Basal responses of the L-type Ca^{2+} and hyperpolarization-activated currents to autonomic agonists in the rabbit sino-atrial node

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1. The dose dependence of the cholinergic agonist acetylcholine (ACh) and the β-adrenergic agonist isoprenaline (Iso) were determined for the hyperpolarization-activated current (I_f) and the L-type Ca^{2+} current ($I_{Ca,L}$) in single cells isolated from the rabbit sino-atrial (SA) node.

2. ACh inhibited I_f by a negative shift of its activation curve with a maximal effect of −9·9 mV; half-maximal effect was produced by 0·019 μM ACh. High ACh concentrations were required to inhibit $I_{Ca,L}$ only partially (31 % inhibition at 300 μM).

3. In contrast, I_f and $I_{Ca,L}$ responded to Iso over a similar dose range, with concentrations for half-maximal enhancement of 0·0136 and 0·0070 μM, respectively.

4. The effects on spontaneous activity of ACh (range 0·001–0·03 μM) and Iso (range 0·001–1 μM) were investigated. ACh decreased the slope of diastolic depolarization at concentrations similar to those inhibiting I_f (>50% at 0·03 μM). Iso enhanced diastolic depolarization at concentrations similar to those affecting both I_f and $I_{Ca,L}$ (half-maximal effect at 0·027 μM).

5. In a ramp-clamp protocol simulating diastolic depolarization, the threshold for activation of inward nifedipine-sensitive current was −41·22 ± 0·68 mV. Although enhancing $I_{Ca,L}$, Iso did not affect this threshold.

6. Half-maximal ACh concentrations for inhibition of automaticity and I_f are similar and are lower than the threshold concentrations for modulation of $I_{Ca,L}$; this argues against a role of $I_{Ca,L}$ in direct muscarinic modulation of pacemaking. In contrast, modulation of I_f, $I_{Ca,L}$ and automaticity occur at similar Iso concentrations. The difference between maximum diastolic potential (−61·95 ± 0·93 mV) and the threshold for Iso-stimulated $I_{Ca,L}$ (−39·54 ± 1·03 mV) suggests that this current plays a role only at later stages of diastolic depolarization.

Characterization of the response to neurotransmitters of the currents contributing to sino-atrial automaticity is necessary for the understanding of the processes underlying modulation of heart rate. While the sensitivity to ACh of the hyperpolarization-activated current (I_f) and the ACh-activated K^+ current ($I_{K,ACh}$) were compared in a previous study (DiFrancesco, Ducouret & Robinson, 1989), little information is available concerning modulation of other currents, potentially involved in sino-atrial pacemaking, such as the high-threshold Ca^{2+} current ($I_{Ca,L}$) (Hagiwara, Irisawa & Kameyama, 1988). The purpose of the present study is to compare the sensitivity to muscarinic and β-adrenergic stimulation of I_f, $I_{Ca,L}$ and automaticity in isolated sino-atrial (SA) myocytes.

METHODS

Dissociation procedure

Hearts were removed from rabbits weighing 0·8–1 kg which were killed by cervical dislocation under inhalation anaesthesia, produced by cotton wool soaked with 200 mg tribromoethanol (Aldrich) dissolved in 10 ml ether. The animals were then

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exanguinated. Myocytes were isolated using the procedure described previously (DiFrancesco & Tromba, 1988*b*) and patch-clamped in the whole-cell or perforated-patch configurations at a temperature of 35·5–36·0 °C. Records were taken in the control solution, during superfusion with test solutions and after return to control; whenever a clear wash-out of effects of each exposure could not be obtained the data were discarded. Cell resistance and capacity were 1256·2 ± 90·2 MΩ ($n = 25$) and 29·13 ± 1·69 pF ($n = 30$), respectively. The resistance of pipettes filled with intracellular solution was 2–5 MΩ. Series resistance was between 4 and 6 MΩ in ruptured-patch, and between 10 and 20 MΩ in perforated-patch configurations and was corrected up to about 75%. Data were recorded on a videorecording system during the experiment, then analysed off-line by either replaying the data directly into a computer, or indirectly by means of a digital oscilloscope.

