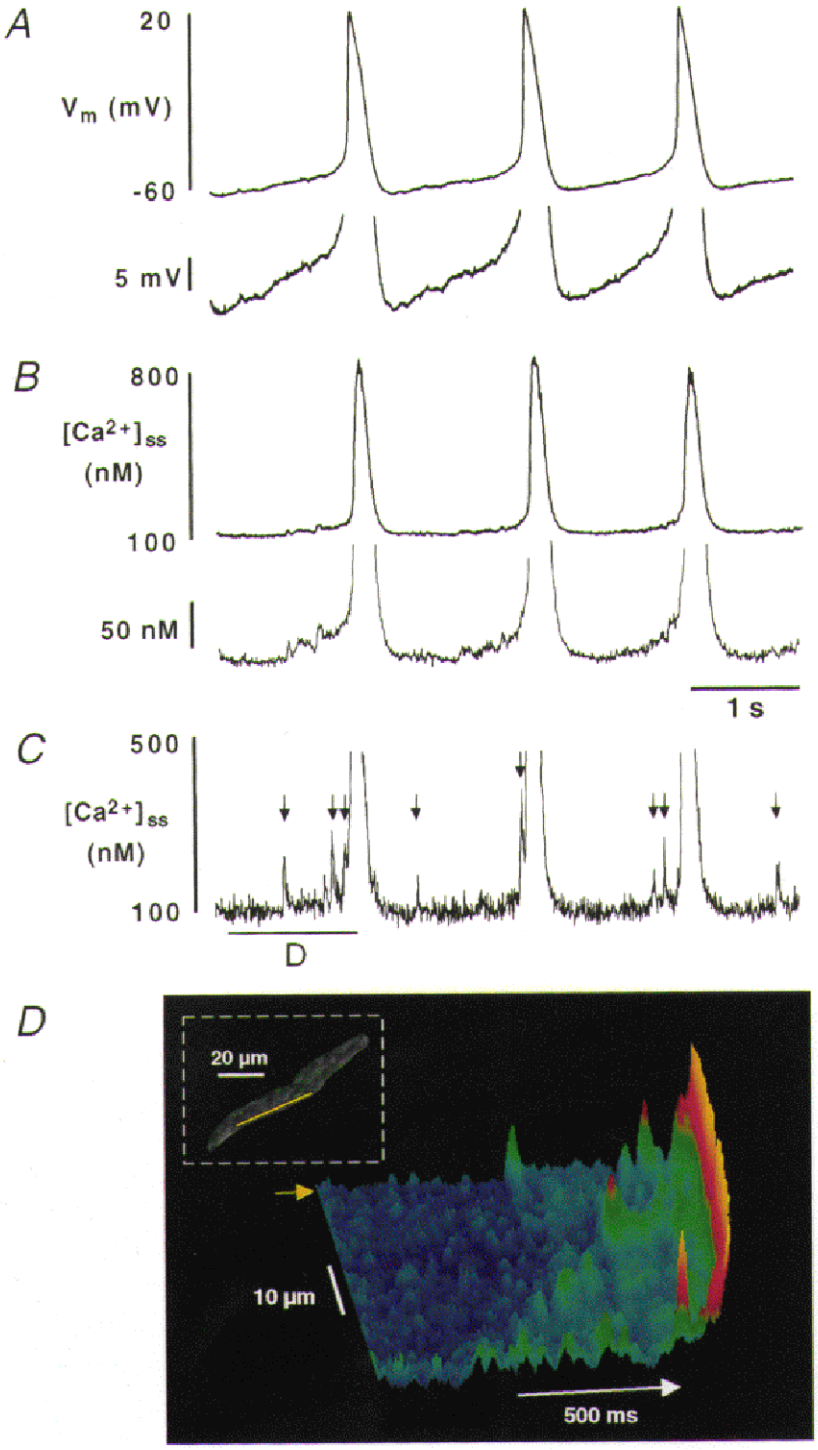

Figure 1. Ca^{2+} sparks precede the action potential in spontaneously beating latent atrial pacemaker cells

