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19. Only estimates of I_{tr} obtained by the I_{tr}-inactivation procedure are shown in Table 1. I_{tr} estimated by the leak-subtraction procedure was also unaffected by the oligonucleotide treatment.
20. We did not detect expression of voltage-dependent Na^+ or transient K^+ channels in oocytes injected with heart RNA that induced Ca^{2+} currents.
21. In fact, oligonucleotides complementary to mRNAs of some channels sometimes enhanced the expression of other channels (Table 3) (I. Lotan *et al.*, unpublished data), possibly due to weakening of the competition among different RNA species on the translational machinery of the oocytes (*15*); destruction of one species would be expected to potentiate the expression of others.
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Muscarinic Modulation of Cardiac Rate at Low Acetylcholine Concentrations

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Slowing of cardiac pacemaking induced by cholinergic input is thought to arise from the opening of potassium channels caused by muscarinic receptor stimulation. In mammalian sinoatrial node cells, however, muscarinic stimulation also inhibits the hyperpolarization-activated current (I_f), which is involved in the generation of pacemaker activity and its acceleration by catecholamines. Acetylcholine at nanomolar concentrations inhibits I_f and slows spontaneous rate, whereas 20 times higher concentrations are required to activate the acetylcholine-dependent potassium current ($I_{K,ACh}$). Thus, modulation of I_f, rather than $I_{K,ACh}$, is the mechanism underlying the muscarinic control of cardiac pacing at low (nanomolar) acetylcholine concentrations.

SINUS NODE AUTOMATICITY IS NORmally modulated by vagal tone. The action of acetylcholine on K^+ conductance was identified as early as 1958 (*1*) and interpreted at that time as the main basis for the slowing of cardiac pacemaking by the vagus. However, later experiments raised questions concerning the significance of this mechanism in mediating cardiac rhythm under conditions of modest muscarinic receptor activation. In particular, it was observed that during short duration vagal stimulation or exposure to nanomolar concentrations of muscarine, a slowing of spontaneous heart rate occurred without any membrane hyperpolarization (*2*). In addition, no increase in K^+ flux was detected under these conditions (*3*). These data suggest that other mechanisms may be involved in the muscarinic control of cardiac rate.

Acetylcholine (ACh) reduces the slow inward Ca^{2+} current (*4*), and this has been suggested to contribute to the observed effects of ACh on cardiac rhythm (*2, 5*). However, in sinoatrial (SA) node cells, the "pacemaker" current I_f also is strongly depressed by ACh (*6*). ACh acts via inhibition of adenylate cyclase and a decreased production of adenosine 3′,5′-monophosphate (cAMP) to shift the I_f activation curve to more negative potentials (*7, 8*). Thus, the possibility arises that I_f inhibition has a role in the vagal modulation of normal cardiac rhythm. To investigate this, we have compared the action of ACh on I_f and $I_{K,ACh}$ in isolated SA node myocytes.

Rabbit SA node myocytes were isolated by treatment with collagenase and elastase and whole-cell voltage or current clamped (*9*). We used freshly isolated cells plated on petri dishes and superfused with a Tyrode solution containing 140 m*M* NaCl, 5.4 m*M* KCl, 1.8 m*M* $CaCl_2$, 1.0 m*M* $MgCl_2$, 20 m*M* *d*-glucose, and 5.0 m*M* Hepes-NaOH, *p*H 7.4. We added $BaCl_2$ (1 m*M*) and $MnCl_2$ (2 m*M*) to better distinguish I_f changes during voltage clamp steps, when indicated. The temperature in the bath was 35° to 36°C. The internal dialyzing solution contained 10 m*M* NaCl, 130 m*M* potassium aspartate, 2.0 m*M* Mg–adenosine triphosphate (ATP), 0.1 m*M* guanosine triphosphate (GTP), 1.0 m*M* EGTA, and 10 m*M* Hepes-KOH, *p*H 7.2. Test solutions were delivered by a superfusion device consisting of a wide-tipped pipette that could be positioned near the cell under study and that allowed fast (2 to 3 s) solution changes.

Superfusion of myocytes with ACh from 0.003 to 30 μ*M* had differential effects on the currents I_f and $I_{K,ACh}$ that changed with concentration. I_f was activated by hyperpolarizing steps from a holding potential of −35 mV (Fig. 1). Addition of 0.01 μ*M* ACh resulted in a reduction of I_f at −85 mV, consistent with a shift of the I_f activation curve to more negative voltages (*6, 7*). The size of the current I_f was reduced more by 0.1 μ*M* ACh (middle) and was only slightly affected by further increasing the ACh concentration to 1 μ*M* (lower). On the other hand, an increase in K^+ permeability, as detected in changes in the holding current and in the instantaneous current at the onset of voltage steps, could only be observed at 0.1 μ*M* or higher ACh concentrations. In all of the seven cells studied by this protocol, I_f inhibition by ACh occurred at concentrations at least one order of magnitude below that at which $I_{K,ACh}$ activation was observed.