Experimental solutions

Cells were continuously superfused with normal Tyrode solution containing (mM): NaCl, 140; KCL, 5·4; $CaCl_2$, 1·8; $MgCl_2$, 1; D-glucose, 5·5; Hepes-NaOH, 5; adjusted to pH 7·4. A fast superfusion pipette allowed solution changes within 1 s (DiFrancesco & Tromba, 1988*b*). For studies of I_f, the external solution contained 1 mM $BaCl_2$ and 2 mM $MnCl_2$, except in those experiments where $I_{Ca,L}$ and I_f were measured simultaneously. $I_{Ca,L}$ was dissected by use of a modified Tyrode solution where the normal $CaCl_2$ was reduced to 0·5 mM, and 2 mM $BaCl_2$ and 0·1 mM $NiCl_2$ were included. Often, I_f and $I_{Ca,L}$ were measured simultaneously (see Fig. 2). The presence of Ni^{2+} and Ba^{2+} allowed blockade of the T-component and an increase of the L- *versus* T-component (Bean, 1985), as well as a Ba^{2+}-induced block of overlapping K^+ currents, including $I_{K,ACh}$. Furthermore, 1–10 μM nifedipine or nitrendipine was used, and $I_{Ca,L}$ defined as the difference current (Bean, 1985). Current decrease due to run-down was minimized by waiting for the measured current to settle to a stable level (usually a few minutes), and, when present, was compensated for during measurements. This was performed by linearly correcting for changes in the current amplitude occurring between control and wash-out recordings.

In a later set of experiments, perforated patches were used to further limit run-down. This allowed $I_{Ca,L}$ to be recorded in normal Tyrode solution. Pipettes were tip-filled normally and back-filled by a pipette solution (see composition below) containing 260 μM amphotericin. The cell responses to neurotransmitters were similar in the two sets of experiments.

Unless otherwise indicated, the pipette solution for $I_{Ca,L}$ experiments contained (mM): NaCl, 10; aspartic acid, 80; CsOH, 70; CsCl, 40; $MgCl_2$, 2; ATP-Na salt, 2; creatine phosphate, 5; GTP, 0·1, EGTA, 5; $CaCl_2$, 2 (calculated free Ca^{2+}, 10^{-7} M); Hepes-KOH, 10; pH 7·2. In perforated-patch experiments ATP-Na and GTP were omitted from the pipette solution. All chemicals were purchased from Sigma.

Measurement of the shift of the I_f activation curve

The presence of even reduced current run-down prevents the accurate estimation of changes of the degree of activation of I_f by protocols requiring several minutes, such as the construction of the full activation curve (DiFrancesco, Ferroni, Mazzanti & Tromba, 1986). We thus measured voltage shifts of the I_f activation curve

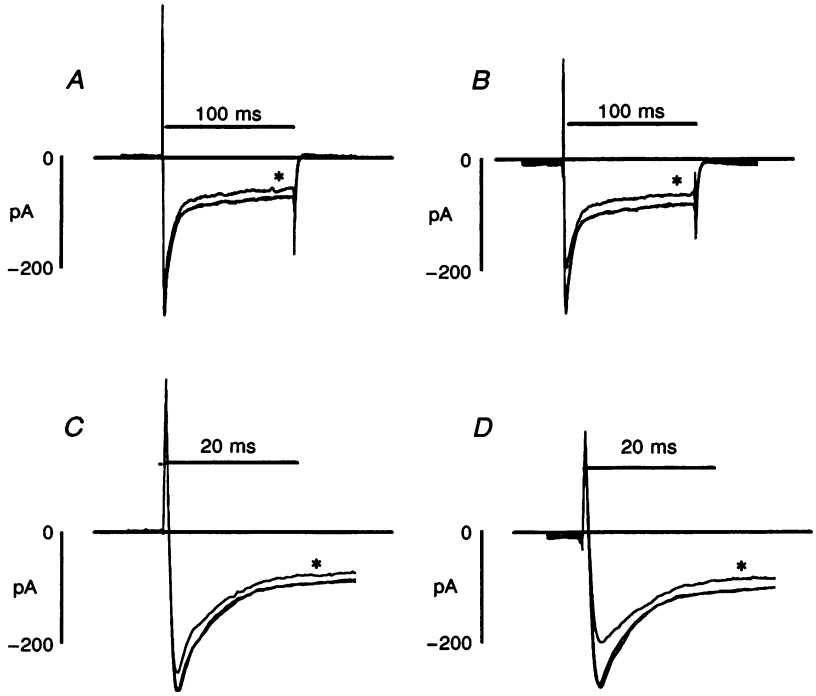

Figure 1. Inhibition of $I_{Ca,L}$ by high concentrations of ACh

$I_{Ca,L}$ was activated on depolarization from a holding potential of −35 to 0 mV. *A* and *B*, superimposition of traces recorded in a control solution, during exposure to ACh (*, 10 μM in *A* and 100 μM in *B*) and after wash-out. All traces were obtained by subtraction of currents recorded in the presence of 5 μM nifedipine. *C* and *D*, same traces as in *A* and *B*, plotted on expanded time scale for better resolution.

