Expression of T- and L-type calcium channel mRNA in murine sinoatrial node

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Abstract At the cellular level, cardiac pacemaking which sets the rate and rhythm of the heartbeat is produced by the slow diastolic depolarization. Several ion channels contribute to this pacemaker depolarization, including T-type and L-type calcium currents. To evaluate the molecular basis of the currents involved, we investigated the cellular distribution of various low voltage activated (LVA) and high voltage activated (HVA) calcium channel mRNAs in the murine sinoatrial (SA) node by in-situ hybridization. The most prominently expressed LVA calcium channel in the SA node is $Ca_v3.1$, whereas $Ca_v3.2$ is present at moderate levels. The dominant HVA calcium channel transcript is $Ca_v1.2$; only traces of $Ca_v1.3$ mRNA are detectable in SA myocytes of mice.

Key words: Electrophysiology; Calcium channel; T-type; L-type; In-situ hybridization; Cardiac ion channel

1. Introduction

Electrical excitation of the mammalian heart originates from specialized pacemaker myocytes located in restricted areas. The normal heartbeat in mammals is maintained by the primary cardiac pacemaking tissue, the sinoatrial (SA) node. Spontaneous activity in SA node cells results from a characteristic phase of their action potential, the slow diastolic depolarization [1,2]. During this phase, which follows the termination of an action potential, the membrane slowly depolarizes until the threshold for a new action potential is reached. The slow diastolic depolarization of cardiac pacemaker cells has been characterized to a large extent by electrophysiological methods; it is thought to depend on a number of ion channel currents, including (i) the hyperpolarization activated inward current I_f, (ii) an inward T-type calcium current $I_{Ca,T}$, (iii) an inward L-type calcium current $I_{Ca,L}$ and (iv) possibly a net inward background conductance which becomes prominent during the decay of the delayed rectifier K^+ conductance [3–7]. The role of I_{Ca} in pacemaker depolarizations has been discussed extensively [8–10]. While I_f drives the initial phase of the diastolic depolarization, the calcium currents are proposed to contribute to the last third of the diastolic depolarization and to the upstroke of the action potential [11]. So far, the molecular basis of these calcium currents is unclear. The expression of the high voltage activated (HVA) calcium channels, $Ca_v1.2$ and $Ca_v1.3$, in heart is well established (for a review, see [12]). Recently, a number of genes encoding low voltage activated (LVA) calcium channels have been cloned. Two of these T-type calcium channel isoforms ($Ca_v3.1$ and $Ca_v3.2$) have been shown to be localized in heart by Northern blot analysis [13–15]. In the present study, we determined the expression of the mRNA of the above LVA and HVA calcium channels in murine SA node using an in-situ hybridization technique.

2. Materials and methods

2.1. Microdissection of murine SA nodes

Adult male Balb/c mice were injected with 300 I.E. heparin i.p. and killed by cervical dislocation. To obtain the SA node, whole hearts were removed and immediately placed in Tyrode's solution (137 mM NaCl, 5.4 mM KCl, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 11.8 mM Na-HEPES, 10 mM glucose; pH 7.4) prewarmed to 37°C. A small piece of tissue ($\sim$1 mm^2) containing the SA node was microdissected from the junction of the superior vena cava (SVC) and right atrium (RA) using a stereo microscope. After dissection, the isolated SA node showed spontaneous contractions at a low frequency (30–60 beats per min). The tissue was fixed in 4% paraformaldehyde at 4°C for 4 h, dehydrated in a graded series of ethanol, cleared with toluene and paraffin-embedded. Sections were cut at 10 μm on a sliding microtome, mounted on Superfrost Plus slides (Menzel-Gläser), heated to 60°C for 3 h and stored at room temperature under dry conditions.

2.2. In-situ hybridization

The sections were deparaffinized in toluene and rehydrated in a series of ethanol (100, 95, 70 and 50%). Slides were pretreated for hybridization as described previously [16] and then prehybridized for 3 h at 42°C in hybridization buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.3 M NaCl, 50 mM DTT, 1× Denhardt's solution, 10% Dextran and 50% deionized formamide). Hybridization proceeded for 16 h at 55°C using each radionucleotide-labeled probe at a specific activity of 5×10^6 cpm/ml. The sections were washed twice in 2×SSC, 1 mM DTT, 1 mM EDTA, then incubated in RNAse A (20 μg/ml) for 30 min at room temperature to remove the unbound probe. Subsequently, a high stringency wash was done using two changes of 0.1×SSC, 1 mM DTT, 1 mM EDTA at 65°C. After dehydration, the slides were exposed to BiomaxMR film (Kodak) for 7 days. For resolution of cellular labeling, slides were coated with liquid film emulsion NTB2 (Kodak) and developed after 6 weeks. Sections were counterstained with hematoxylin–eosin and examined by dark- and bright-field microscopy. [^{35}S]UTP-labeled cRNA probes were transcribed in vitro from fragments including nucleotides (nt) 6128–6481 of murine $Ca_v3.1$ (part of the carboxy-terminal region, [14]) and nt 2355–2628 (loop between repeat II and III) of murine $Ca_v1.2$. We used degenerate primers to polymerase chain reaction-amplify probes of murine $Ca_v1.3$ and $Ca_v3.2$ from a mouse heart cDNA library; the fragments corresponded to amino acids 777–858 and 1024–1119 of human $Ca_v1.3$ [17] and $Ca_v3.2$ [13], respectively. All probes were located in regions that are unique between the various calcium channel isoforms. In-situ hybridizations were always performed with the corresponding sense cRNA probes on adjacent sections. These control hybridizations showed no signals (Figs. 2B, 3B, 4B and 5B).

