**Go controls the hyperpolarization-activated current in embryonic stem cell-derived cardiocytes**

**Ye CP, Duan SZ, Milstone DS, Mortensen RM.** Go controls the hyperpolarization-activated current in embryonic stem cell-derived cardiocytes. *Am J Physiol Heart Circ Physiol* 294: H979–H985, 2008. First published December 21, 2007; doi:10.1152/ajpheart.00293.2007.—Hyperpolarization current (I_H) is an important player in controlling heart rate and is stimulated by cAMP and inhibited by members of the pertussis toxin-sensitive G-protein G3i/G3o family. We have successfully derived cardiocytes from embryonic stem cells lacking G3i or G3o and G3o. We have established that both basal and isoproterenol-stimulated activities of I_H in these cardiocytes have typical nodal-atrial characteristics and are unaffected by targeted gene inactivation of the G proteins G3i or G3o and G3o. Under basal conditions, both G3i and G3o are required for muscarinic inhibition of I_H activity via a mechanism that involves the generation of nitric oxide, whereas, with prior stimulation by β-agonists, only G3i is required and G3o and nitric oxide production are not. Our findings establish an essential role for G3o in the antidiadenergic effect of muscarinic agent on I_H.

G proteins; ion channels; nitric oxide; muscarinic receptors

**ALTERATION IN THE CARDIAC SINOATRIAL NODE SPONTANEOUS DISCHARGE RATE IS A CENTRAL MECHANISM FOR THE CONTROL OF HEART RATE.** Heart rate is stimulated by sympathetic stimulation through β-adrenergic receptors and the activation of G3i and inhibited by parasympathetic activity through M3 muscarinic acetylcholine receptors and the activation of G3o/G3i. Understanding the pathways that control heart rate will lead to the better understanding of arrhythmias of the sinoatrial node associated with aging and cardiovascular diseases. There has been considerable debate about the specific signal transduction pathways and ion channels that regulate heart rate in response to M2 muscarinic stimulation (3, 4, 10, 11, 24, 28).

Activation of the inwardly rectifying potassium current, (I_K-ACh), has been hypothesized to play a major role in the negative chronotropic effects of parasympathetic stimulation (24). However, the importance of I_K-ACh has been challenged because no correlation has been established between the dose response of this channel and cardiomyocyte slowing (12). In embryonic stem (ES) cell-derived cardiocytes, gene inactivation of αo2 or αo3 eliminates the muscarinic stimulation of I_K-ACh but does not affect cardiomyocyte slowing (26). Inactivation of I_K-ACh by G protein-activated inwardly rectifying K+ channel 4 knockout, which eliminates basal and stimulated I_K-ACh activity, was shown to nearly eliminate the heart rate variability and to decrease the negative chronotropic effects of parasympathetic stimulation (27). These results show that I_K-ACh plays a critical role in regulation of heart rate and heart rate variability but that other pathways and ion channels are also involved in regulation of the negative chronotropic effects. I_H, the hyperpolarization-activated nonselective cation current, has been proposed to contribute to the negative chronotropic effects of parasympathetic stimulation (1, 10, 21). It has been reported that an I_H mutation results in reduced heart rates in zebrafish embryos (4). Like the L-type Ca2+ channel, the activity of I_H is modulated by G-protein-regulated second messenger systems (6, 29). A role for the adenylyl cyclase/cAMP system in transmitting β-adrenergic and muscarinic receptor input has been extensively documented (2, 25). More recently, nitric oxide (NO) was shown to stimulate basal I_H activity directly and to inhibit the isoproterenol (Iso)-stimulated activity of I_H in preparations of isolated sinoatrial nodal cells (15, 16, 31). Furthermore, the effects of acetylcholine and NO appear to involve distinct regulatory mechanisms (5, 31).

Because it has not been defined which G3i/Go protein is responsible for the negative chronotropic effects on I_H by parasympathetic stimulation, we hypothesize that specific G proteins (G3o2, G3o3, and/or G3o) control I_H activity under different conditions (basal or adrenergic stimulation). We have used cardiocytes derived from wild-type (WT) and mutant mouse ES cells in which specific genes for G proteins have been inactivated (26) to study the muscarinic regulation of I_H.