Simultaneous recordings of membrane voltage (V_m; *A*) and subsarcolemmal Ca^{2+} concentration ($[Ca^{2+}]_{ss}$; *B*). The lower traces in each panel show amplified recordings of diastolic voltage (*A*) and $[Ca^{2+}]_{ss}$ (*B*). The amplified traces in *B* show an increase in $[Ca^{2+}]_{ss}$ during the late pacemaker depolarization preceding the action potential ('Ca^{2+} pedestal'). $[Ca^{2+}]_{ss}$ started to increase at threshold potentials of about −58 mV. *C*, local $[Ca^{2+}]$ transients recorded by averaging over a short distance (1 μm) of the scanned line intersecting with a site displaying localized Ca^{2+} release (arrow in *D*). The arrows mark individual Ca^{2+} sparks. *D*, surface plot representation of the linescan image illustrating changes in $[Ca^{2+}]_{ss}$ during the diastolic depolarization preceding the first action potential (indicated by the horizontal bar in *C*). The inset in *D* indicates the positioning of the line.

Ni^{2+} (25–50 μM), a blocker of cardiac T-type Ca^{2+} channels (Hagiwara *et al.* 1988; Zhou & Lipsius, 1994; Lee *et al.* 1999), decreased the slope of diastolic depolarization (Fig. 3*A*), prolonged pacemaker cycle length by 230% compared with control and suppressed the diastolic Ca^{2+} pedestal which preceded the $[Ca^{2+}]_{ss}$ transients triggered by the pacemaker action potentials (Fig. 3*B*). Under voltage clamp conditions, Ni^{2+} also inhibited LVCR in the voltage range between −60 and −45 mV (Fig. 3*C*) but was without effect on Ca^{2+} release triggered by $I_{Ca,L}$ at potentials positive to −45 mV (not shown). At the same time, no significant effect of Ni^{2+} was observed on peak $I_{Ca,L}$ or $[Ca^{2+}]_{ss}$ transients triggered by step depolarizations or action potentials. Finally, Fig. 3*D* illustrates that in a latent atrial pacemaker cell the diastolic increase in $[Ca^{2+}]_i$ initiated at −58 mV was paralleled by a slow inward current in response to voltage ramps. The onset of the inward current dip coincided with the rise in $[Ca^{2+}]_{ss}$. The slow ramp depolarization and the relatively large amplitude of this inward current makes it unlikely that it is due to activation of $I_{Ca,T}$. A similar Ca^{2+} release-dependent current that develops during the late phase of the pacemaker potential has been previously identified in these cells as $I_{Na\text{-}Ca}$ (Zhou & Lipsius, 1993). Similar results were obtained in six pacemaker cells.

Further experiments indicated that LVCR is specific for pacemaker cells. As shown in Fig. 4*A*, non-pacemaker atrial myocytes completely lacked LVCR when stimulated with a ramp–step protocol. When atrial cells were stimulated with a continuous voltage ramp from −70 to 0 mV (Fig. 4*B*), Ca^{2+} release was invariably initiated at potentials positive to −40 mV ($n = 20$), consistent with Ca^{2+} release triggered by $I_{Ca,L}$. With the continuous ramp protocol (Fig. 4*B*), Ca^{2+} release triggered by $I_{Ca,L}$ was associated with a slow transient inward current characteristic of $I_{Na\text{-}Ca}$. In addition, the inhibitory effect of ryanodine on the electrical activity of SA node pacemaker cells (Lipsius, 1989; Rigg & Terrar, 1996; Li *et al.* 1997) suggests that LVCR also operates in SA node pacemakers. In Fig. 4*C* and *D*, single SA node

