Ionic Mechanisms Underlying the Positive Chronotropy Induced by β 1-Adrenergic Stimulation in Guinea Pig Sinoatrial Node Cells: a Simulation Study

Yukiko HIMENO¹, Nobuaki SARAI², Satoshi MATSUOKA¹, and Akinori NOMA¹

¹Department of Physiology and Biophysics, Graduate School of Medicine, and ²Nano-Medicine Merger Education Unit, Kyoto University, Yoshida-Konoe, Sakyo-ku, Kyoto 606-8501, Japan

Abstract: Positive chronotropy induced by β 1-adrenergic stimulation is achieved by multiple interactions of ion channels and transporters in sinoatrial node pacemaker cells (SANs). To investigate the ionic mechanisms, we updated our SAN model developed in 2003 and incorporated the β 1-adrenergic signaling cascade developed by Kuzumoto *et al.* (2007). Since the slow component of the delayed rectifier K^+ current (I_{Ks}) is one of the major targets of the β 1-adrenergic cascade, we developed a guinea pig model with a large I_{Ks} . The new model provided a good representation of the experimental characteristics of SANs. A comparison of individual current during diastole recorded before and after β 1-adrenergic stimulation clearly showed the negative shift of the L-type Ca^{2+} current (I_{CaL}) takeoff potential, enlargement of the sustained inward current (I_{st}), and the hy-

perpolarization-activated nonselective cation current (I_{ha}) played major roles in increasing the firing frequency. Deactivation of I_{Ks} during diastole scarcely contributed to the time-dependent decrease in membrane K^+ conductance, which was the major mechanism for slow diastolic depolarization, as indicated by calculating the instantaneous equilibrium potential (lead potential). This was because the activation of I_{Ks} during the preceding action potential was negligibly small. However, I_{Ks} was important in counterbalancing the increase in I_{CaL} and the Na^+/Ca^{2+} exchange current (I_{NaCa}), which otherwise compromised the positive chronotropic effect by elongating the action potential duration. Enhanced Ca^{2+} release from the sarcoplasmic reticulum failed to induce an obvious chronotropic effect in our model.

Key words: β 1-adrenergic receptor, cardiac pacemaker model, sinoatrial node, sympathetic nerve stimulation, simulation.

Sympathetic stimulation of SA node pacemaker cells (SANs) is essential for increasing heart rate when a larger blood supply is required for the body. The autonomic neurotransmitter, noradrenaline, is released from nerve terminals, binds to the β 1-adrenergic receptor, and initiates intracellular signal transduction in SANs, which causes the increased firing frequency of spontaneous action potentials. This positive chronotropy is due to a variety of functional modifications of ion channels and ion transporters. To date, electrophysiological and pharmacological studies have provided experimental evidence to show that ion channels and transporters are modified by the β 1-adrenergic stimulation. To clarify the contributions of each current, however, an integrative analysis is required because positive chronotropy is induced by multiple interactions of all ion channels and transporters, which have different kinetics and respond differently to β 1-adrenergic stimulation. In 2003, we developed a SAN model that included spontaneous action potential generation and intracellular

ion homeostasis, including Ca^{2+} dynamics [1, 2]. Using this model, we proposed the principal ionic mechanisms underlying the spontaneous action potential. In the current study, we revised the model by updating the ion channels and incorporating the mitochondria and β 1-adrenergic cascade models described by Matsuoka *et al.* (2004), Terashima *et al.* (2006), Takeuchi *et al.* (2006), and Kuzumoto *et al.* (2007) [3–6] to analyze the roles of each target ion current, including the L-type Ca^{2+} current (I_{CaL}), the sustained inward current (I_{st}), the hyperpolarization-activated cation current (I_{ha}), and the slow component of the delayed rectifier K^+ current (I_{Ks}) in the positive chronotropy during β 1-adrenergic stimulation. Our new model also includes an alteration of Ca^{2+} dynamics during stimulation by calculating a modification of the Ca^{2+} pump (SERCA) on the sarcoplasmic membrane. Thus we discuss the hypothesis that Ca^{2+} release from the sarcoplasmic reticulum (SR) plays a crucial role in regulating pacemaker activity [7–10].

Received on Dec 25, 2007; accepted on Jan 16, 2008; released online on Jan 19, 2008; doi:10.2170/physiolsci.RP015207

Correspondence should be addressed to: Akinori Noma, Department of Physiology and Biophysics, Graduate School of Medicine, Kyoto University, Yoshida-Konoe, Sakyo-ku, Kyoto 606-8501, Japan. Tel: +81-75-753-4357, Fax: +81-75-753-4349, E-mail: noma@card.med.kyoto-u.ac.jp

It is well established that the decay in K^+ conductance during slow diastolic depolarization is crucial for the generation of spontaneous action potentials [11, 12], and this K^+ conductance is mainly supported by the rapid component of the delayed rectifier K^+ current (I_{Kr}) in rabbit. However, Matsuura *et al.* (2002) [13] demonstrated that the guinea pig SANs show a significant amplitude of I_{Ks} during the depolarizing pulse, and also I_{Ks} tail on repolarization, which was not the case in rabbit SANs (see also Anumonwo *et al.* (1992) [14]). Furthermore, I_{Ks} is one of the major targets of the β_1 -adrenergic cascade. Therefore in this study we examined if I_{Ks} in guinea pig could be substituted for I_{Kr} in contributing to pacemaker depolarization during β_1 -adrenergic chronotropy. We constructed a guinea pig SAN model with reference to the experimental data for I_{Kr} and I_{Ks} obtained from guinea pig SANs by Matsuura *et al.* [13]. As far as we know, this is the first attempt to investigate the mechanism of the positive chronotropy of firing rate in a SAN model including intracellular β_1 -adrenergic signaling cascades.