by a quicker method (DiFrancesco *et al.* 1989). From −35 mV, steps of constant amplitude were applied in the control solution at a rate of 0·166 Hz to a voltage E near the mid-activation point. During superfusion of the test solution, the holding potential was shifted manually (by an amount S_m), until the I_f trace overlapped that in the control solution. Since after adjustment the current was recorded at $E + S_m$, and not at E as in control, a correction was introduced to calculate the shift (S) of the I_f activation curve from the measured holding potential displacement (S_m). Superimposition of the two traces realizes the condition:

$$G_f(E - E_f)y_\infty(E) = G_f(E - E_f + S_m)\, y_\infty(E + S_m - S), \quad (1)$$

where G_f is conductance, E_f reversal potential, and $y_\infty(E)$ the steady-state activation variable at voltage E. Developing to the first term of Taylor series the right-hand term of eqn (1) and rearranging yields the relation:

$$S = S_m(1 + (y_\infty/y'_\infty)/(E - E_f)), \quad (2)$$

which was used for calculation of S with the values $y_\infty = 0{\cdot}5$, $E_f = -12{\cdot}24$ mV (DiFrancesco & Mangoni, 1994) and $y'_\infty = dy_\infty/dE = -0{\cdot}10811$ mV^{-1} (DiFrancesco & Noble, 1989). This procedure allowed quick evaluation of shifts with a resolution of tenths of a millivolt, due to the steepness of the I_f activation curve.

RESULTS

Effect of ACh on the currents I_f and $I_{Ca,L}$

In Fig. 1 the action of 10 and 100 μM ACh on $I_{Ca,L}$ activated by stepping to 0 mV from a holding potential of −35 mV in a single cell is shown.

The peak current was inhibited by 12·5 and 31·2% at 10 and 100 μM ACh, respectively. At the dose of 1 μM, ACh inhibited $I_{Ca,L}$ by only 4·3% (data not shown). The

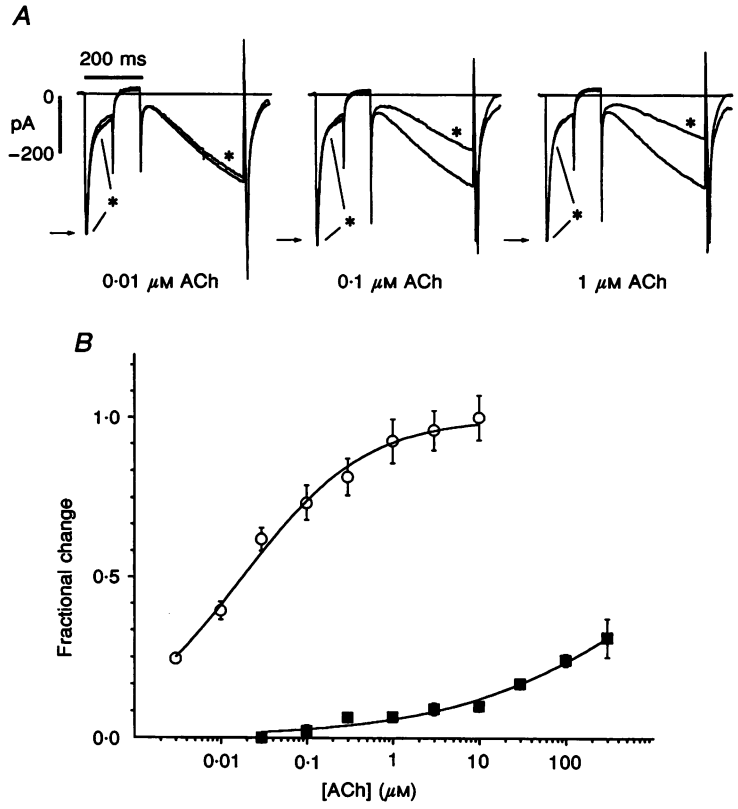

Figure 2. Dependence of I_f and $I_{Ca,L}$ upon ACh

A, $I_{Ca,L}$ and I_f were recorded simultaneously in the same cell (see text for details) in control and during superfusion with ACh at increasing concentrations. Arrows and asterisks indicate currents in control and ACh solutions, respectively. Digital subtraction of traces recorded in nifedipine (5 μM) was performed during depolarization to 0 mV in order to dissect the L-component of the Ca^{2+} current. Return traces coincided with the control traces and are omitted for clarity. *B*, dose–response relation for the ACh-induced I_f (○) and $I_{Ca,L}$ (■) inhibition. Plotted values (mean ± S.E.M.) represent normalized fractional decreases ($I_{Ca,L}$) or normalized shifts of the activation curve in millivolts (I_f). Continuous lines represent best fitting of data points by the Hill equation $y/y_{max} = A_{n_H}/(K' + A_{n_H})$, where y is the measured change, y_{max} its asymptotic value, A is agonist concentration, K' is the apparent dissociation constant and n_H is the Hill number. Best-fitting parameters were: $y_{max} = -9{\cdot}898$ mV, EC_{50} $(= (K')^{1/n_H}) = 0{\cdot}0194$ μM, $n_H = 0{\cdot}618$ (I_f); $y_{max} = 1$ (corresponding to the prediction of full current inhibition by saturating ACh), $EC_{50} = 2921$ μM; $n_H = 0{\cdot}348$ ($I_{Ca,L}$).