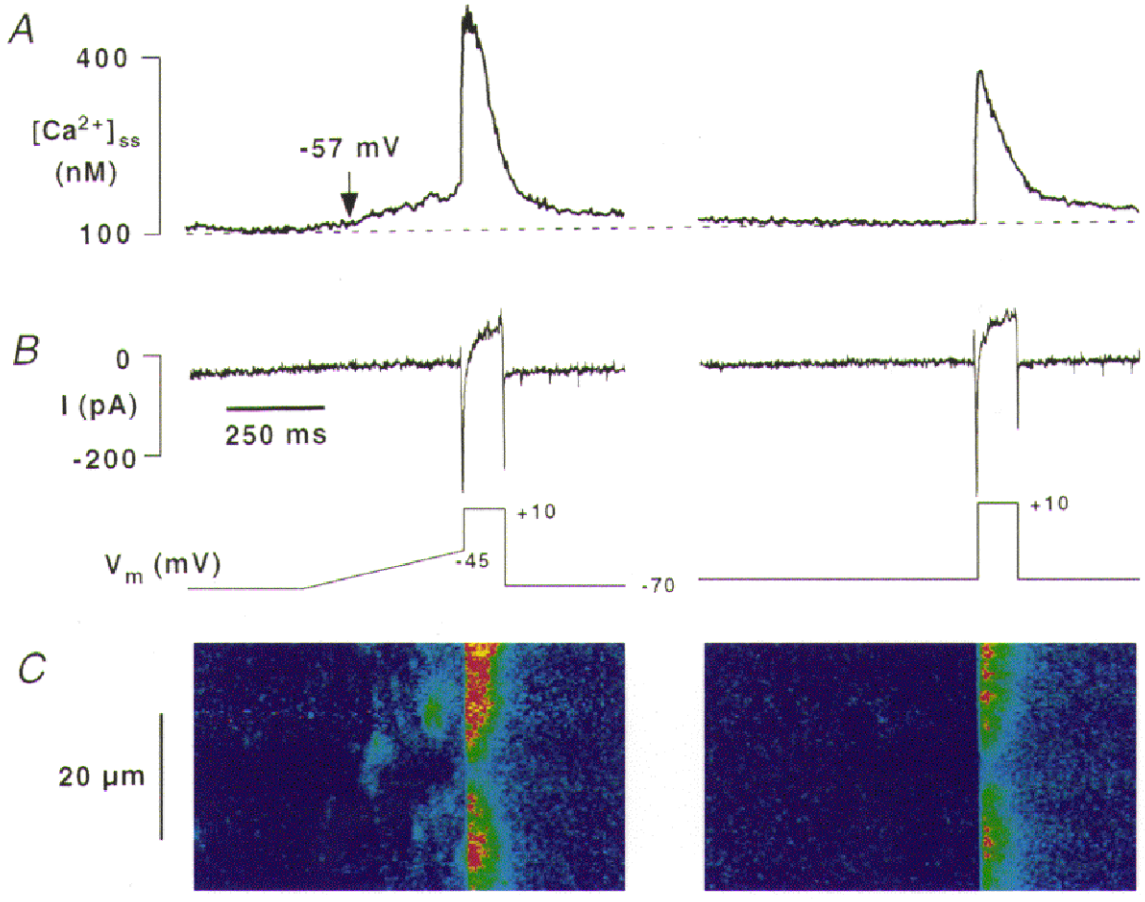

Figure 2. Pacemaker Ca^{2+} sparks are triggered by a voltage-dependent mechanism

Changes in $[Ca^{2+}]_{ss}$ (line plot presentation, *A*; linescan image, *C*) and membrane currents (*B*) in a latent atrial pacemaker cell in response to the voltage clamp protocol shown below the current trace. A depolarizing voltage clamp ramp from −70 to −45 mV (400 ms) prior to the voltage step to +10 mV (150 ms) elicited LVCR which reproduced the diastolic Ca^{2+} pedestal seen in spontaneous pacemaker cells (left panels). In the same cell, when the voltage ramp was omitted (right panels), no sparks were detected prior to the $[Ca^{2+}]_{ss}$ transient elicited by the step depolarization. Prolongation of the interval between beats also did not result in detection of spontaneous Ca^{2+} sparks (not shown).