**MATERIALS AND METHODS**

**Cell lines and culture.** The generation of mutant (αo2 and αo3/αo3-null) mouse D3 ES cell lines has been described previously (26, 30). Briefly, constructs with a neomycin resistance gene inserted in a required exon were used to target the endogenous αo2 and αo3 genes in WT D3 ES cells. The αo3-null cell line was transfected with a hygromycin containing an αo2 construct to produce the cell line lacking both αo2 and αo3 (αo2/αo3-null). Both WT and mutant D3 ES cell lines were maintained and differentiated as before. After 14–18 days of differentiation, spontaneously contracting cardiomyocytes were dissociated by collagenase digestion or mechanically for study of I_H.

**Electrophysiological measurements.** All recordings were performed at ambient temperature (22–24°C). Action potentials were recorded with the use of a nystatin-perforated whole cell configuration in current-clamp mode (19). Pipettes with a tip diameter of 1–2 μm

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were filled with a solution containing (in mM) 90 potassium aspartate, 50 KCl, 4 MgCl₂, 10 HEPES (pH 7.4), and 100 μg/ml nystatin. After stable basal whole cell recordings, the bath was perfused with agent-containing (Iso or carbachol) solution, and the recordings continued for 15–20 min.

Conventional whole cell recordings in the voltage-clamp mode were performed for characterization of \(I_f\). The standard bath solution contained (in mM) 150 NaCl, 5.4 KCl, 0.5 MgCl₂, 1.8 CoCl₂, 2 mM HEPES (pH 7.4), and 10 glucose. Other currents (calcium, potassium, BaCl₂, and 30 mV) were expressed as means ± SE, and statistical comparisons between groups were performed by Student’s t-test using the same software.

**RESULTS**

Mutant ES cells lacking α₂o (α₂o-null) and both α₂/α₃ (α₂/α₃-null) were isolated and characterized previously (26, 30). Western blots confirmed gene inactivation for the individual knockout cell lines and reflected no compensatory changes in expression of other G-protein subunits (data not shown). These results are the same as reported before (26, 30). Compared with WT cells, α₂o-null and α₂/α₃-null cells showed no significant differences in growth characteristics or in their ability to differentiate into cardiocytes.

**Spontaneous action potential.** Two weeks after the induction of differentiation, the cells were put in normal Tyrode solution and observed under microscope. Only those individual spindle-shaped cells that continued to contract after 5 min were chosen for the present study. Spontaneous action potentials recorded from WT and mutant cells displayed the characteristic shape of atrial-nodal cardiocytes (Fig. 1). In the basal condition, the action potentials occurred at frequencies that were not significantly different among the cell lines. Addition of the β-adrenergic agonist Iso (20 nM) increased the rate of diastolic depolarization and increased the firing rate in all cell lines. The muscarinic agonist carbachol (50 nM) decreased the Iso-stimulated firing rate of action potentials in WT and α₂/α₃-null cells (Fig. 1A). Addition of carbachol alone also increased the firing rate in Iso-stimulated WT cells to lower than basal condition (Fig. 1A). Carbachol alone decreased the firing rate in all cell lines (38 ± 2 beats/min for WT, 43 ± 5 beats/min for α₂/α₃-null, 40 ± 3 beats/min for α₂o-null; \(P > 0.05\) for comparisons between different cell lines), consistent with previous data on α₂o-null cells and α₂-null cells (26). Although the isolated hearts from α₂o-null mice respond to carbachol alone with slightly less sensitivity (14), it would be difficult to detect this small change

![Fig. 1](http://ajpheart.physiology.org/)
in cell culture. Furthermore, changes in diastolic depolarization rate in all cell lines followed the same trend as the firing rate under different conditions.

**Characterization of If:** Examples of whole cell current traces (Fig. 2A) revealed a slowly activating inward current evoked by hyperpolarization. The voltage dependence of current activation is illustrated in the activation curve (Fig. 2B). Under basal conditions, identical whole cell currents were also obtained from \( \alpha_{2}/\alpha_{3}\)-null or \( \alpha_{\omega}\)-null cells (traces not shown). \( V_{50} \) results were \(-70.3 \pm 0.4 \text{ mV} (n = 10) \) for WT, \(-71.0 \pm 0.7 \text{ mV} (n = 7) \) for \( \alpha_{2}/\alpha_{3}\)-null, and \(-70.7 \pm 0.6 \text{ mV} (n = 8) \) for \( \alpha_{\omega}\)-null; \( k \) results were \(-8.5 \pm 0.5 \text{ mV} (n = 10) \) for WT, \(-8.8 \pm 0.9 \text{ mV} (n = 7) \) for \( \alpha_{2}/\alpha_{3}\)-null, and \(-8.3 \pm 0.7 \text{ mV} (n = 8) \) for \( \alpha_{\omega}\)-null cells. No differences were shown among the cell lines.