MATERIALS AND METHODS

Parameter set in the guinea pig SAN model. The extracellular ion concentrations (in mM) were 141 for Na^+ , 5.4 for K^+ , 1.8 for Ca^{2+} , 140 for Cl^- , and 10 for large anionic compounds (LA). LA was membrane-impermeable. A schematic diagram of the SAN model is shown in Fig. 1. General abbreviations are listed in the Table S1, the equations describing the kinetic behavior of individual functional units are in Tables S2–15, and steady state variables such as ionic currents, flux, and metabolite concentrations are given in Table S16 in the online supplemental material. The basic structure of the guinea pig model is the same as that of the SAN model based mainly on rabbit data [2]. Assuming that the membrane potential (V_m) is homogeneous within a single cell, we calculated dV_m/dt from the

total current of ion channels and ion exchangers (I_{tot}) normalized for the input membrane capacitance (C_m) and the current applied through the electrode (I_{ext}) as follows (see also Table S4),

$$dV_m/dt = -(I_{tot} + I_{ext}) \quad (1)$$

$$I_{tot} = I_{Na} + I_{CaL} + I_{CaT} + I_{st} + I_{ha} + I_{K1} + I_{Kr} + I_{Ks} + I_{KACH} + I_l + I_{NaCa} + I_{NaK} + I_{PMCA} \quad (2)$$

See Tables S7, S8, S9, and S11 for definitions and equations for each current. The total background current (I_l) is defined as

$$I_l = I_{bNSC} + I_{Cab} + I_{Kpl} + I_{l(Ca)} + I_{KATP} + I_{Clb} \quad (3)$$

See Table S10 for definitions and equations for each background current. The following functions were newly added to the model. First, ATP production by oxidative phosphorylation, creatine kinase, and adenylate kinase [3, 15] (Tables S5 and S15) was implemented. The ATP production rate was adjusted to obtain a steady state [ATP] of 7.0 mM, which was in the range of 5–10 mM [16]. A ratio $[NADH]/([NAD]+[NADH]) = 0.29$ was within the range of values obtained experimentally in guinea pig ventricular myocytes [17]. Second, the mechanisms of Cl^- homeostasis and volume regulation [4, 5] were incorporated by including ion flux via a $Na^+/K^+/2Cl^-$ cotransporter 1 (J_{NKCC1}), background Cl^- current (I_{Clb}), water flux, and LA. The magnitudes of I_{Clb} and water flux were scaled according to the ratio of input capacitance between SANs and ventricular cells (Tables S4 and S9). The intracellular Cl^- concentration was set at about 30 mM by adjusting the activity of J_{NKCC1} . Since the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel current (I_{CFTR}) was reported to be absent [18] and no data were available for I_{VRCC} in SANs, they were not included in our model. Equations for the protein kinase A (PKA) and the cAMP effect on I_{st} and I_{ha} were newly implemented in the

Fig. 1. Schematic diagram of the guinea pig SA node cell model.

Fig. 2. Comparison of I_{CaL} between simulation and experiment. **(A)** The upper panel shows the reconstructed I_{CaL} with a test pulse to 0 mV from a holding potential of -50 mV under control conditions (○) and with 0.1 μM ISO (●), and the lower panel shows corresponding experimental data with test pulses from -40 to +50 mV [24]. **(B)** Simulated peak I-V relationships of I_{CaL} with (red line) and without (black line) 0.1 μM ISO are compared with experimental I-V relationships [24] in the lower panel (with [●] and without [○] 0.1 μM ISO). **(C)** Simulated concentration-response relationship ($n_H = -1.39$, $K_D = 0.0158$ μM) for the enhancement of I_{CaL} by ISO measured with a test pulse to 0 mV from a holding potential of -50 mV.

SAN model. And third, the β1-adrenergic signaling module [6] (Tables S6 and S12) was incorporated in the model. Target proteins of β1-adrenergic signaling, including I_{Ks} , are colored in red in the diagram (Fig. 1). One significant feature of guinea pig SANs is a larger I_{Ks} , compared to I_{Kr} as indicated by Anumonwo *et al.* (1991) [14] and Matsui *et al.* (2002) [13], in contrast to rabbit SANs, which has an almost negligible I_{Ks} [19]. In regard to the kinetic equations for I_{Kr} , we adopted those developed for I_{Kr} in rabbit by Ono and Ito (1995) [20].

Ordinary differential equations were integrated using the Euler method with an adaptive time step, and simultaneous equations were solved by Broyden's method, a quasi-Newton procedure. The model was constructed with a Java-based simulation platform, simBio [21]. The source code is available at <http://www.sim-bio.org/>.

Ionic channels and transporters modulated in the β1-adrenergic signaling cascade model. In our SAN model, I_{CaL} , I_{sr} , I_{ha} , I_{Ks} , I_{NaK} , the plasma membrane Ca^{2+} -ATPase (PMCA) current (I_{PMCA}) and the SR Ca^{2+} pump (SERCA) current (I_{SRU}), are enhanced by β1-adrenergic stimulation. Such stimulation activates adenylate cyclase via G-protein (G_s) and elevates the cAMP level. The increased cAMP activates PKA by unleashing its catalytic subunits to phosphorylate the above target proteins, except for I_{ha} , which is directly modulated by the concentration of cAMP. In the simulation, the activities of I_{CaL} , I_{sr} , I_{ha} , I_{Ks} , I_{NaK} , I_{PMCA} and $SERCA$ (I_{SRU}) were modulated as a function of the concentration of PKA catalytic subunits or cAMP, as follows.

(1) I_{CaL} : Equations for I_{CaL} were basically the same as those used in the ventricular cell model [6]. The increase of I_{CaL} and negative shift of current-voltage (I-V) relationships by β1-adrenoceptor stimulation were reconstructed by defining model equations as functions of [PKA], the concentration of free catalytic subunit of PKA. The fully activated amplitude of I_{CaL} was multiplied by f_{PKA} , which is given as (Table S7),

$$f_{PKA} = \max \cdot ([PKA] - PKA0)^{nH} / \{ ([PKA] - PKA0)^{nH} + K_m PKA^{nH} \} + 1 \quad (4)$$

where the value for the concentration of PKA in the absence of β1-adrenergic stimulation ($PKA0 = 0.000136$ mM) was obtained from the original paper by Kameyama *et al.* (1985) [22] and other values for the maximum amplitude (max), Hill coefficient (nH) and the equilibrium constant for PKA ($K_m PKA$) were determined to reconstruct the recorded increase of I_{CaL} by ISO described by Zaza *et al.* (1996) [23] and Ke *et al.* (2007) [24] in SANs (17.5, 1 and 0.0065 mM, respectively).