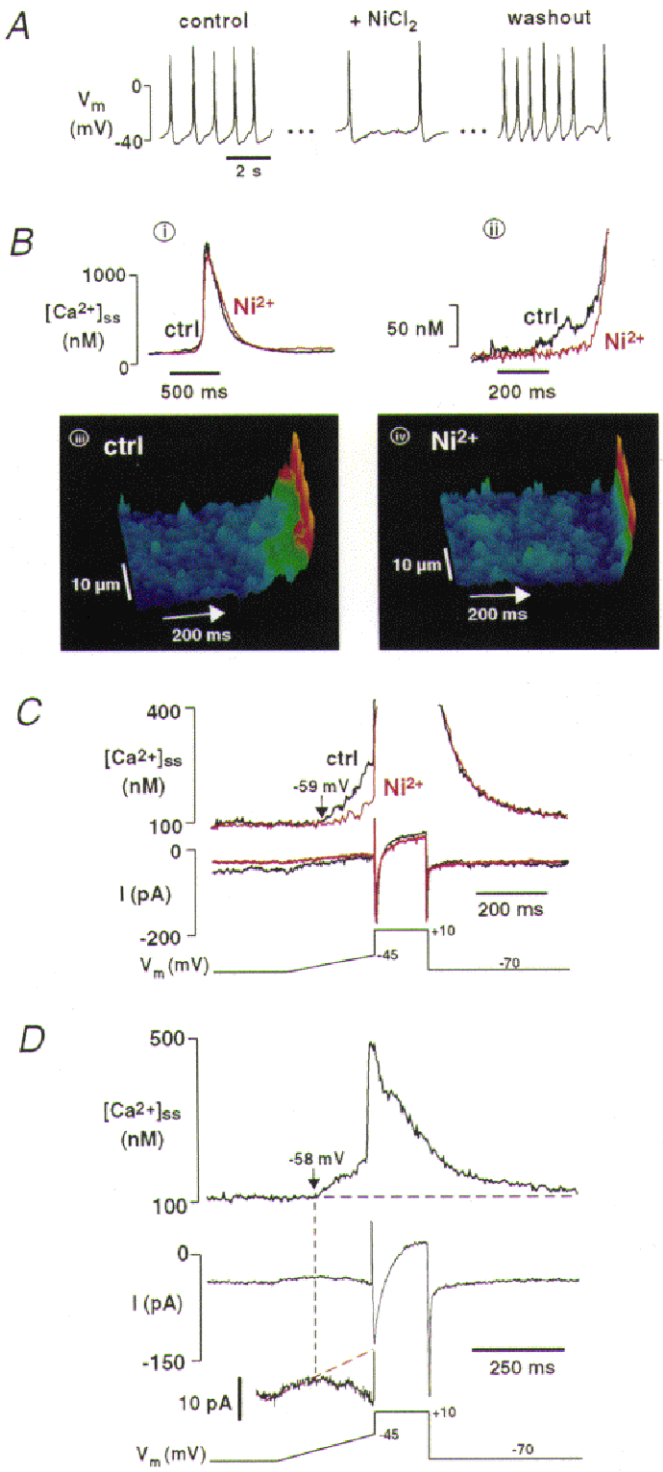

Figure 3. Nickel inhibits diastolic Ca^{2+} release in spontaneously active and voltage clamped pacemaker cells

