—**Short Communications**—

A TTX-sensitive transient Na^+ current recorded in morphologically identified primary pacemaker cells

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Primary pacemaker cells are functionally dominant in pacemaking of the sinoatrial (SA) node in the mammalian heart. They exhibit the most rapid diastolic depolarization (phase 4), but display a slow upstroke (phase 0) of spontaneous action potentials (APs)[1,2]. In the rabbit heart, such typical nodal cells morphologically resemble the "P-cells" (pale cells) that are found in the SA node of dog and man[3].They are irregular or spindle shaped, 15～30 μm in length and 5～8 μm in diameter. In comparison with latent or subsidiary pacemaker cells, which are triggered to fire by the primary pacemakers, the latter contain fewer and more loosely organized myofilaments; a greater proportion of the cytoplasm is occupied by the nucleus, and a larger number of cavernous invaginations are present in the sarcolemma[4–6].

Although the basis for the slower upstroke of spontaneous APs in primary pacemaker cells, unlike in other regions, is predominantly the contribution of the L-type Ca^{2+} current[7], there is still some controversy as to whether tetrodotoxin (TTX)-sensitive Na^+ channels (i_{Na}) are present in primary pacemaker cells. The slower upstroke has been attributed to either the absence of i_{Na}, or the presence of i_{Na} but these channels are unavailable at the depolarized phase 4 potential. Indeed, very few studies by the patch-clamp technique have investigated the TTX-sensitive transient Na^+ current (I_{Na})[8–11], probably because of difficulties both in the yield of viable single nodal cells and in attaining a satisfactory voltage clamp of I_{Na} with fast kinetics. In preliminary studies, Giles and van Ginneken[8] found no evidence of I_{Na} in pacemaker cells isolated from the center of the rabbit SA node, even when long (500 ms) prepulses to − 90 mV were applied. Honjo and Boyett[9] reported that I_{Na} was absent in smaller cells with a cell capacitance (C_m) of < 28.5 pF. On the other hand, Denyer and Brown[10] revealed that I_{Na} was nearly always observed in small pacemaker cells, which had a mean C_m of 28.3 pF, and which were isolated from the leading central nodal area, and that TTX (30 μM) attenuated the upstroke velocity of the spontaneous APs. Nevertheless, in none of these studies was it established whether the recordings were from primary or latent pacemaker cells. The purpose of the present study was to overcome these uncertainties by using electron microscopy to identify primary pacemaker cells; the whole-cell voltage− and current-clamp methods were applied to isolated *primary* pacemaker cells to confirm, directly, the presence of I_{Na}.

Details of the cell isolation have previously been published[11]. Single nodal cells were isolated enzymatically from the "central nodal region" of an adult male New Zealand White rabbit. The nodal cells were oval-shaped in the Ca^{2+}-containing medium and beat rhythmically. The whole-cell ruptured patch-clamp technique (Dagan Model 8900, Minneapolis, MN; 10−kHz bandwidth, 1−GΩ (feedback resistor) was used to record I_{Na}. Patch electrodes (2～3 MΩ) were filled with (mM): 130 CsCl, 5.0 MgATP, 5.0 Na_2CrPO_4, 5.0 EGTA and 5.0 HEPES (pH 7.2 with CsOH). The external solution contained (mM): 20 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.8

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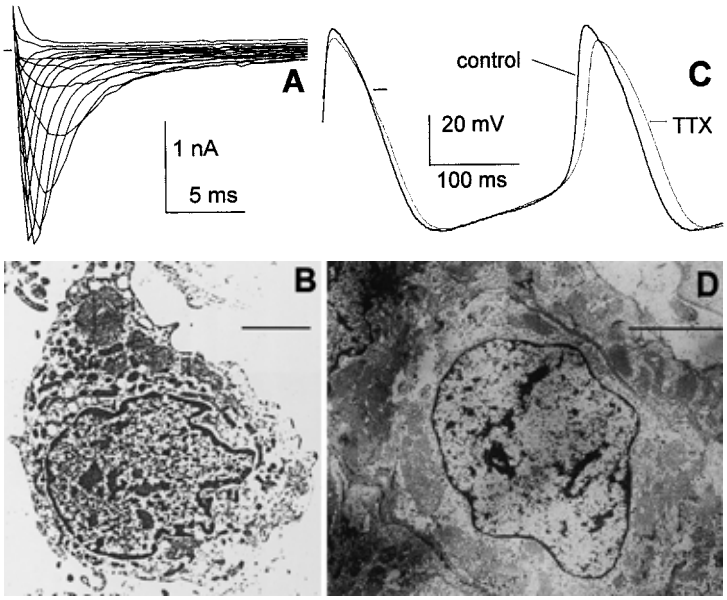

Fig. 1 **A**: Original current traces of TTX-sensitive transient Na^+ current (I_{Na}) in a nodal cell (cell capacitance : C_m = 21.5 pF). Step depolarization was applied to test potentials of − 68 mV to +37 mV in 5− or 10−mV increments (holding potential = − 98 mV, temperature = 22.8℃). Leak current was not compensated. **B**: The transmission electron photomicrograph (TEM) identifies the cell in which the I_{Na} was recorded as a primary pacemaker cell, i.e. a "P-cell". The scale bar indicates 2 μm. **C**: TTX (30 μM) depolarized the take-off potential and decreased the upstroke velocity of typically dominant spontaneous action potentials of the primary pacemaker cell (C_m = 23.2 pF, temperature = 36.1℃). **D**: A TEM of the P-cell from the SA node tissue in a rabbit heart. The scale bar, 2 μm.