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3. Results and discussion

3.1. T-type calcium channel isoforms in murine SA node

The molecular identity of the calcium channel isoforms underlying T-type and L-type currents in the SA node has not been elucidated yet. As a first step into this issue, we used in-situ hybridization to determine the expression profile of calcium channel transcripts in SA myocytes. The murine SA node is a flattened, elongated structure measuring about 250 μm in width. It is located above the crista terminalis in the wall of the SVC near its ostium into the RA. Histologically, the SA node cells stain palely in hematoxylin–eosin and have no striations, in contrast to the surrounding working myocardium. In the mouse, the SA node is enmeshed in collagenous connective tissue and has a prominent SA nodal artery (SNA), which serves as a landmark for identification (Fig. 1). Northern analysis has shown [13–15] that the $Ca_v3.1$ and $Ca_v3.2$ T-type calcium channels are present in heart, whereas the $Ca_v3.3$ isoform is a brain-specific channel [18]. Therefore, we examined the SA expression of the $Ca_v3.1$ and $Ca_v3.2$ channel. $Ca_v3.1$ is the dominantly expressed LVA calcium channel in murine SA node (Fig. 2A,C). We estimated that $Ca_v3.1$ transcripts are enriched 30-fold in the SA node above their level in the atrial working myocardium. $Ca_v3.2$ transcripts are also enriched in the SA node (Fig. 3A,C). However, the expression level of the $Ca_v3.2$ transcripts is low compared to that of $Ca_v3.1$. The $Ca_v3.1$ and $Ca_v3.2$ transcripts were clearly expressed in SA nodal myocytes and not in neuronal cells which are commonly observed in the subepicardial tissue near the node and within the nodal area. $Ca_v3.1$ and $Ca_v3.2$ transcripts were not observed in the smooth muscle wall of the nodal artery. Both isoforms are present in the atrial myocytes outside the conduction system (Figs. 2A and 3A). This result is consistent with reports describing T-type calcium currents in working myocytes isolated from this cardiac compartment [19,20].

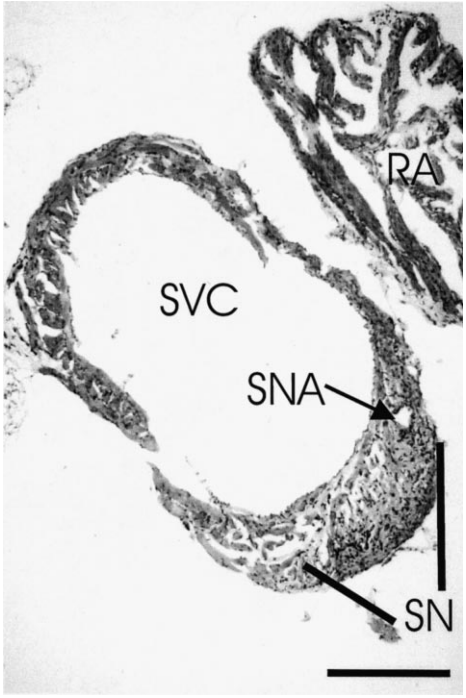

Fig. 1. Hematoxylin–eosin stained paraffin-section through the murine SA node region. SNA: sinus node artery; SVC: superior vena cava; RA: right atrium. The SA node (SN) corresponds to the area surrounding the SNA. Scale bar: 200 μm.