A typical \( I_{f} \) in sinoatrial node cells has lower amplitude with lower concentrations of \( Na^{+} \) and higher amplitude with higher concentrations of \( K^{+} \) (1, 10, 20). To test whether \( I_{f} \) in ES cell-derived cardiocytes have these characteristics, we recorded tail currents in bath solutions containing different concentrations of \( Na^{+} \) and \( K^{+} \). The representative currents are shown in Fig. 2C. The \( V_{c} \) determined from the current-voltage curves of these tail currents (Fig. 2D), demonstrated a shift in \( V_{c} \) from \(-26.2 \) to \(-37.3 \text{ mV} \) when \( Na^{+} \) was lowered from 150 to 90 mM. In contrast, an increase in extracellular \( K^{+} \) from 5.4 to 20 mM shifted \( V_{c} \) to a more positive potential, confirming that the current described above is a nonselective cation current carried by both \( Na^{+} \) and \( K^{+} \). These characteristics are similar to those of \( I_{f} \) in sinoatrial node cells.

This inward current was also found to be sensitive to cesium and not altered by externally applied barium (data not shown). Overall, the electrophysiological characteristics of \( I_{f} \) expressed in the ES cell-derived cardiocytes were similar to the \( I_{f} \) from sinoatrial nodal cells (7, 9, 10). Furthermore, our data showed that these basal characteristics were not altered by inactivation of the pertussis toxin (PTX)-sensitive \( \alpha_{2}/\alpha_{3}\)-null subunits. \( I_{f} \) currents were observed in \( \sim 75-80\% \) of spontaneously contracting cardiocytes tested (a total of 108 cells from 22 preparations), and this percentage did not vary significantly between WT and mutant cells.

**Inhibition of basal \( I_{f} \) requires multiple G proteins.** We examined the ability of the muscarinic agonist carbachol to modulate basal \( I_{f} \) activity in WT and \( \alpha\)-subunit-null ES cell-derived cardiocytes. The basal \( I_{f} \) traces elicited using the same voltage protocols for WT and mutant cells were quite similar. The voltage dependence of current activation is illustrated in the activation curve (Fig. 3). Externally applied carbachol (1 \( \mu M \)) reduced basal \( I_{f} \) amplitude at \(-70 \text{ mV} \) and shifted the activation curve to a more negative \( V_{50} \) in WT cells; the voltage shift, \( \Delta V_{50} \) (carbachol \( V_{50} \) – basal \( V_{50} \)), is \(-6.30 \pm 1.33 \text{ mV} (n = 4) \) for WT cells (Fig. 3A). Carbachol had no significant effect on the current of either \( \alpha_{2}/\alpha_{3}\)-null or \( \alpha_{\omega}\)-null cardiocytes; the \( \Delta V_{50} \) is \(-1.47 \pm 0.82 \text{ mV} (n = 4) \) for \( \alpha_{2}/\alpha_{3}\)-null cells and \(-0.85 \pm 0.78 \text{ mV} (n = 5) \) for \( \alpha_{\omega}\)-null cells (Fig. 3, B and C). A consistent pattern was observed for the mutant cell lines when the current amplitudes at \(-70 \text{ mV} \) were compared. The current amplitude at \(-70 \text{ mV} \) was measured because it is close to \( V_{50} \), at which the inhibition by carbachol has larger effects than at full-activation voltage.