inhibition was fully reversed for each ACh concentration upon wash-out. Since the sensitivity to ACh of the I_f current in SA node cells is reported to be higher (DiFrancesco *et al.* 1989), the action of ACh on the two currents I_f and $I_{Ca,L}$ was compared with a protocol allowing simultaneous recording of both components. The protocol consisted of depolarizing steps from a holding potential of −40 to 0 mV (activating $I_{Ca,L}$ preferentially over $I_{Ca,T}$), followed by hyperpolarizations to −100 mV (activating I_f), applied sequentially every 4 s. To dissect $I_{Ca,L}$ from the T-component we also used an appropriate external solution (see Methods), and subtracted from control and ACh traces, traces during perfusion with 5 μM nifedipine. As shown in Fig. 2*A*, whereas I_f was moderately affected by 0·01 μM ACh and strongly inhibited by > 0·1 μM ACh, $I_{Ca,L}$ was hardly modified by even the highest ACh dose.

A more complete investigation of the dose dependence of ACh-induced inhibition of I_f and $I_{Ca,L}$ is shown in Fig. 2*B*. The $I_{Ca,L}$ dose–response relation was constructed from twenty-six cells. Inhibition of $I_{Ca,L}$ did not reach saturation with doses as large as 300 μM, at which the fractional decrease was 31·1 ± 6·4% ($n = 3$). The concentration for half-maximal inhibition of $I_{Ca,L}$ was calculated to be 2921 μM (see legend). Values for the I_f dose–response relationship were measured as described in the Methods, and their fitting yielded a maximal shift of −9·9 mV and a half-maximal inhibition concentration of 0·0194 μM (30 cells). Comparison of the dose–response curves indicates that much higher ACh concentrations are required to inhibit $I_{Ca,L}$ than to inhibit I_f; although less markedly, I_f is also known to be more sensitive to ACh than $I_{K,ACh}$ (half-maximal activation by ACh at 0·26 μM, DiFrancesco *et al.* 1989).

Effect of Iso on the currents I_f and $I_{Ca,L}$

To determine the relative role of ionic currents affected by β-adrenergic stimulation, the dose–response relation of Iso on I_f and $I_{Ca,L}$ was investigated next. Examples of the effect of three different doses of Iso on I_f and $I_{Ca,L}$ in one cell are shown in Fig. 3*A*.

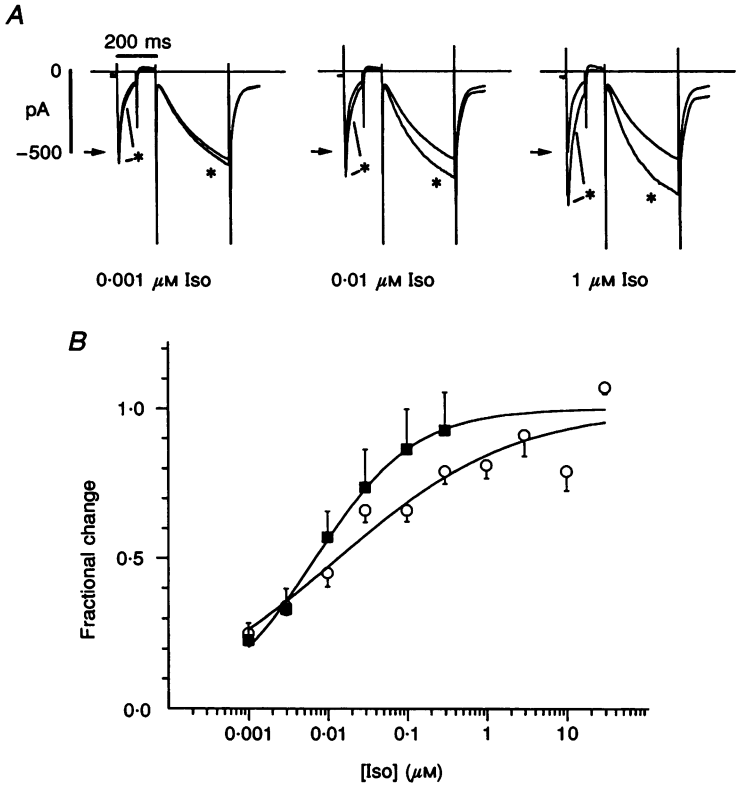

Figure 3. Dependence of I_f and $I_{Ca,L}$ upon Iso

A, $I_{Ca,L}$ and I_f were recorded simultaneously in the same cell in control and during superfusion with Iso at increasing concentrations (for details see text and legend of Fig. 1). Arrows and asterisks indicate currents in control and ACh solutions, respectively. *B*, dose–response relationships of Iso-induced I_f (○) and $I_{Ca,L}$ (■) stimulation. Data points were fitted with the same equation as in Fig. 2. Values for the $I_{Ca,L}$ curve represent normalized fractional increases in 55 cells, whereas those for the I_f curve represent normalized shifts of the activation curve in millivolts in 102 cells (mean ± S.E.M.). Only single exposures were used for constructing the $I_{Ca,L}$ curve to avoid interference from response run-down. Best-fitting parameters were: $y_{max} = 9{\cdot}62$ mV; EC_{50} ($= (K')^{1/n_H}$) = 0·0136 μM; $n_H = 0{\cdot}392$ (I_f); $y_{max} = 54{\cdot}19\%$; $EC_{50} = 0{\cdot}0070$ μM; $n_H = 0{\cdot}691$ ($I_{Ca,L}$). S.E.M. bars drawn in one direction only for clarity.