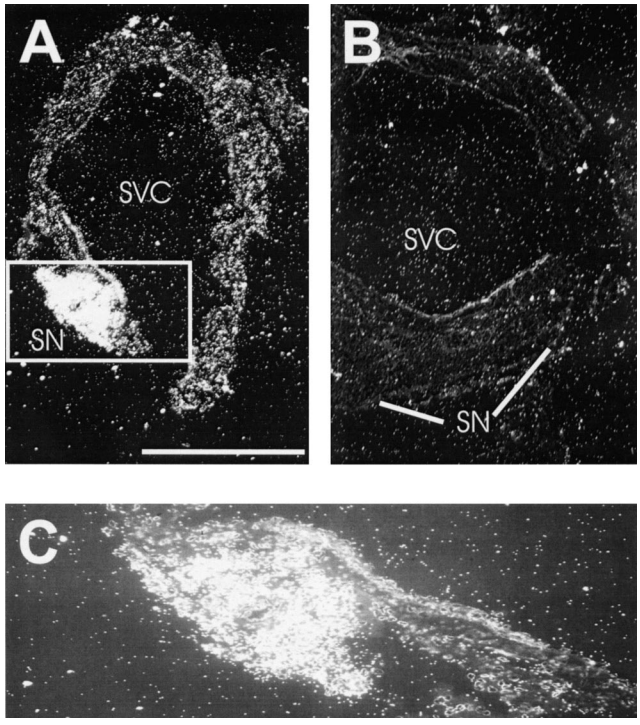

Fig. 2. Dark-field micrographs of hybridizations with ^{35}S-labeled antisense (A) and sense (B) cRNA riboprobes directed against $Ca_v3.1$. C: Magnification of the boxed area marked in (A). Exposure to film emulsion was 8 weeks. Scale bar: 400 μm.

The relative importance of the two T-type calcium channels can be assessed from their differences in the recovery from inactivation. The expressed $Ca_v3.1$ and $Ca_v3.2$ channel recover with a fast ($\sim$80 ms) and a slow (1–2 s) time constant [19]. Eighty percent of the $Ca_v3.1$ current recovered with the fast time constant, whereas 84% of the $Ca_v3.2$ current amplitude recovered with the slow time constant [19]. The $I_{Ca,T}$ of SA nodal cells recovers from inactivation with a time constant of about 140 ms [5]. This comparison suggests, therefore, that the majority of the SA $I_{Ca,T}$ is caused by $Ca_v3.1$. Furthermore, this suggestion agrees excellently with the in-situ hybridization data. In addition to $Ca_v3.1$, $Ca_v3.2$ seems to contribute to the native $I_{Ca,T}$, although to a lesser degree.

3.2. L-type calcium channel isoforms in murine SA node

It is well established that the $Ca_v1.2$ and $Ca_v1.3$ L-type calcium channels are expressed in heart [12,21]. However, it was not known whether or not these channels were present in SA myocytes. In-situ hybridization with a $Ca_v1.3$-specific probe revealed minimal to weak expression of the $Ca_v1.3$ mRNA in the SA node (Fig. 4). Furthermore, and in contrast to the LVA calcium channels, the $Ca_v1.3$ mRNA was not specifically enriched in the SA node region. The weak, but detectable autoradiographic signal was equally distributed all over the SA area and the adjacent working myocardium including the smooth muscle cells of the nodal artery. Identical results (not shown) were obtained with a second probe

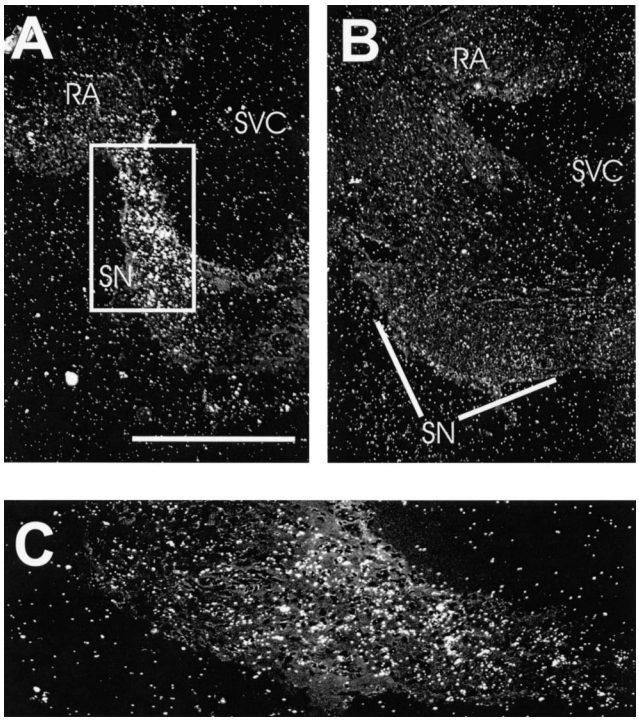

Fig. 3. Dark-field micrographs of hybridizations with ^{35}S-labeled antisense (A) and sense (B) cRNA riboprobes directed against $Ca_v3.2$. C: Magnification of the boxed area shown in (A). Scale bar: 400 μm.

corresponding to amino acids 676–858 of the human $Ca_v1.3$ sequence [17]. These in-situ hybridization results are in agreement with RNAse protection studies [21] showing a low abundance of this channel type mRNA in rat atria. However, the low-level expressed $Ca_v1.3$ channel might be of functional significance in the SA node, since it was reported that $Ca_v1.3$ knockout mice have sinus node arrhythmia at rest and normal sinus rhythm at exercise [22].