**Inhibition of \( \beta\)-agonist-stimulated \( I_{f} \) requires \( \alpha_{i} \), but not \( \alpha_{o} \), \( \beta\)-Adrenergic agonists increase \( I_{f} \) activity in native nodal cells, and muscarinic agonists oppose the stimulation (9, 29).** We examined the ability of carbachol to oppose \( \beta\)-adrenergic stimulation (Iso) of \( I_{f} \) in WT and \( \alpha\)-subunit-null cardiocytes. Experiments were performed with the same protocol shown in Fig. 2A. Current amplitude at baseline (no additions), 3 min after exposure to Iso (0.5 \( \mu M \)), and 3 min after addition of both Iso and carbachol (1 \( \mu M \)) were measured at each hyperpolarizing voltage step. At \(-70 \text{ mV} \), Iso stimulated an approximately twofold increase in current amplitude; however, there was a much smaller increase at \(-110 \text{ mV} \). This voltage dependency of the adrenergic stimulation of \( I_{f} \) in these ES cell-derived cardiocytes is similar to that in ventricular myocytes (17). The activation curves \((G/G_{\max} \text{ vs. } \text{ voltage})\) are shown in Fig. 4. Data were analyzed with the Boltzmann equation; the effects of Iso and Iso plus carbachol on \( V_{50} \) and the amplitude of \( I_{f} \) were shown in Fig. 4. See also Fig. 2D. Characteristics of hyperpolarization current \((I_{p})\) in ES cell-derived cardiocytes. Conventional whole cell recordings in the voltage-clamp mode were performed. An individual cardiocyte was voltage-clamped from a holding potential of \(-35 \text{ mV} \) to various hyperpolarizing voltages (\(-45 \) to \(-110 \text{ mV} \) in \( 5-\text{mV} \) increments). A: representative current traces. Dotted line indicates the steady-state current at \(-35 \text{ mV} \). B: normalized specific conductance \((G/G_{\max})\) plotted vs. hyperpolarizing voltage. Half-maximal activation voltage \((V_{50})\) and slope factor \((k)\) are derived by fitting data in a Boltzmann equation (solid line). C: tail-current analysis for the \( I_{f} \) reversal potential. D: relationship between the tail current \((pA/pF)\) and test voltage steps in different extracellular solutions. Standard condition = 5.4 mM extracellular \( K^{+} \) and 150 mM extracellular \( Na^{+} \). Low \( Na^{+} = 2.5 \text{ mM extracellular } K^{+} \) and 90 mM extracellular \( Na^{+} \). High \( K^{+} = 20 \text{ mM extracellular } K^{+} \) and 130 mM extracellular \( Na^{+} \).
were summarized. We found no significant differences in the effects of Iso on $V_{50}$ or $k$ between the WT and $\alpha_{2}/\alpha_{13}$-null cells. The positive shift induced by Iso in $V_{50}$ was 9.1 mV for WT, $\alpha_{2}$-null, and $\alpha_{o}$-null cells, respectively ($n = 4$ for each cell type). All of the activation curves for Iso-stimulated currents had steeper voltage dependence, indicated by the inverse slope factor $k$, in the range of $-8.0$ to $-7.7$ mV for all cell types. In the majority of experiments, currents reached the maximum amplitude less than 1 min after exposure to Iso.

In WT cells, carbachol completely reversed the stimulatory effect of Iso on $I_{f}$ and reduced the current below its basal level.

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**Fig. 3.** Effects of Carb on $I_{f}$ of WT (A), $\alpha_{2}/\alpha_{13}$-null (B), and $\alpha_{o}$-null (C) cells. Activation curves are shown for normalized specific conductance ($G/G_{\text{max}}$) vs. hyperpolarizing voltage for WT, $\alpha_{o}$-null, and $\alpha_{2}/\alpha_{13}$-null cells. Curves were fit to data points by the Boltzmann distribution. Symbols and bars represent means ± SE for 4–6 individual cells. **Top**: representative traces. In A, Carb curve is significantly different from the basal curve (2-way ANOVA: $P < 0.0001$; Bonferroni posttests: **$P < 0.01$, #P < 0.001$). Carb curve and basal curve are not statistically different by 2-way ANOVA in either B ($P = 0.06$) or C ($P = 0.072$).