A, Ni^{2+} (50 μM) reversibly slowed the rate of a spontaneously beating latent atrial pacemaker cell by selectively decreasing the slope of the late phase of diastolic depolarization. *B*, Ni^{2+} (50 μM) inhibited the diastolic Ca^{2+} pedestal (ii, and surface plots in iii and iv) without a significant reduction of the $[Ca^{2+}]_{ss}$ transient triggered by the action potential (i). Spontaneous $[Ca^{2+}]_{ss}$ transients were aligned to the point of maximum rate of rise. ctrl, control. *C*, in another voltage clamped pacemaker cell, Ni^{2+} (25 μM) inhibited LVCR initiated at −59 mV. Peak $I_{Ca,L}$ elicited by the step depolarization was unaffected by Ni^{2+}. *D*, $[Ca^{2+}]_{ss}$ and membrane current in response to a ramp–step depolarization in a third latent atrial pacemaker cell. During the ramp depolarization the current deviated from the expected linear change in background current (dashed line). The onset of inward current coincided with the rise in $[Ca^{2+}]_{ss}$ indicative of $I_{Na\text{-}Ca}$ stimulation.

pacemaker cells isolated from the same cat hearts were voltage clamped using the ramp–step (*C*) or continuous ramp (*D*) protocols. LVCR was detected in both cases at threshold voltages of −55 mV (*C*) and −52 mV (*D*). Using the continuous ramp protocol (*D*), the Ca^{2+} release appeared biphasic, compatible with Ca^{2+} release triggered by low voltage-activated $I_{Ca,T}$ (−52 mV) and high voltage-activated $I_{Ca,L}$ (−36 mV). The larger Ca^{2+} release triggered at more positive voltages by $I_{Ca,L}$ was associated with a transient stimulation of inward $I_{Na\text{-}Ca}$ (Fig. 4*D*). It therefore appears that LVCR is specific for pacemaker cells and is not found in regular atrial muscle cells. Similar results were obtained in three SA node pacemaker cells.

DISCUSSION

In both SA node (Hagiwara *et al.* 1988) and latent atrial pacemakers (Zhou & Lipsius, 1994) activation of $I_{Ca,T}$ contributes to the last third or late phase of diastolic depolarization. Likewise, the contribution of SR Ca^{2+} release to both SA node (Lipsius, 1989; Rigg & Terrar, 1996; Li *et al.* 1997) and latent atrial (Rubenstein & Lipsius, 1989; Zhou & Lipsius, 1993) pacemaker function has been implied from the observation that ryanodine, an alkaloid that blocks SR Ca^{2+} release (Rousseau *et al.* 1987), inhibits the late phase of diastolic depolarization. The present report directly demonstrates, in both SA node and latent atrial pacemaker cells, that highly localized subsarcolemmal Ca^{2+} release from the SR, i.e. Ca^{2+} sparks, occurs concurrently with the late phase of diastolic depolarization. Moreover, several findings indicate that diastolic Ca^{2+} spark activity was triggered by $I_{Ca,T}$: (1) diastolic Ca^{2+} sparks were elicited at low voltages compatible with $I_{Ca,T}$ and clearly distinguished from $I_{Ca,L}$-triggered Ca^{2+} release (positive to −40 mV); (2) diastolic Ca^{2+} release, as well as diastolic depolarization, were inhibited by micromolar concentrations of Ni^{2+}, a blocker of cardiac T-type Ca^{2+} channels (Hagiwara *et al.* 1988; Zhou & Lipsius, 1994; Lee *et al.* 1999). Moreover,