The voltage shift of gating was given by biasing V_m with the following V_{shift} in all equations defining the rate constants of the voltage-dependent gate (Table S7):

$$V_{shift} = 62.5 \cdot ([PKA] - PKA0)^{nH} / \{ ([PKA] - PKA0)^{nH} + K_m PKA^{nH} \} \quad (5)$$

Parameters for Ca^{2+} -dependent inactivation gate were adjusted to obtain the experimental inactivation time course of I_{CaL} , as shown in Fig. 2A [24]. In both simulation and

Fig. 3. Comparison of I_{st} . (A) Voltage clamp records of I_{st} with (●) and without (○) 0.1 μ M ISO in simulations (upper panel) and experiments [30] (lower panel). (B) Simulated peak I-V relationships of I_{st} with (red line) and without (black line) 0.1 μ M ISO. The holding potential was -80 mV. (C) ISO concentration- I_{st} relationship compared to experimental data (mean $\pm$ SEM) [26].

experiment, the amplitudes were approximately doubled by adding 0.1 μ M isoprenaline (ISO). The negative shift of the peak I-V relationship was approximately 7 mV (Fig. 2B, upper panel), as obtained experimentally in guinea-pig SANs (Fig. 2B, lower panel) [24] and in various types of cells [25]. In the dose-response curve in Fig. 2C, saturated increase of I_{CaL} was 116% of the control.

(2) I_{st} : The increase of I_{st} was reconstructed by defining the limiting amplitude as a function of [PKA] (Table S7). The fully activated amplitude of I_{st} was multiplied by f_{PKA} , which is given by the same equation for I_{CaL} (Eq. 4), but values for max and K_m PKA were different from those of I_{CaL} (1.0 and 0.000065 mM, respectively). In Fig. 3, cur-

rents in the presence of 0.1 μ M ISO (●) were approximately doubled compared to the control (○) in both the simulation and the experiment. The I-V relationships in Fig. 3B were reconstructed with and without ISO by plotting the amplitude of the initial peak I_{st} measured at the beginning of depolarizing pulses. The reversal potential of I_{st} was +12 mV. The amplitudes of the current were increased in a dose-dependent manner as shown in Fig. 3C, in agreement with the experimental data of Toyoda *et al.* (2006) [26].

(3) I_{ha} : The positive shift in voltage-dependent activation of I_{ha} was defined as a function of the concentration of free cAMP, [cAMP] (Table S7). As for I_{CaL} , the voltage

Fig. 4. Comparison of I_{ha} . (A) Voltage clamp records of I_{ha} with (●) and without (○) ISO in simulations (upper panel) and experiments [27] (lower panel). ISO concentrations were 0.1 μ M in the simulation and 1 μ M experimentally. Traces were elicited by 2 s test pulses to -80 mV from a holding potential of -35 mV. (B) Simulated activation-voltage relationships of I_{ha} with (red line) and without (black line) 1 μ M ISO are compared with experimental I-V relationships in the lower panel (with [●] and without [○] 1 μ M ISO) [28]. Data were obtained at the end of 4 s hyperpolarizing pulses to various test potentials from a holding potential of -40 mV.

Fig. 5. Comparison of I_{Ks} . **(A)** Current traces during 4 s voltage clamp steps to membrane potentials in the range -40 to $+40$ mV from a holding potential of -50 mV and expanded tail currents elicited after depolarizing steps to -30 , -10 , $+10$, $+30$, $+50$, and $+70$ mV in simulation (upper panel) and in experiments by Matsuura *et al.* (2002) (lower panel) [13]. I_{Kr} was blocked in the simulation and was blocked with $5 \mu\text{M}$ E-4031 in the experiment. The tail current was fitted by a sum of two ex-

ponential functions with time constants of $\tau_f = 61.0$ ms and $\tau_s = 122.7$ ms at -50 mV, compared to 44.4 and 151.5 ms in the experiment. **(B)** I-V relationships for I_{Ks} tail currents obtained with (red line) and without (black line) application of $1.0 \mu\text{M}$ ISO are compared with experimental I-V relationships [29] in the lower panel (with [●] and without [○] $1 \mu\text{M}$ ISO). Data points were obtained at the end of 4 s depolarizing pulses to various test potentials from a holding potential of -50 mV.

shift of gating was given by biasing V_m with V_{shift} (calculated as follows) in all equations defining the rate constants of the voltage dependent gate,

$$V_{shift} = 15 \cdot ([\text{cAMP}] - \text{cAMP0})^{nH} / \{([\text{cAMP}] - \text{cAMP0})^{nH} + K_m \text{cAMP}^{nH}\} \quad (6)$$

where values for the concentration of cAMP in the absence of β1-adrenergic stimulation (cAMP0), nH and the equilibrium constant for cAMP (K_m cAMP) were 0.000294 mM, 1 and 0.0002 mM, respectively. [cAMP] represents the concentration of free cAMP. In Fig. 4A, I_{ha} activated at -80 mV in the presence of $0.1 \mu\text{M}$ ISO ($[\text{cAMP}]_i = 4.88 \mu\text{M}$) was larger than the control, as in experiments [27] shown in the lower panel. The simulated activation time course of I_{ha} in the presence of ISO was accelerated slightly. The voltage-shift of activation in the simulation was similar to that in the experimental data, as shown in Fig. 4B [28]. The $V_{1/2}$ of the sigmoid curves obtained in the control and in the presence of $1 \mu\text{M}$ ISO were -69.1 mV and -43.7 mV, respectively, in the simulation (upper panel) and -61.9 mV and -47.1 mV, respectively, in experiments (lower panel). It should be noted that I_{ha} could be activated over the voltage range of slow diastolic

depolarization in the presence of β1-adrenergic stimulation.

(4) I_{Ks} : An I_{Ks} model of guinea-pig SANs is a modification of the recently developed new ventricular model [6] (Table S8). Channel gating was described by voltage-dependent and Ca^{2+} -dependent activation kinetics, and β1-adrenergic stimulation modifies the voltage gate and the current amplitude, as in the ventricular cell model. The voltage dependency and the permeability of the ventricular I_{Ks} model was modified according to experimental data obtained by Matsuura *et al.* (2002) [13]. Figure 5A compares the time courses of I_{Ks} activation and deactivation induced by the pulse protocol indicated at the top of the figure. The very slow component of activation was not observed in simulation, but the initial activation time course was similar to those in the experiment. The deactivation time course over the diastolic potential range also agreed with experimental data; it was fitted well by a sum of two exponential components, as in the experiment. The same equations for the PKA effect in the ventricular β1-adrenergic signaling model developed by Kuzumoto *et al.* [6] as shown in Eqs. 7–9 were used in the SAN model. Activated PKA phosphorylated the main subunit of I_{Ks} , KCNQ1 and increased the amplitude of I_{Ks} and shifted its voltage-de-

pendency in a negative direction. The fully activated amplitude of I_{Ks} was multiplied by f_{PKA} , which is given as a function of a fractional concentration of phosphorylated KCNQ1, F_KCNQ1p ,

$$F_KCNQ1p = [KCNQ1p] / [KCNQ1_{tot}], \quad (7)$$

$$f_{PKA} = 0.565 / \{1 + (0.4 / F_KCNQ1p)^6\} + 0.435. \quad (8)$$

Similarly, the voltage shift was defined by F_KCNQ1p as,

$$V_{shift} = 1.35 \cdot \{(F_KCNQ1p / 0.0947) - 1\}. \quad (9)$$

In Fig. 5B, I_{Ks} tail current increased 1.5- to 2-fold by 1 μ M ISO in the voltage range of action potential, which agreed with experimental data [29].