$MgCl_2$, 0.3 NaH_2PO_4, 4.0 4−aminopyridine, 106 tetraethylammonium, 1.2 $NiCl_2$, 4.0 CsCl, 5.5 dextrose, and 5.0 HEPES; pH 7.4 with HCl. The temperature was 23 ± 0.2℃. All voltage-gated currents other than I_{Na} were completely blocked. The electrode capacitance and series resistance were compensated as much as possible to obtain a sufficient voltage clamp of I_{Na} with a voltage escape of < 5%. The perforated-patch whole-cell current clamp method with nystanin was employed to record spontaneous APs. The nystanin (300 μg/m*l*)-containing pipette solution was the same as that described above except that K^+ replaced Cs^+. The bathing solution contained (mM): 126 NaCl, 5.4 KCl, 2.5 $CaCl_2$, 0.8 $MgCl_2$, 0.3 NaH_2PO_4, 5.5 dextrose, and 5.0 HEPES; pH 7.4 with NaOH. In these experiments the temperature was 36 ± 0.2℃. After patch perforation was completed (15～30 min) so that a stable minimal series resistance was obtained, regular spontaneous APs were recorded. Electrophysiological experiments to record I_{Na} or spontaneous APs were performed only in smaller nodal cells (C_m < 25 pF), with higher beat rates (>180/min). If C_m was larger than 25 pF at the beginning of each experiment, we discarded the data. Computer software (*pClamp 7* ; Axon Instruments) was used to acquire and analyze the data.

The single cells were prepared for the study by transmission electron microscopy (TEM) immediately (<30 min) after the electrophysiological experiments. When a patch electrode was removed from the surface of a cell that was attached to the dish bottom, the ruptured or perforated holes of the membrane were completely restored. After its location was clearly marked on the outside of the dish bottom, the bathing solution was discarded and the single cell was fixed in 3% glutaraldehyde in 67 mM Na-cacodylate buffer (pH 7.3) overnight at 4℃. Following 3 washes in cacodylate buffer, the cell was post-fixed in 1% osmium tetroxide in 67 mM cacodylate buffer. After dehydration in graded concentrations of ethanol, the cell was embedded in Epon, sectioned by a microtome, stained with 4% aqueous uranyl acetate and Reynold's lead citrate, and observed on a Hitachi TEM.

Typical current traces of I_{Na} could be recorded from a nodal cell (C_m = 21.5 pF) (**Fig. 1 A**). As the holding potential was − 98 mV, the threshold was approximately − 63 mV and the peak current measured be-

tween −28 and −33 mV was −2.1 nA (−97.7 pA/pF). The reversal potential was +22 mV. The characteristics of the inward current were representative of I_{Na}, and were completely and reversibly blocked by 30 μM TTX. TEM findings of this cell revealed a typical primary pacemaker cell, i.e. a "P-cell" (**Fig. 1 B**), although some parts of the cytoplasm were degenerated with vacuolation after the electrophysiological experiment using the EGTA-containing pipette solution and the external solution with low Na^+ concentration (20 mM). Much of the cytoplasm was occupied by a centrally located nucleus, with surrounding mitochondria, glycogen granules and a few myofilaments without Z-lines. The findings virtually compared with the TEM image of the P-cell from a rabbit central SA node tissue (**Fig. 1 D**). We performed the TEM study in four other nodal cells that had I_{Na}. The TEM findings were essentially the same and the cells were morphologically identified as primary pacemaker cells. The effects of TTX on spontaneous APs were examined in another nodal cell (C_m = 23.2 pF). Control APs showed the typical characteristics of dominant pacemaker cells. TTX (30 μM) depolarized the take-off potential to phase 0 (−43 mV to −39 mV), and suppressed the upstroke velocity (from 7.5 to 3.9 V/s) (**Fig. 1 C**). TEM examination of the cell also demonstrated the features of primary pacemaker cells as shown in **Fig. 1 B**.

There is functional heterogeneity in mammalian SA node tissue and/or cells[1,2,9]. Electrophysiologically identified dominant or primary pacemaker cells do not necessarily have morphological features of typical nodal cells[1]. This is the first report that clearly demonstrates that primary pacemaker cells morphologically identified by TEM indeed do *have* I_{Na} that functionally contributes to the upstroke velocity of spontaneous APs. The result appears contradictory to the widely accepted conviction that I_{Na} is absent from the primary pacemaker cell. Larger SA node cells with C_m greater than 30 pF, which had I_{Na}, were morphologically identified as subsidiary pacemaker cells or transitional cells by TEM studies (data not shown). The functional dominance of the primary pacemaker cells is determined by the fastest firing rate of spontaneous APs *in vivo*, but not by morphological features[1], and also not by the absence of I_{Na}.

It is assumed that the distribution of the nodal cells that have I_{Na} is inhomogeneous in each region of the SA node. The central dominant area could have the lowest density of primary pacemaker cells *with* I_{Na}, whereas this density might increase toward the transitional region. Larger SA node cells with I_{Na} increase toward the peripheral region. However, the density of i_{Na} channels in each cell is essentially equal throughout the SA node[12]. Further investigations are warranted to identify differences in the distribution of cells having I_{Na} by means of immunohistochemical methods, i. e. with specific antibodies to i_{Na} channels.

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