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**Fig. 4.** Antiadrenergic effect of Carb in WT (A), $\alpha_{2}/\alpha_{13}$-null (B), and $\alpha_{o}$-null (C) cells with activation of $I_{f}$ by Iso. Curves were plotted as described in MATERIALS AND METHODS and Fig. 3. Effect of Carb on Iso-stimulated $I_{f}$ current amplitude was determined by stimulating cells with 0.1 µM Iso for at least 5 min followed by Carb at the indicated concentrations. Results are expressed as the percentage of Iso-stimulated current amplitude before the addition of Carb. Symbols and bars represent means ± SE for 4–6 individual cells. **Top**: representative traces. In A, Iso curve is significantly different from the basal curve (2-way ANOVA: $P < 0.0001$; Bonferroni posttests: *$P < 0.05$, #P < 0.001$). Iso + Carb curve is also significantly different from the Iso curve (2-way ANOVA: $P < 0.0001$; Bonferroni posttests: **$P < 0.01$, &P < 0.001$). In B, Iso curve is significantly different from the basal curve (2-way ANOVA: $P < 0.0001$; Bonferroni posttests: #P < 0.001$). Iso + Carb curve is not significantly different from the basal curve (2-way ANOVA: $P = 0.11$); instead, it is significantly different from the Iso curve (two-way ANOVA: $P < 0.0001$; Bonferroni posttests: &P < 0.01, $SP < 0.001$). In C, Iso curve is significantly different from the basal curve (2-way ANOVA: $P < 0.0001$; Bonferroni posttests: *$P < 0.05$, #P < 0.001$). Iso + Carb curve is not significantly different from the basal curve (2-way ANOVA: $P < 0.0001$; Bonferroni posttests: &P < 0.001), but the Iso + Carb curve is not significantly different from the Iso curve (2-way ANOVA: $P = 0.19$).
causing a \(-4.1 \pm 1.3\) mV shift in \(V_{50}\) from basal (Fig. 4A). This was similar to the results seen with addition of carbachol to unstimulated cells. In cells lacking both \(\alpha_{\text{i2}}\) and \(\alpha_{\text{i3}}\), carbachol reversed the Iso-induced increase in \(I_I\) but did not decrease current activity below the basal level (Fig. 4B). In sharp contrast, carbachol had no effect on the Iso-stimulated current in cells lacking \(\alpha_{\text{i0}}\) (Fig. 4C). A dose-response curve generated from similar experiments showed that, even at higher concentrations of carbachol \((10^{-5} \text{M})\), the Iso-stimulated current was not significantly reduced in the \(\alpha_{\text{i0}}\)-null cardiocytes, as seen in \(\alpha_{\text{i2}}/\alpha_{\text{i3}}\)-null cells (Fig. 5). Without Iso stimulation, both \(\alpha_{\text{i0}}\)-null and \(\alpha_{\text{i2}}/\alpha_{\text{i3}}\)-null cells showed significantly less sensitivity to a range of carbachol concentrations (Fig. 5).

Because of previous controversial reports about the role of NO in \(\alpha_{\text{i0}}\)-mediated pathways, we investigated the effects of a NO synthase inhibitor, \(\text{l-NMMA}\). Pretreatment of WT cells with \(\text{l-NMMA (0.1 mM)}\) ameliorated the inhibitory effect of carbachol on basal \(I_I\). The activation curves showed that the NO synthase inhibitor did not alter the basal characteristics of \(I_I\) \((V_{50}\) or \(k)\) but that it did prevent carbachol from inducing a leftward shift in the activation curve (Fig. 6A). We also examined the effect of \(\text{l-NMMA}\) on the anti-\(\beta\)-adrenergic effect of carbachol in WT cells (Fig. 6B). Again, the NO synthase inhibitor had no effect on basal or Iso-stimulated current characteristics (current amplitude, \(V_{50}\), and \(k)\). Pretreatment of the WT cells with \(\text{l-NMMA}\) did not prevent the anti-\(\beta\)-adrenergic effect of carbachol. However, the NO synthase inhibitor prevented carbachol from further reducing \(I_I\) activity below the basal level.

**DISCUSSION**

To our knowledge, this is the first report to define the G-protein-signaling requirements for muscarinic-receptor modulation of \(I_I\) using cardiocytes lacking specific PTX-sen-
sitive G proteins. ES cell-derived cardiocytes have been demonstrated to recapitulate many characteristics of sinoatrial node cells (1, 10, 20). More importantly, they are much easier to be genetically modified than adult pacemaker cells, which are very difficult to isolate.