(5) I_{NaK} , $I_{SR}U$ and I_{PMCA} : Because of insufficient data for I_{NaK} , $I_{SR}U$, and I_{PMCA} in guinea-pig SANs, we used essentially the same equations for kinetics as those in the ventricular cell model [6] (Table S11).

RESULTS

Whole cell current and current-voltage relationship in the SAN model

The simulated membrane currents and current-voltage relationships obtained from the guinea pig SAN model (upper panel) are compared with experimental results (lower panel) from Guo *et al.* (1997) [30] in Fig. 6. Hyperpolarizing pulses from the holding potential of -50 mV activated the slowly increasing inward current, I_{ha} , which became visible from -70 to -80 mV. The activation time courses seemed similar, except for faster activation in the experiment at -110 mV. Depolarizing pulses to various potentials induced transient inward current, which was attributed to I_{CaL} , and slowly increased the outward current, I_{Ks} . The activation threshold of I_{CaL} was -40 mV, and the potential of the peak inward current in the I-V curve was -10 mV in both the simulation and experiment (●). The voltage range of delayed rectification in the simulation also agreed well with the experimental I-V relationship for the late current (○). On repolarization, outward tail currents were observed as a result of the deactivation of I_{Ks} and I_{Kr} . The contribution of I_{Kr} to the tail current was comparable to that of I_{Ks} ; the ratio of I_{Kr}/I_{Ks} was 0.74 after the depolarizing pulse to $+20$ mV. This is in contrast to the major contribution of I_{Ks} to the onset current during the depolarizing pulse; I_{Kr}/I_{Ks} was 0.02 during the depolarizing pulse to $+20$ mV because of the strong inward rectification of I_{Kr} .

Ionic mechanisms of spontaneous action potentials

The spontaneous activity of the cell was successfully reproduced as a result of the coordination of all the ion channels and transporters in the model. The action potential parameters are compared in Table 1. Both the maxi-

Fig. 6. Membrane currents in the guinea pig SAN model and in experiments. Membrane current traces induced by depolarizing (from -40 to $+20$ mV, upper traces) and hyperpolarizing (from -60 to -110 mV, lower traces) pulses (left panel) from a holding potential at -50 mV and the current-voltage relationship (right panel) from simulation (top) and from the experimental data of Guo *et al.* (1997) (bottom). The peak inward current on depolarization or the initial current level on hyperpolarization (●) and the late current levels measured near the end of 500 ms pulses (○) were plotted against V_m .

mum diastolic potential and the overshoot are in good agreement with those in the experiments. The maximum rate of rise in the simulation is within the range of experimental data reported by Guo *et al.* (1997) [30] and Toyoda *et al.* (2005) [26], but slightly larger than that of Rigg *et al.* (2000) [31], and the spontaneous frequency agrees well with Rigg *et al.* (2000) [31]. To understand the mechanism underlying pacemaker depolarization, we adopted the “lead potential, V_L ” analysis introduced by Sarai *et al.* (2003) [2]. The lead potential is defined by the equivalent circuit shown in Fig. 7A, where dV_m/dt is given as

$$\frac{dV_m}{dt} = -\frac{G_m \cdot (V_m - V_L)}{C_m}. \quad (10)$$

Both V_L and G_m vary automatically in the SAN model ac-

Table 1. Action potential parameters of single guinea pig SANs. In the simulation, parameters were obtained in the steady state for control data and 30 s after the application of 0.01 μM and 0.1 μM ISO.

Reference		Maximum diastolic potential (mV)	Overshoot (mV)	Total amplitude (mV)	Maximum rate of rise (V/s)	Frequency (/min)
Rigg <i>et al.</i> (2000) [31]	Control	-57 ± 2	16 ± 4	73	1.5 ± 0.4	132 ± 19
	[ISO] 0.1 μM	-62 ± 3	27 ± 4	89	3.5 ± 0.6	284 ± 14
Guo <i>et al.</i> (1997) [30]	Control	-58.7 ± 3.8	17.9 ± 4.8	76.6	3.6 ± 1.3	191 ± 76.6
Toyoda <i>et al.</i> (2005) [26]	Control	-64.3*	17.5*	81.8	7.0 ± 1.2	178 ± 8
Matsuura <i>et al.</i> (2002) [13]	Control	-64.3 ± 1.8*	22.5*	86.8	–	205 ± 11
Simulation	Control	-64.81	12.93	77.74	4.33	131.9
	[ISO] 0.01 μM	-65.5	13.69	79.19	5.117	159.9
	[ISO] 0.1 μM	-66.12	15.04	81.16	7.29	177.6

*Data were corrected for a liquid junction potential of 5 mV between the aspartate-rich pipette solution and the Tyrode solution. The results were expressed as the mean ± SEM except for results obtained by Guo *et al.*, which were expressed as the mean ± SD.

cording to the time-dependent changes in membrane ionic conductance (G_X) and the equilibrium potential (E_X) for each ionic species (X):

$$G_m = G_K + G_{Na} + G_{Ca} + G_{Cl}, \quad (11)$$

$$V_L = \frac{G_K E_K + G_{Na} E_{Na} + G_{Ca} E_{Ca} + G_{Cl} E_{Cl} - I_{NaK} - I_{PMCA}}{G_m}. \quad (12)$$

When G_K , G_{Na} , G_{Ca} , and G_{Cl} were calculated from the total K^+ , Na^+ , Ca^{2+} , and Cl^- fluxes (Table S4), respective-

ly, along the time course of the spontaneous action potential, V_L changed as shown in the control panel in Fig. 7B (V_L in red and V_m in gray). When dV_m/dt is positive, V_L is more positive than V_m , so that an inward current is generated and the membrane depolarizes, with the opposite effect of when dV_m/dt is negative. V_L crossed V_m at all flexion points where dV_m/dt is zero at the overshoot potential and the maximum diastolic potential.