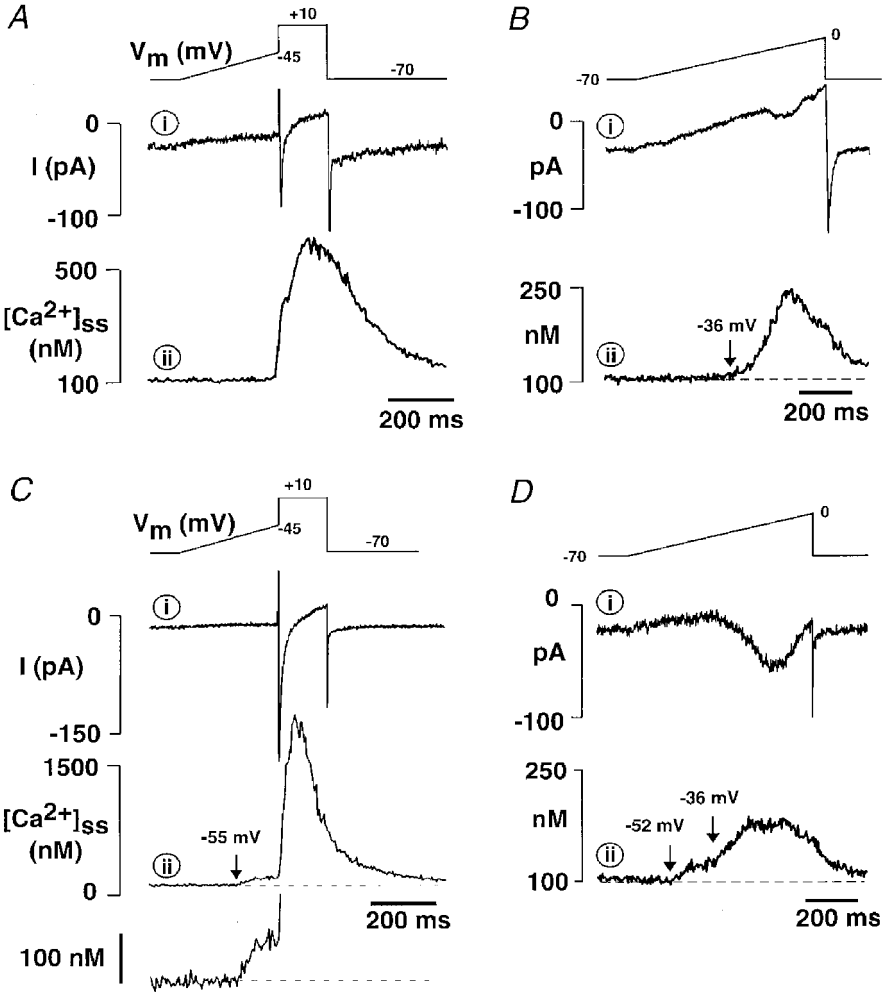

Figure 4. LVCR is present in primary pacemakers from the SA node, but absent in non-pacemaker atrial muscle cells

Response of membrane current (i) and $[Ca^{2+}]_{ss}$ (ii) to a ramp–step depolarization (left panels) or a continuous ramp (right panels) in a non-pacemaker atrial muscle cell (*A* and *B*) and a SA node pacemaker cell (*C* and *D*). In the regular atrial muscle cell, Ca^{2+} release was triggered at −36 mV. In the SA node pacemaker cell, two components of Ca^{2+} release were elicited at −52 and −36 mV.

the fact that atrial myocytes lack LVCR and only exhibit Ca^{2+} release triggered by $I_{Ca,L}$ emphasizes the unique role of LVCR in pacemaker cells. The absence of this mechanism in working atrial myocytes can be explained by the fact that atrial pacemaker cells possess a 5-fold higher density of $I_{Ca,T}$ than non-pacemaker atrial cells (Zhou & Lipsius, 1994). This higher $I_{Ca,T}$ density in pacemaker cells increases the probability that T-type Ca^{2+} channels will trigger Ca^{2+} release from subsarcolemmal Ca^{2+} stores. Although the mechanism is present in both SA node and latent atrial pacemaker cells, its relative contribution to pacemaker function may be more prominent in latent than SA node pacemaker function. This is based on our previous findings which indicate that inhibition of SR Ca^{2+} release by ryanodine slows pacemaker rate significantly more in latent atrial than SA node pacemaker cells (Lipsius, 1989), and that latent atrial pacemaker cells exhibit a more prominent presence and size of subsarcolemmal SR cisternae compared with SA node pacemaker cells (Rubenstein *et al.* 1987). The present results also suggest that LVCR stimulates inward $I_{Na\text{-}Ca}$, which would depolarize the pacemaker potential to threshold (Fig. 3*D*). $I_{Na\text{-}Ca}$ has been reported to contribute significantly to automaticity in latent atrial pacemakers (Zhou & Lipsius, 1993) as well as SA node pacemakers (Campbell *et al.* 1992). The depolarizing effect of $I_{Na\text{-}Ca}$ is expected to elicit further voltage-dependent activation of $I_{Ca,T}$–Ca^{2+} spark activity, resulting in a positive feedback mechanism contributing to the late phase of diastolic depolarization.