We had previously established that G_{i2} and G_{i3} (but not G_o) are required for activation of I_f (26). Muscarinic inhibition of the Iso-activated L-type calcium channel (I_{f,L-Ca}) requires G_o-dependent regulation of NO-dependent second messenger systems (30). For I_{f,L-Ca}, expression of both G_{i2} and G_{i3} is also required for the normal rapid inhibition of Iso-stimulated channel activity. We have now established that G_o is also essential for the antiadrenergic effect of carbachol on I_f and that the signaling mechanism does not appear to involve NO. Finally, muscarinic inhibition of basal I_f activity appears to require NO generation and all three PTX-sensitive G proteins: G_o, G_{i2}, and G_{i3}. The points at which these pathways converge have not yet been established. In some cell systems, NO may be a convergence point. Regulation of cAMP is less likely to be a convergence point, since it has been reported that inactivation of cAMP is less likely to be a convergence point, since it has been reported that inactivation of α_o but not α_o affects cAMP (18, 23).

For I_f and the L-type calcium channel, there has been considerable debate and disagreement about the role of NO in inhibition by muscarinic receptor activation. Interestingly, we find that the same G-protein subunit (α_o) is required for inhibition of I_f whether there is prior β-adrenergic stimulation or not. However, G_o and NO synthesize activity are only required when inhibiting I_f below the basal state and not when inhibiting I_f stimulated by β-adrenergic agonists. The involvement of a second messenger system in the basal state is consistent with the involvement of cAMP in the muscarinic inhibition of I_f in sinoatrial node myocytes observed by Accili et al. (3). It is not clear whether a second messenger system is involved in the presence of β-agonists. Thus pathways may differ based on the repertoire of other receptors. Therefore, the exact experimental conditions may influence the importance of NO in the pathway.

In these terminally differentiated ES cell-derived cardiocytes, the action potentials were characterized by a diastolic depolarization phase with the membrane potential slowly increasing from −65 to −40 mV, a voltage range well covered by the activation curves of I_f recorded in this study. Characteristics of the I_f expressed by these nodal-atrial-like cardiocytes are similar to those of I_f defined in adult sinoatrial node cells or cardiac ventricular myocytes, in aspects such as the trace of the tail currents and the amplitude change by concentrations of Na^+ and K^+ (1, 10, 20, 32). Muscarinic agonists decrease I_f activity via mechanisms involving their inhibitory effect on cellular cAMP levels (7, 13, 22, 25). The activation by Iso and inhibition by carbachol have larger effects at V_{So} than at full-activation voltage, confirming that β-adrenergic and muscarinic agonists modulate current kinetics (2). It has been reported that acetylcholine inhibits I_f in Purkinje fibers only after β-stimulation, whereas it also inhibits basal I_f in the sinoatrial node (8). The present results showed that the modulations of I_f by adrenergic and muscarinic agonists in the ES cell-derived cardiocytes are the same as those seen in the native I_f in sinoatrial nodal cells.

We also established that the principal electrophysiological characteristics of I_f were not altered in the α-subunit-null cell lines and that the stimulatory effect of the adrenergic agonist Iso was unaffected by inactivation of these PTX-sensitive G proteins. We did note a marked alteration in muscarinic modulation of basal I_f activity in cell lines lacking α_o or α_{i2}/α_{i3}. In both mutants, the ability of carbachol to reduce I_f activity below basal levels was blunted. This effect was similar to that observed with the NO synthase inhibitor L-NMMA, suggesting that one or more of these PTX-sensitive G proteins may inhibit basal I_f activity via NO-dependent mechanisms.

Absence of α_o completely prevented the anti-β-adrenergic effect of carbachol on I_f, whereas absence of both α_{i2} and α_{i3} did not significantly alter the inhibition by carbachol of Iso-stimulated currents. These data demonstrate that pathways regulated by G_o signaling are critical for the anti-β-adrenergic effect of carbachol on I_f and that G_{i2}/G_{i3} signaling is not required for this response. Furthermore, the inability of L-NMMA to alter the antiadrenergic effect of carbachol on I_f suggests that NO is not the principal second messenger involved.

These results establish a critical role for α_o-containing heterotrimers in the muscarinic regulation of negative chronotropy and I_f. The specific G proteins and second messenger systems required for regulation of heart rate, contractility, and cardiac ion channels (I_f, I_{f,L-Ca} and I_{f,K-ACb}) are different and can vary depending on the cell system analyzed.

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