To evaluate the contribution of individual currents to the slow diastolic depolarization by V_L analysis, the gating parameter for a given current was fixed for 275 ms (un-

Fig. 7. The “lead potential, V_L ” and contribution of currents to slow diastolic depolarization. (A) The equivalent circuit for whole-cell electrophysiology. (B) V_L (blue line) was calculated and superimposed on V_m (gray line) and V_L (red line) in control. Except in the control recording, for the calculation of V_L the gating parameters of I_{Kr} , I_{Ks} , I_{CaL} , I_{st} , and I_{ha} were fixed at values obtained at the maximum diastolic potential (150 ms) and released after a period of 275 ms (unshaded).

Fig. 8. Simulated channel opening probabilities for I_{Kr} and I_{Ks} . (A) V_m . (B) Opening probability of I_{Kr} . (C) Current amplitude of I_{Kr} . (D) Opening probability of I_{Ks} . (E) Current amplitude of I_{Ks} . Note the difference in scales between the probabilities for I_{Kr} and I_{Ks} .

shaded period) at the value obtained at the maximum diastolic potential. Note that the calculation of V_L (blue line) was independent from that of V_m (gray line); thus the time course of V_m is the same in all panels. When the gating parameter of I_{Kr} was fixed, V_L became flat below V_m , indicating that I_{Kr} is mainly responsible for slow diastolic depolarization. In contrast, when the gating parameter of I_{Ks} was fixed, the time course of V_L was only moderately modified if compared with the control V_L . I_{CaL} played an important role in accelerating the last phase of slow diastolic depolarization, which was in agreement with its activation threshold at around -40 mV. I_{sr} played a crucial role in determining the depolarizing rate in the latter half of slow diastolic depolarization, as is evident in the panel for I_{sr} . Fixing I_{ha} gating had almost no effect on V_L . These findings are in good agreement with a previous study in the “rabbit” SAN model [2].

To gain a deeper insight into the contribution of I_{Ks} to the rate of slow diastolic depolarization in comparison to I_{Kr} , open probabilities and amplitudes of I_{Ks} and I_{Kr} were compared during slow diastolic depolarization (Fig. 8). The driving force for outward K^+ flux increased in a time-dependent manner, but the open probability of I_{Kr} de-

creased gradually from 0.46 to 0.10 (Fig. 8B); thus the current amplitudes of I_{Kr} remained fairly constant in the late phase of pacemaker depolarization (Fig. 8C). As expected from the deactivation time course of I_{Ks} in the voltage clamp experiment (Fig. 5), the open probability of I_{Ks} also decreased during diastolic depolarization (Fig. 8D). However, the amplitude of I_{Ks} was very small after the time of the maximum diastolic potential. This was because the open probability of I_{Ks} during the action potential was only 0.007 at the maximum, which was 1/58.8 of I_{Kr} because of its slow activation kinetics. Furthermore, the reversal potential of I_{Ks} was less negative than that of I_{Kr} , giving a smaller electrochemical driving force for I_{Ks} during diastole. Thus I_{Ks} could not contribute to the decay in the whole cell K^+ conductance during slow diastolic depolarization (Fig. 8E).

Time course of responses to $\beta 1$ -adrenergic stimulation

The time course of the $\beta 1$ -adrenergic effect is one of the critical factors in controlling arterial blood pressure or in evaluating autonomic regulation using the R-R interval variability. Here the time course of firing frequency of SANs was examined in terms of underlying reaction cascade in the model (Fig. 9).

The concentration of cAMP increased exponentially within the initial 20 s after the onset of ISO application, as shown in the top panel of Fig. 9A, and its saturation level was determined by the phosphodiesterase (PDE) activity. The fraction of the free catalytic subunit of PKA showed almost the same time course as that of cAMP (Fig. 9A, middle panel). Approximately 13% of the activated catalytic subunit was inactivated by the binding of protein kinase inhibitor (PKI) at 30 s after the application of 0.01 μ M ISO. The increase of firing frequency saturated much earlier than those of cAMP or PKA activation after ISO application (Fig. 9A, bottom panel). This dissociation of the time course in firing frequency from that in cAMP or PKA is due to the nonlinear relationship between the channel activation and the firing frequency; however, the details of the mechanism are currently unclear. We were only able to test the secondary effect of a modified intracellular Na^+ concentration ($[Na^+]_i$), which increased almost linearly to 5.13 mM, from 4.45, within the initial 30 s of ISO application. Indeed, the outward I_{NaK} , modulated by activated PKA, was gradually enlarged by the increase in $[Na^+]_i$ (Fig. 9B, top panel). When the amplitude of I_{NaK} was temporarily decreased to the initial level (Fig. 9B, middle panel) by arbitrarily decreasing its magnitude factor, the firing frequency was increased. The enhancement of I_{NaK} by increasing the concentration of intracellular Na^+ was one of the mechanisms responsible for the early saturation in the acceleration of firing frequency.

The relationship between the increase in firing frequency and ISO concentration is shown in Fig. 9C. The firing

Fig. 9. Simulation of β1-adrenergic action on increase in cAMP, activation of PKA, and increase in firing frequency. **(A)** Time courses for the increase in cAMP and the activation of PKA by 0.01 μM ISO are shown in the top and middle panels, respectively. A simultaneous recording for the change in firing frequency is shown in the bottom panel. **(B)** The time course of enhancement of I_{NaK} after the application of 0.01 μM ISO is shown in the top panel. In the middle panel, the amplitude of I_{NaK} was decreased to the control current at time zero by re-

ducing the number of Na^+/K^+ pumps at 25 s *in silico*. The time course of the firing frequency with the reduction of the amplitude of I_{NaK} is shown in the bottom panel. **(C)** ISO dose-dependent increase in firing frequency. Data were obtained 30 s after ISO application. Symbols with error bars indicate experimental data (mean ± SEM) in a guinea pig multicellular preparation by Rigg *et al.* (2000) with (○) and without (●) 2 μM ryanodine [31].

frequency was measured 30 s after the application of ISO, which increased the frequency by 41.5% from the control rate at the maximum dose (10 μM) of ISO. Experimental data from Rigg *et al.* (2000) [31] were superimposed on the simulation result. The curve obtained from simulation ($n_H = 0.586$, $K_D = 0.0098$ μM) almost coincided with the experimental result in the presence of 2 μM ryanodine (○) in the low concentration range below 0.01 μM, but the slope was shallower than that obtained without ryanodine (●). The approximate half-maximal dose for ISO, 0.01 μM, was used in further analyses in this study.