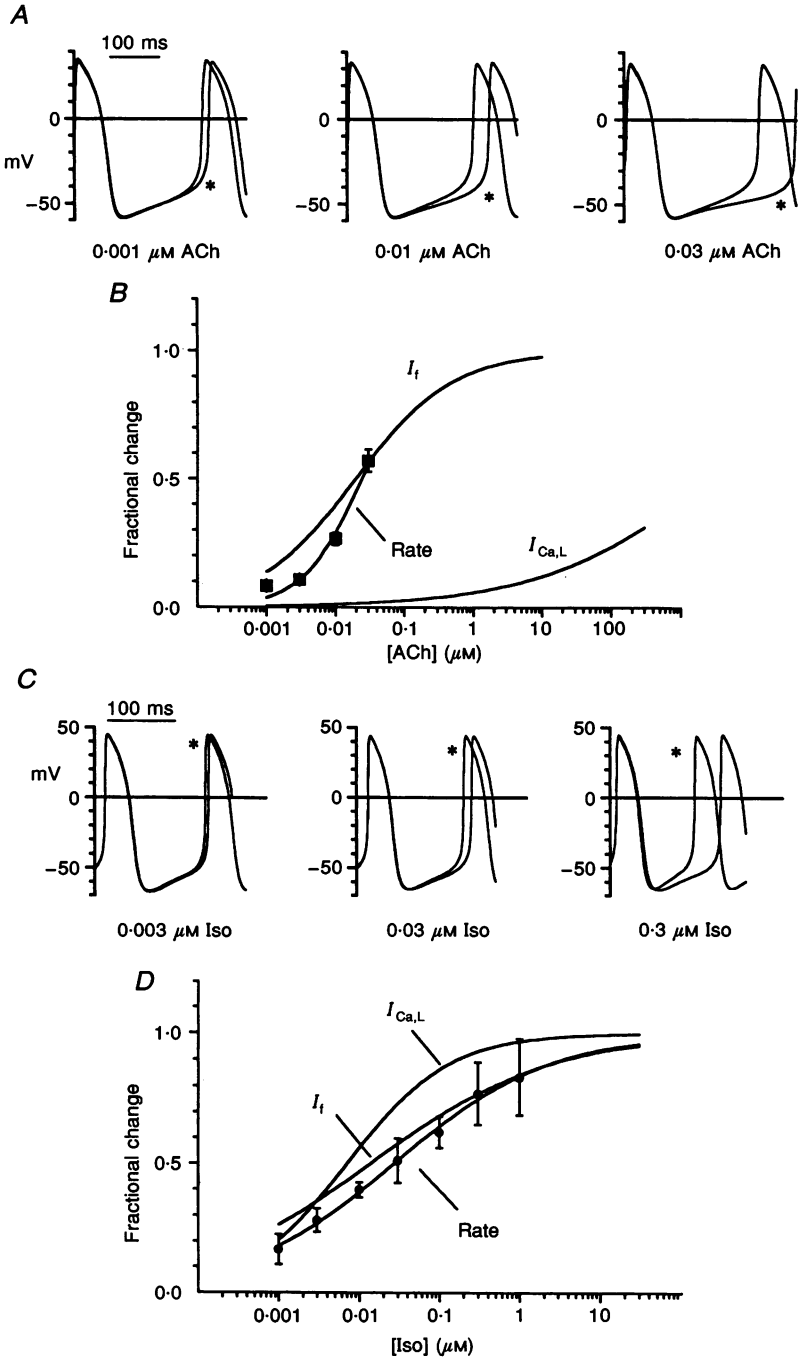

Figure 4. Effect of ACh and Iso on diastolic depolarization rate

A and *B*, action of ACh on diastolic rate. *A*, spontaneous activity recorded in a cell superfused by the control solution and after addition of increasing ACh concentrations (*). *B*, fractional diastolic depolarization rate decrease plotted against ACh concentration. Mean diastolic depolarization rate was measured between MDP and the take-off potential, identified as the point of maximal curvature (peak of second derivative) of action potentials. Five or more records were used for averaging each control and test trace, and the data plotted represent the fractional decrease (mean ± S.E.M.) from 6 cells. The best fit of the experimental data with the same equation as in Fig. 2 yielded an EC_{50} value of 0·0240 μM and an n_H value of 1·02 (maximal inhibition corresponded to $y_{max} = 1$). Dose–response curves for I_f and $I_{Ca,L}$, taken from Fig. 2, are also shown for comparison. *C* and *D*, action of Iso on diastolic rate. *C*, action potentials recorded before and during increasing Iso concentrations in one cell. *D*, dose–response relationship for the fractional diastolic rate against Iso concentration. Mean ± S.E.M. values from $n = 64$ cells are plotted. Best fitting of experimental data yielded the following parameters: $y_{max} = 0·8905$, $EC_{50} = 0·0268$ μM, $n_H = 0·454$. Also drawn for comparison are dose–response curves of I_f and $I_{Ca,L}$ taken from Fig. 3.

The comparison in Fig. 3*B* of dose–response relations for I_f and $I_{Ca,L}$ stimulation by Iso shows that, unlike the situation with ACh, the Iso relations cover similar ranges. The concentrations of Iso giving half-maximal I_f and $I_{Ca,L}$ stimulation are 13·6 and 7·0 nM, respectively.

Modulation of pacemaking rate by ACh and Iso

The evidence in Fig. 2 above for a specific action of low ACh doses on I_f suggests that the action of low ACh doses on spontaneous rate may be useful in estimating the I_f contribution to pacemaker activity.