Single T-type channel openings may be sufficient to trigger Ca^{2+} sparks (Stern, 1992; López-López *et al.* 1995). Local elevation of $[Ca^{2+}]_{ss}$ will stimulate Na^+–Ca^{2+} exchange carrier molecules in the vicinity of the SR Ca^{2+} release sites, providing additional inward current for as long as $[Ca^{2+}]_{ss}$ stays elevated. A Ca^{2+} spark (~20–50 ms at 33 °C) outlasts a single T-type Ca^{2+} channel opening (mean open time, 2·3 ms; Balke *et al.* 1992). Therefore, stimulation of $I_{Na\text{-}Ca}$ both amplifies the unitary T-type current event and prolongs in time inward current flow, thereby allowing efficient temporal summation of $I_{Ca,T}$–Ca^{2+} spark events. The fact that inhibition of SR Ca^{2+} release by ryanodine markedly prolongs pacemaker cycle length in latent atrial (Zhou & Lipsius, 1993) and SA node (Lipsius, 1989; Rigg & Terrar, 1996; Li *et al.* 1997) pacemaker cells in the presence of intact, functional T-type Ca^{2+} channels, emphasizes the importance of this mechanism in normal pacemaker function. In ryanodine-treated cells, $I_{Ca,T}$ alone cannot sustain the steep slope of the pacemaker depolarization. The currents associated with a single 'pacemaker spark' are below the resolution of whole-cell recordings. We can, however, speculate that with a membrane resistance of ~2 GΩ (Wu *et al.* 1991) and estimates of Ca^{2+} fluxes underlying Ca^{2+} sparks of ~3 pA (Blatter *et al.* 1997) only a few local events, well within the range of spark frequencies observed in spontaneously beating cells, are necessary to depolarize the late diastolic slope to threshold (ΔV, ~5–10 mV).

The present findings provide another example of subcellular Ca^{2+} release events that control membrane excitability. For example, in smooth muscle cells Ca^{2+} sparks can activate Ca^{2+}-dependent K^+ channels to cause hyperpolarization (Nelson *et al.* 1995) or Ca^{2+}-activated Cl^- channels to cause depolarization (ZhuGe *et al.* 1998). Moreover, interstitial cells of Cajal, the gastrointestinal pacemakers, exhibit rhythmic Ca^{2+} release from IP_3-dependent Ca^{2+} stores activating a Ca^{2+}-dependent cation current that drives the pacemaker depolarization (Thomsen *et al.* 1998). Ca^{2+} release in these cells, however, appears to be driven by an oscillator within the endoplasmic reticulum/Ca^{2+} store. In contrast, Ca^{2+} release and $I_{Na\text{-}Ca}$ in cardiac pacemaker cells serve as a feedback amplifier tightly coupled to the plasma membrane pacemaker mechanism via activation of $I_{Ca,T}$. Because intracellular Ca^{2+} release is sensitive to a variety of second messenger signalling mechanisms as well as variations in SR Ca^{2+} load, this mechanism is a potential site for regulation of heart rate by autonomic neurotransmitters, hormones or drugs. Moreover, because latent atrial pacemakers appear to rely more on SR Ca^{2+} release for their pacemaker mechanism than SA node pacemakers, drugs or disease processes that enhance intracellular Ca^{2+} content and/or release may be expected to enhance latent atrial pacemaker automaticity. These findings, therefore, may provide insight into the etiologies and potential treatments of atrial arrhythmias resulting from enhanced forms of automaticity.

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