The parameters of action potentials in simulations in ISO (0.01 and 0.1 μM) are compared in the Table 1 with experimental data (0.1 μM) in isolated guinea pig SANs from Rigg *et al.* [31]. The maximum diastolic potential, overshoot, total amplitude, maximum rate of rise, and frequency increased in both the simulation and the experiment in the presence of ISO. The increase in firing frequency in the simulation was smaller than that in the experiment.

In the current model, J_{NKCC1} and I_{Clb} are not modified by β1-adrenergic stimulation. However, $[Cl^-]_i$ increased due to changes in I_{Clb} , which is dependent on V_m , from a control level of 30.04 mM to 30.06 mM after the application of ISO for 2 h. Because the change in $[Cl^-]_i$ was small, we concluded that the change in the cell volume was negligibly small.

Ionic mechanisms of spontaneous action potential under β1-adrenergic stimulation

V_m and currents obtained in control (black line) and 30 s after 0.01 μM ISO application (red line) are superimposed individually at the time point of the maximum rate of rise in Fig. 10. The slope of slow diastolic depolarization was significantly increased by ISO application to 0.126 mV/ms, from 0.087, when measured over the linear portion of the diastolic depolarization. This increase in the depolarizing rate (0.039 mV/ms) can decrease the duration taken for ~25 mV depolarization from the maximum diastolic potential (−65 mV) to the takeoff potential of the action potential

Fig. 10. Comparison of V_m and ionic currents (I_{CaL} , I_{st} , I_{ha} , I_{Kr} and I_{Ks}) before (black lines) and 30 s after the application of 0.01 μ M ISO (red lines). The maximum rates of rise of the initial action potential are superimposed. The peak of the I_{CaL} is off the scale.

(−40 mV) by 88 ms. The negative shift of I_{CaL} activation ($v_{shift} = 1.7$ mV with 0.378 μ M catalytic subunit of PKA) is marginal ($1.7/25 = 6.8\%$) if compared with the 25 mV magnitude of the diastolic depolarization. Thus we conclude that the increase in the slope of diastolic depolarization is mainly responsible for the positive chronotropy. A prolongation of the action potential by about 15.4 ms partially compromised the positive chronotropic effect of the increased rate of diastolic depolarization.

Fig. 11. Modulation of I_{NaCa} by β_1 -adrenergic stimulation. Intracellular Ca^{2+} concentration, I_{NaCa} and V_m are shown in the top, middle, and bottom panels, respectively. The traces were recorded for control conditions (black lines) and 30 s after the application of 0.01 μ M ISO (red lines).

The increase in the rate of diastolic depolarization is due to increases in both I_{st} and I_{ha} . If measured over the period from the maximum diastolic potential to the take-off potential, the ratio of the increase of I_{st} / I_{ha} measured by the time integral of the increase of each current during slow diastolic depolarization was 1.19, indicating almost equal contributions of these currents to the positive chronotropy.

The increase in the outward I_{Kr} during diastole is secondarily caused by the positive shift of V_m . On the other hand, the amplitude of I_{Ks} increased by up to 150% at the peak directly by the PKA catalytic subunit and activation of the Ca^{2+} -dependent gate. The current amplitude of I_{Ks} was still negligibly small during diastolic depolarization after β_1 -adrenergic stimulation.

Increase in I_{NaCa} and positive chronotropy during β_1 -adrenergic stimulation

The increase in the amplitude of I_{CaL} by β_1 -adrenergic stimulation increased the Ca^{2+} content of the cell, especially within the SR, which then increased the magnitude of the Ca^{2+} transient by 56% at the peak, thereby increas-

Fig. 12. Sequential events underlying the spontaneous action potential. LCR: Local subsarcolemmal ryanodine receptor-mediated Ca^{2+} release.

ing the magnitude of I_{NaCa} during the Ca^{2+} transient. However, because of the PKA-dependent activation of Ca^{2+} uptake by SERCA, the decay of the Ca^{2+} transient accelerated during β 1-adrenergic stimulation, so that $[\text{Ca}^{2+}]_i$ and therefore the magnitude of inward I_{NaCa} were almost superimposable with control data during diastolic depolarization (Fig. 11, middle panel). Thus the above modulation of I_{NaCa} does not contribute to the positive chronotropy in the model. On the contrary, the increase in I_{NaCa} during the action potential hampered the positive chronotropic effect of β 1-adrenergic stimulation by prolonging the action potential duration. These conclusions differ from those in Kurata *et al.* (2002) [32] and Maltsev *et al.* (2004) [33] and will be discussed in the next section.

DISCUSSION

I_{Ks} and I_{Kr} in the guinea pig SAN cell model

I_{Ks} has recently been suggested to be a large component of I_K in guinea pig SANs [13, 14], and therefore we investigated the contribution of I_{Ks} to slow diastolic depolarization by simulation. This analysis showed that the decay of K^+ conductance by the deactivation of I_{Kr} , but not by I_{Ks} , only influenced the lead potential, V_L , and played a major role in determining pacemaker depolarization in guinea pig SANs, as also found in other mammalian species, including rabbit [11, 12, 19, 34]. We clearly showed the slight possibility of involvement of I_{Ks} in slow diastolic depolarization because the activation of I_{Ks} during the preceding action potential was negligibly small. On the other hand, the activation of I_{Kr} was rapid, and the amplitude of I_{Kr} was larger than I_{Ks} , during the repolarizing phase of action potential (Figs. 8 and 10). Even the amplitude of I_{Kr} , measured as the E-4031 sensitive component of the tail current in the voltage clamp experiment, was only half (approximately 51% with 100 ms test pulse) that of I_{Ks} in Fig. 2 in Matsuura *et al.* (2002) [13]. Our simulation sug-

gested that the increase of I_{Ks} is useful in inhibiting the prolongation of the action potential by counterbalancing the increase in I_{CaL} and I_{NaCa} during β 1-adrenergic stimulation. We assumed that I_{Kr} was not modulated by β 1-adrenergic stimulation in our model, but it has been reported that the deactivation rate of I_K is accelerated by ISO in rabbit SANs. The authors suggested that the rate of slow diastolic depolarization would be accelerated by this mechanism during β 1-adrenergic stimulation [35]. However, it was difficult to reproduce simultaneous negative shifts in the I-V relationship and an increase in decay rate because negative shifts in the voltage dependency of activation were usually accompanied by a decrease in deactivation rate in the conventional voltage-dependent gate model. Therefore this mechanism was not included in our model.