In Fig. 4*A* an example of the changes induced by increasing ACh concentrations on the spontaneous activity in an SA node cell are shown. Each panel represents a superimposition of two representative traces taken just before and during superfusion with ACh. The fractional decrease of the slope of diastolic depolarization from six cells is plotted in Fig. 4*B* against ACh concentration. Measurements were limited to the ACh concentrations lower than 0·3 μM because higher doses arrested spontaneous activity. The position of the diastolic depolarization rate curve on the ACh concentration axis is compared with that of the dose–response relations for I_f and $I_{Ca,L}$ taken from Fig. 2. This comparison can only be roughly indicative, given the complex relation between currents and rate during activity, but does show that the diastolic depolarization rate curve lies at ACh concentrations that, while inhibiting I_f, are insufficient to affect $I_{Ca,L}$.

The dose dependence of the effect of Iso on the slope of diastolic depolarization is illustrated in Fig. 4*C* and *D*. Figure 4*C* shows the effect of increasing Iso concentrations on the rate of diastolic depolarization. Average quantitative

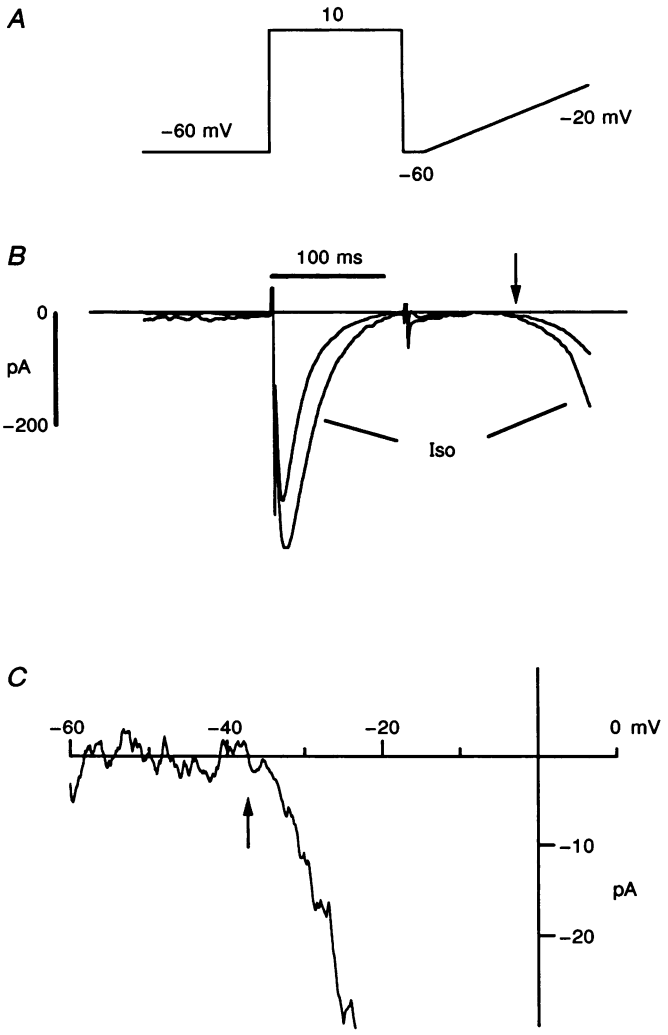

Figure 5. Effect of Iso on $I_{Ca,L}$ activation during ramp depolarization

A, voltage-clamp protocol used to simulate a cardiac cycle with a step (−60, 10, −60 mV) and a ramp (from −60 to −20 mV; 0·2 V s^{-1}). *B*, nifedipine (10 μM) difference traces in the control solution and during superfusion with 0·1 μM Iso. *C*, expanded plot of the ramp portion of the difference between traces in Iso and control as a function of membrane potential. Arrows in *B* and *C* mark the point at which the control and Iso current traces start to diverge.

results are plotted in Fig. 4*D*, and compared with the Iso dose–response relations for I_f and $I_{Ca,L}$, taken from Fig. 3. It is apparent that the rate of diastolic depolarization is modulated by Iso over the same range of concentrations as the two ionic currents.