The mRNA of the $Ca_v1.2$ calcium channel could be detected clearly in both atrial and SA node myocytes as well as in the arterial smooth muscle cells (Fig. 5). The relative

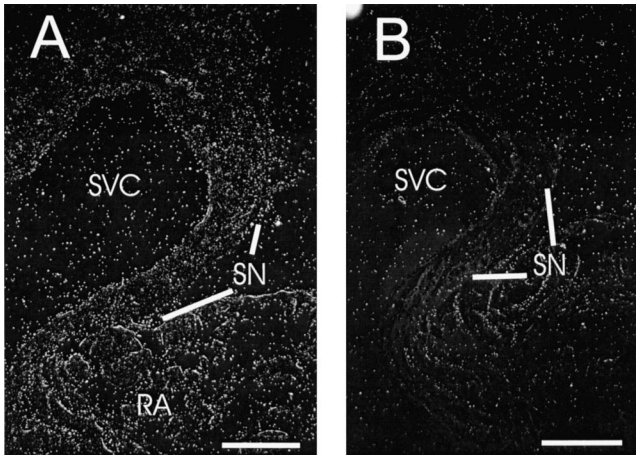

Fig. 4. Dark-field micrograph showing the cellular localization of $Ca_v1.3$ channel transcripts in the murine SA-node and atrium. A: Antisense. B: Sense. Scale bar: 200 μm.

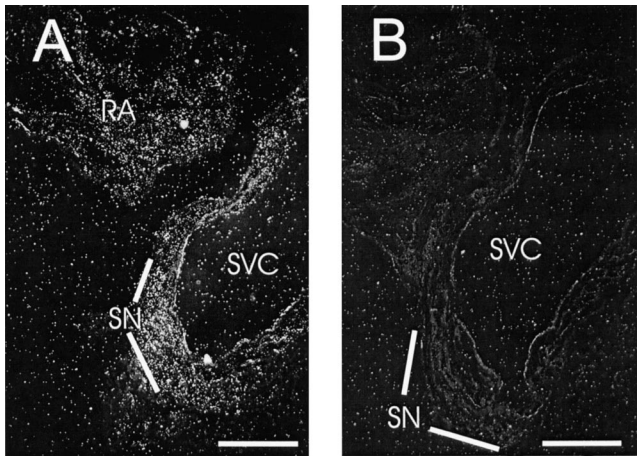

Fig. 5. Dark-field micrographs of hybridizations with ^{35}S-labeled antisense (A) and sense (B) cRNA riboprobes directed against $Ca_v1.2$. Scale bar: 200 μm.

abundance of $Ca_v1.2$ transcripts was higher than that of the $Ca_v1.3$ mRNA (compare Figs. 4 and 5). Unlike $Ca_v1.3$, the signal of $Ca_v1.2$ showed a small but yet significant enrichment in the SA node region.

3.3. Conclusion

The exact mechanism of the slow diastolic depolarization in SA node cells is still a matter of controversy, mostly because of the difficulty to assess by electrophysiological means the contributions of the various ion channels to the pacemaker depolarization. According to the 'DiFrancesco–Noble'-model [1], the initial phase of the pacemaker depolarization is carried by the hyperpolarization-activated I_f current. At a membrane potential positive to −60 mV, the T-type current activates [5] and induces calcium release from a subsarcolemmal store [11]; the L-type calcium current with its more positive activation threshold close to −30 mV would be responsible for the upstroke of the new action potential [2].

Based on mRNA expression studies and electrophysiological characterization, we have proposed that the HCN4 and possibly HCN2 channels are responsible for the I_f current in murine SA node [23]. The present study defines the molecular basis of L- and T-type calcium currents in cardiac pacemaker myocytes. In-situ hybridization with several known calcium channel sequences shows a high expression of the T-type $Ca_v3.1$ gene, a modest expression of the T-type $Ca_v3.2$ gene, a significant expression of the L-type $Ca_v1.2$ gene and a very weak expression of the L-type $Ca_v1.3$ gene. One may speculate that both HCN4 and $Ca_v3.1$ and HCN2 and $Ca_v3.2$ colocate in the same cells. As already shown for thalamic neurons [24], depolarization by HCN channels would activate the $Ca_v3.1$ (and possibly $Ca_v3.2$) calcium channel, which then triggers the last part of the diastolic depolarization and the action potential carried by $Ca_v1.2$ and $Ca_v1.3$. Future work is required to support this hypothetical scheme, e.g. the detailed analysis of knock-out mice for each of the ion channel isoforms.

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