Intracellular Ca^{2+} concentration and pacemaker activity

It is still a matter of debate whether intracellular Ca^{2+} dynamics affect pacemaker activity [9, 10, 36]. The mechanism in which the inward I_{NaCa} was enhanced by the time-dependent local intracellular Ca^{2+} concentration and accelerated pacemaker depolarization has been reproduced only in the model developed by Kurata *et al.* (2002) [32]. In this model, it was assumed that the subsarcolemmal domain of 1% of the cell volume increased the Ca^{2+} concentration-dependent enhancement of I_{NaCa} during the last 60 ms of slow diastolic depolarization, and that activated I_{CaT} increased the local subsarcolemmal Ca^{2+} concentration in addition to SR Ca^{2+} release during slow diastolic depolarization. However, without the effect of the subsarcolemmal domain (as in our model), Ca^{2+} released from the SR diffuses to the cytosol instantaneously and does not enhance I_{NaCa} . Similarly, the inclusion of diastolic spontaneous local Ca^{2+} release from the SR, as measured by Bogdanov *et al.* (2001) [37], in a model includ-

ing the subsarcolemmal space increases the rate of slow diastolic depolarization by activating I_{NaCa} [33]. To assess the validity of different models, we need to determine experimentally if the subsarcolemmal Ca^{2+} concentration is changed when EGTA is applied to inhibit the global (myoplasmic) Ca^{2+} transient. This is because most of the spontaneous action potentials have been recorded using the ruptured patch clamp mode with EGTA in the pipette solution. The amplitude of I_{CaT} is still uncertain in guinea pig SANs, and further experiments are needed. It is also important to consider variability in the distributions of ionic channels [36] according to the location and size of the cells [38] when the contribution of the SR to pacemaker activity is being discussed [39].

Spontaneous activity and mechanisms of positive chronotropy during $\beta 1$ -adrenergic stimulation in SANs

The positive chronotropic effect of $\beta 1$ -adrenergic stimulation is summarized in Fig. 12, using the scheme of sequential events during the spontaneous action potential. The negative shift of I_{CaL} activation, which supports the initial rising phase and the overshoot of the action potential [40], was only supplemental to the $\beta 1$ -adrenergic chronotropy at moderate agonist concentrations. The increase in the I_{CaL} magnitude prolongs the duration of action potentials and thereby decreases the firing frequency, as suggested by Sarai *et al.* (2003) [2]. In turn, the increased intracellular Ca^{2+} transient under $\beta 1$ -adrenergic stimulation partly compensated for the increase in the action potential duration by inactivating I_{CaL} and activating I_{Ks} . The increase in I_{ha} and I_{st} contributed equally to accelerating slow diastolic depolarization. The contribution of I_{NaCa} to modifying slow diastolic depolarization was almost negligible in our model, even in the presence of $\beta 1$ -adrenergic agonist. However, such currents might play a crucial role in increasing the rate of depolarization only if I_{NaCa} is strongly coupled with SR Ca^{2+} release by assuming a very limited subsarcolemmal space, as in the model of Kurata *et al.* (2002) [32] and in the modified version of this model in Maltsev *et al.* (2004) [33].

The authors thank Professor T. Powell, Associate Professor T. Mitsuiye, and members of the Cell/Biodynamics Simulation Project, Kyoto University, for their suggestions. This work was supported by the Leading Project for Biosimulation from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