Voltage dependence of the effect of Iso on $I_{Ca,L}$

In the light of the results in Fig. 3, indicating a similar dose dependence of I_f and $I_{Ca,L}$ on Iso, we wanted to verify the extent of $I_{Ca,L}$ (defined as the nifedipine-sensitive current) contribution to the inward current induced by Iso in the diastolic range. To address this question, a voltage-clamp protocol was employed that combined a voltage step with a voltage ramp. The step (from −60 to +10 mV for 125 ms) served to rapidly depolarize and repolarize the cell, as in an action potential, while the ramp (slope of 0·2 V s^{-1}) mimicked the diastolic depolarization. The voltage protocol (Fig. 5*A*) was conducted before and during exposure to nifedipine, and a nifedipine-sensitive difference current, in the absence and presence of 100 nM Iso, was measured.

Figure 5*B* illustrates the difference current in the control solution and in the presence of Iso. The large inward difference current during the initial step depolarization, is increased by Iso and decays by the end of the step. At the start of the ramp there is little difference current, but an inward current develops later during the ramp. In Fig. 5*C* the ramp portion of the Iso-induced (nifedipine sensitive) current is replotted at higher gain as a function of voltage during ramp application. No increase of the nifedipine difference current by Iso occurred in this cell until the membrane potential was positive to about −37 mV (arrow). The threshold of $I_{Ca,L}$ activation during the ramp was −41·22 ± 0·68 mV ($n = 5$) and the threshold for Iso-activated current was −39·54 ± 1·03 mV ($n = 5$). The comparison between these values and the maximum diastolic potential measured in spontaneously beating cells (−61·95 ± 0·93 mV; $n = 29$) suggests that, if Iso stimulation of $I_{Ca,L}$ does contribute to diastolic depolarization, it is only during its terminal phase, when the membrane potential is approaching the activation threshold for the subsequent action potential. The threshold potential for $I_{Ca,L}$ activation during the ramp is in agreement with that measured during diastolic depolarization in action potential clamp experiments (Doerr, Denger & Trautwein, 1989).

DISCUSSION

Sensitivity of I_f and $I_{Ca,L}$ to ACh and Iso

Our results indicate that the currents I_f and $I_{Ca,L}$ are similarly sensitive to Iso, but differ markedly in their sensitivity to ACh. It is interesting to attempt to reconcile these differing responses in the light of what is known about the intracellular signal transduction cascades involved. The effects of Iso and ACh on both I_f and $I_{Ca,L}$ are mainly mediated by modulation of intracellular cAMP levels (DiFrancesco & Tromba, 1988*a*). As is known to occur in other cardiac myocytes (Trautwein & Hescheler, 1990), modulation of $I_{Ca,L}$ further requires activation, by cAMP, of protein kinase A (PKA). Conversely, in the SA node I_f can be directly stimulated by intracellular cAMP (DiFrancesco & Tortora, 1991). In this case, one can make an analogy to drug receptor interactions, and speculate that the basal cAMP concentration, in SA node cells, would be near the K_D for the interaction of cAMP with the I_f channel, but barely at the threshold for activation of PKA. In such a case, while I_f would be affected by changes of cAMP in both directions, $I_{Ca,L}$ would respond only to increases of cAMP concentration above the basal level. This hypothesis does not conflict with the possibility that phosphorylation or direct G-protein interaction may play an additional role in I_f modulation (Yatani & Brown, 1990; Yu, Chang & Cohen, 1993). The alternative hypothesis that the cAMP pool affecting $I_{Ca,L}$ may be confined to a compartment relatively inaccessible to regulation by muscarinic agonists should also be considered.

Previous evidence (DiFrancesco & Tromba, 1988*b*; Brown & Denyer, 1989) indicated that $I_{Ca,L}$ inhibition could be obtained at ACh concentrations of 0·3 and 0·05 μM, respectively. These data represent single exposures to ACh doses near the threshold found by us (see Fig. 2) and within the limits of cell-to-cell variability are not incompatible with our data. In a set of measurements similar to that of this work, Petit-Jacques *et al.* (Petit-Jaques, Bois, Bescond & Lenfant, 1993) report $I_{Ca,L}$ inhibitions stronger than those found in our experiments (for example, 44·3% at 1 μM ACh). While the reason for the differing results is not clear, the low degree of $I_{Ca,L}$ inhibition by ACh obtained in the current work is not due to a reduced receptor sensitivity, since in the same cells I_f was strongly reduced by agonist doses ineffective on $I_{Ca,L}$ (see Fig. 2).