- Matsuoka S, Sarai N, Kuratomi S, Ono K, Noma A. Role of individual ionic current systems in ventricular cells hypothesized by a model study. *Jpn J Physiol.* 2003;53:105-23.
- Sarai N, Matsuoka S, Kuratomi S, Ono K, Noma A. Role of individual ionic current systems in the SA node hypothesized by a model study. *Jpn J Physiol.* 2003;53:125-34.
- Matsuoka S, Sarai N, Jo H, Noma A. Simulation of ATP metabolism in cardiac excitation-contraction coupling. *Prog Biophys Mol Biol.* 2004;85:279-99.
- Terashima K, Takeuchi A, Sarai N, Matsuoka S, Shim EB, Leem CH, Noma A. Modelling Cl^- homeostasis and volume regulation of the cardiac cell. *Philos Transact A Math Phys Eng Sci.* 2006;364:1245-65.
- Takeuchi A, Tatsumi S, Sarai N, Terashima K, Matsuoka S, Noma A. Ionic mechanisms of cardiac cell swelling induced by blocking Na^+/K^+ pump as revealed by experiments and simulation. *J Gen Physiol.* 2006;128:495-507.
- Kuzumoto M, Takeuchi A, Nakai H, Oka C, Noma A, Matsuoka S. Simulation analysis of intracellular Na^+ and Cl^- homeostasis during $\beta 1$ -adrenergic stimulation of cardiac myocyte. *Prog Biophys Mol Biol.* 2007; in press.
- Rigg L, Terrar DA. Possible role of calcium release from the sarcoplasmic reticulum in pacemaking in guinea-pig sino-atrial node. *Exp Physiol.* 1996;81:877-80.
- Li J, Qu J, Nathan RD. Ionic basis of ryanodine's negative chronotropic effect on pacemaker cells isolated from the sinoatrial node. *Am J Physiol.* 1997;273:H2481-9.
- Honjo H, Inada S, Lancaster MK, Yamamoto M, Niwa R, Jones SA, Shibata N, Mitsui K, Horiuchi T, Kamiya K, Kodama I, Boyett MR. Sarcoplasmic reticulum Ca^{2+} release is not a dominating factor in sinoatrial node pacemaker activity. *Circ Res.* 2003;92:e41-4.
- Lakatta EG, Maltsev VA, Bogdanov KY, Stern MD, Vinogradova TM. Cyclic variation of intracellular calcium: a critical factor for cardiac pacemaker cell dominance. *Circ Res.* 2003;92:e45-50.
- Irisawa H, Brown HF, Giles W. Cardiac pacemaking in the sinoatrial node. *Physiol Rev.* 1993;73:197-227.
- Noma A. Ionic mechanisms of the cardiac pacemaker potential. *Jpn Heart J.* 1996;37:673-82.
- Matsuura H, Ehara T, Ding WG, Omatsu-Kanbe M, Isono T. Rapidly and slowly activating components of delayed rectifier K^+ current in guinea-pig sino-atrial node pacemaker cells. *J Physiol.* 2002;540:815-30.
- Anumonwo JM, Freeman LC, Kwok WM, Kass RS. Delayed rectification in single cells isolated from guinea pig sinoatrial node. *Am J Physiol.* 1992;262:H921-5.
- Korzeniewski B, Zoladz JA. A model of oxidative phosphorylation in mammalian skeletal muscle. *Biophys Chem.* 2001;92:17-34.
- Nichols CG, Lederer WJ. Adenosine triphosphate-sensitive potassium channels in the cardiovascular system. *Am J Physiol.* 1991;261:H1675-86.
- Jo H, Noma A, Matsuoka S. Calcium-mediated coupling between mitochondrial substrate dehydrogenation and cardiac workload in single guinea-pig ventricular myocytes. *J Mol Cell Cardiol.* 2006;40:394-404.
- Takano M, Noma A. Distribution of the isoprenaline-induced chloride current in rabbit heart. *Pflugers Arch.* 1992;420:223-6.
- Ito H, Ono K. A rapidly activating delayed rectifier K^+ channel in rabbit sinoatrial node cells. *Am J Physiol.* 1995;269:H443-52.
- Ono K, Ito H. Role of rapidly activating delayed rectifier K^+ current in sinoatrial node pacemaker activity. *Am J Physiol.* 1995;269:H453-62.
- Sarai N, Matsuoka S, Noma A. simBio: a Java package for the development of detailed cell models. *Prog Biophys Mol Biol.* 2006; 90.
- Kameyama M, Hofmann F, Trautwein W. On the mechanism of beta-adrenergic regulation of the Ca channel in the guinea-pig heart. *Pflugers Arch.* 1985;405:285-93.
- Zaza A, Robinson RB, DiFrancesco D. Basal responses of the L-type Ca^{2+} and hyperpolarization-activated currents to autonomic agonists in the rabbit sino-atrial node. *J Physiol.* 1996; 491 (Pt 2): 347-55.
- Ke Y, Lei M, Collins TP, Rakovic S, Mattick PA, Yamasaki M, Brodie MS, Terrar DA, Solaro RJ. Regulation of L-type calcium channel and delayed rectifier potassium channel activity by p21-activated kinase-1 in guinea pig sinoatrial node pacemaker cells. *Circ Res.* 2007;100:1317-27.
- McDonald TF, Pelzer S, Trautwein W, Pelzer DJ. Regulation and modulation of calcium channels in cardiac, skeletal, and smooth muscle cells. *Physiol Rev.* 1994;74:365-507.
- Toyoda F, Ding WG, Matsuura H. Responses of the sustained inward current to autonomic agonists in guinea-pig sino-atrial node pacemaker cells. *Br J Pharmacol.* 2005;144:660-8.
- Bucchi A, Baruscotti M, Robinson RB, DiFrancesco D. $I(f)$ -dependent modulation of pacemaker rate mediated by cAMP in the presence of ryanodine in rabbit sino-atrial node cells. *J Mol Cell Cardiol.* 2003;35:905-13.
- Hagiwara N, Irisawa H. Modulation by intracellular Ca^{2+} of the hyperpolarization-activated inward current in rabbit single sino-atrial node cells. *J Physiol.* 1989;409:121-41.
- Ding WG, Toyoda F, Matsuura H. Blocking action of chromanol 293B on the slow component of delayed rectifier K^+ current in guinea-pig sino-atrial node cells. *Br J Pharmacol.* 2002;137:253-62.
- Guo J, Mitsuiye T, Noma A. The sustained inward current in sino-atrial node cells

- of guinea-pig heart. *Pflugers Arch.* 1997;433:390-6.
31. Rigg L, Heath BM, Cui Y, Terrar DA. Localisation and functional significance of ryanodine receptors during beta-adrenoceptor stimulation in the guinea-pig sinoatrial node. *Cardiovasc Res.* 2000;48:254-64.
32. Kurata Y, Hisatome I, Imanishi S, Shibamoto T. Dynamical description of sinoatrial node pacemaking: improved mathematical model for primary pacemaker cell. *Am J Physiol Heart Circ Physiol.* 2002;283:H2074-101.
33. Maltsev VA, Vinogradova TM, Bogdanov KY, Lakatta EG, Stern MD. Diastolic calcium release controls the beating rate of rabbit sinoatrial node cells: numerical modeling of the coupling process. *Biophys J.* 2004;86:2596-605.
34. Verheijck EE, van Ginneken AC, Bourier J, Bouman LN. Effects of delayed rectifier current blockade by E-4031 on impulse generation in single sinoatrial nodal myocytes of the rabbit. *Circ Res.* 1995;76:607-15.
35. Lei M, Brown HF, Terrar DA. Modulation of delayed rectifier potassium current, i_K , by isoprenaline in rabbit isolated pacemaker cells. *Exp Physiol.* 2000;85:27-35.
36. Musa H, Lei M, Honjo H, Jones SA, Dobrzynski H, Lancaster MK, Takagishi Y, Henderson Z, Kodama I, Boyett MR. Heterogeneous expression of Ca^{2+} handling proteins in rabbit sinoatrial node. *J Histochem Cytochem.* 2002;50:311-24.
37. Bogdanov KY, Vinogradova TM, Lakatta EG. Sinoatrial nodal cell ryanodine receptor and Na^+-Ca^{2+} exchanger: molecular partners in pacemaker regulation. *Circ Res.* 2001;88:1254-8.
38. Boyett MR, Honjo H, Kodama I, Lancaster MK, Lei M, Musa H, Zhang H. The sinoatrial node: cell size does matter. *Circ Res.* 2007;101:e81-2.
39. Boyett MR, Honjo H, Kodama I. The sinoatrial node, a heterogeneous pacemaker structure. *Cardiovasc Res.* 2000;47:658-87.
40. Noma A, Irisawa H. Effects of calcium ion on the rising phase of the action potential in rabbit sinoatrial node cells. *Jpn J Physiol.* 1976;26:93-9.