Lack of Ca^{2+} current sensitivity to the direct action of ACh has been previously reported (Fischmeister & Hartzell, 1986; Hescheler, Kameyama & Trautwein, 1986; Boyett, Kirby, Orchard & Roberts, 1988). This property does not imply, however, that $I_{Ca,L}$ cannot be modulated by ACh indirectly, through β-adrenergic antagonism, as occurs in other preparations. The sensitivity of β-stimulated $I_{Ca,L}$ to ACh has not been thoroughly investigated in the SA node, although in one study (Habuchi, Lu, Morikawa & Yoshimura, 1995) in the presence of 2 μM Iso, ACh was reported to reduce $I_{Ca,L}$ by 7 and 51% at the concentrations of 0·1 and 10 μM, respectively.

Possible other currents

A steady-state, nifedipine-sensitive current modulated by catecholamines that might contribute to diastolic depolarization has been described recently in rabbit SA myocytes (Guo, Ono & Noma, 1995). Such a current was not detected by the protocol employed in this study to identify the nifedipine- and isoprenaline-sensitive contribution to diastole (Fig. 5). Since the 'sustained inward current' is

reported to be activated by voltage clamping from holding potentials of −80 mV (Guo *et al.* 1995), lack of evidence for this component may be due to the different experimental conditions used in our work.

The delayed rectifier current (I_K) is modulated by neurotransmitters through cAMP/PKA-dependent phosphorylation (Yazawa & Kameyama, 1990). Stimulation of I_K would, both enhance the outward current during diastole and hyperpolarize the maximum diastolic potential (Verheijck, Van Ginneken, Bourier & Bouman, 1995); this would, in turn, increase I_f activation. These actions would influence rate in opposite directions, and the net effect would be a function of the relative magnitudes of I_K and I_f, and of their kinetics, at each agonist concentration. Thus, although potentially relevant, modulation of I_K is unlikely to be the main determinant of the monotonic response of SA rate to agonists.

We conclude that, on the basis of this and previous evidence (DiFrancesco *et al.* 1989), among the neurotransmitter-modulated currents present in the SA node and likely to be active during diastolic depolarization, I_f and $I_{K,ACh}$ appear to be, from the data available to date, the major contributors to the effects of muscarinic modulation, the former prevailing at low, and the latter at high agonist concentrations. The very low sensitivity of $I_{Ca,L}$ to ACh contrasts with the view that I_{Ca} inhibition may contribute to direct modulation of pacemaking by muscarinic receptors (Irisawa, Brown & Giles, 1993).

An analysis of the relative importance of different components to neurotransmitter-induced modulation based on the comparison between dose–response relationships can only be qualitative. For example, in correlating rate modulation by moderate ACh doses to changes in I_f and $I_{K,ACh}$ in intact preparations, it should also be considered that all changes in $I_{K,ACh}$ are superimposed on a basal activation of the channel given by intrinsic activity (Ito, Ono & Noma, 1994). A more quantitative estimate of the overall effect on rate of the changes in currents is best achieved by integration of all changes by numerical simulation. Numerical modelling has shown the importance of I_f in buffering rate changes caused by modification of the 'background' component (Noble, Denyer, Brown & DiFrancesco, 1992). Since $I_{K,ACh}$ activation hyperpolarizes the membrane, thus increasing the degree of I_f activation, a similar interplay may exist between these two components in determining the overall response to ACh. The observation that rate modulation by muscarinic and β-adrenergic stimulation persists in the presence of I_f channel blockers (Boyett, Kodama, Honjo, Arai & Suzuki, 1993; Cai, Lei & Brown, 1995) also underlines the fact that rate modulation may result from a fine interplay between different components, rather than from the action on a single ionic current. With regard to the modulation of $I_{Ca,L}$ by ACh, the large concentrations required and the limited effect strongly suggest, even on a qualitative basis, that $I_{Ca,L}$ does not contribute substantially to direct muscarinic rate modulation.

With respect to adrenergic modulation, I_f stimulation is likely to be the main mechanism underlying the increased steepness of the diastolic depolarization phase, and to account therefore for most of the positive chronotropic effect, whereas the L-type Ca^{2+} current modulation may contribute by accelerating the fraction of the diastolic depolarization just preceding action potential takeoff and the upstroke of the action potential itself. Moreover, an increased $I_{Ca,L}$ will lower the threshold of action potential generation. The reported β-adrenergic-induced rate acceleration in the presence of I_f blockers (Cai *et al.* 1995) can thus be explained with an increase in $I_{Ca,L}$.

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