Precise quantitation of this apparent difference in sensitivity of I_f and $I_{K,ACh}$ could

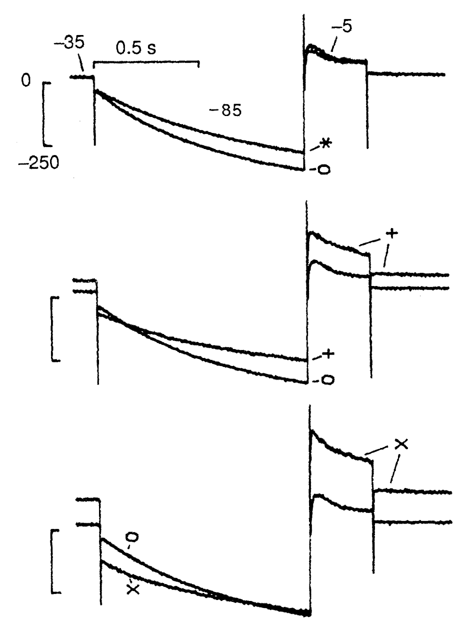

Fig. 1. Separation of the effects of ACh on I_f and $I_{K,ACh}$. Two-pulse protocols were applied every 3 s during superfusion with various doses of ACh. The myocyte was superfused with normal Tyrode solution (○, control) and with Tyrode containing 0.01 (∗), 0.1 (+), and 1 μ*M* (x) ACh. In each case ACh superfusion was maintained until a steady-state effect was achieved, typically 20 s, and was followed by an appropriate washout period.

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not be obtained under these experimental conditions because of the overlap of effects at higher ACh concentrations. We therefore measured the dose-response relation for each current under conditions that permitted their complete separation (Fig. 2). The I_f relation was obtained in the presence of Ba^{2+} to block $I_{K,ACh}$, whereas $I_{K,ACh}$ was measured in a voltage range in which I_f is fully deactivated. As mentioned above, ACh causes a leftward shift of the activation curve of I_f along the voltage axis. The magnitude of this shift can be quantified by adjusting the holding potential during application of a step of fixed amplitude, until the I_f waveform in the presence of ACh is superimposed over the control record. Current traces and the corresponding holding potentials recorded with this protocol in different ACh concentrations from one representative cell are shown in Fig. 2A. The resulting dose-response relation is illustrated in Fig. 2C (triangles). The half-maximal concentration was 0.013 μM. In a separate series of experiments, we measured $I_{K,ACh}$ at the holding potential of −40 mV in the presence of different concentrations of ACh. This holding potential was selected to minimize possible interference of the delayed K^+ current, I_K (*10*) (Fig. 2B). The measured currents were normalized on the basis of cell capacitance. The half-maximal concentration, on the basis of the dose-response relation, was 0.26 μM (Fig. 2C, circles). Thus, the qualitative difference suggested by the earlier experiment was confirmed. There is a 20-fold difference in the half-maximal concentrations of ACh required to inhibit I_f and activate $I_{K,Ach}$, respectively.

We next investigated whether this difference in sensitivity was reflected in the behavior of the cell during spontaneous activity. Activity of spontaneously beating cells was monitored during superfusion with a series of ACh concentrations. ACh (0.01 μM) led to a slowing of the pacemaker rate due to a decreased slope of the diastolic depolarization, consistent with an inhibition of I_f (Fig. 3, top). At this concentration there was no obvious hyperpolarization of the membrane, as expected if $I_{K,ACh}$ is not activated. At 1 μM there was a marked hyperpolarization and a shortening of the action potential (bottom), both effects consistent with strong activation of $I_{K,ACh}$. At the intermediate concentration of 0.1 μM, slight hyperpolarization and intermediate slowing occurred. Similar results were obtained with multiple ACh applications in three cells. Plotting the percent slowing of pacemaker rate (Fig. 2C) as a function of ACh concentration shows that greatest slowing occurs below 0.1 μM ACh (68% decrease), where K^+-conductance activation is minimal and I_f depression is substantial. Superfusion with ACh at 1 μM or higher invariably led to arrest (plotted as 100% frequency inhibition).

Our study describes the full dose-response relation for ACh action on I_f and $I_{K,ACh}$ in single SA node cells. There is a 20-fold difference in the half-maximal concentrations of ACh needed to inhibit the pacemaker current I_f and activate $I_{K,ACh}$. As little as 0.01 μM ACh can induce a significant shift of the I_f activation curve and a slowing of spontaneous rate without activation of $I_{K,ACh}$ or membrane hyperpolarization. The latter effects are observed only at higher concentrations of ACh. In fact, at concentrations below 0.1 μM, where I_f modulation is the more prominent action of ACh, spontaneous rate is slowed by more than a factor of 2, and when concentrations of ACh causing a substantial K^+-current activation are used (1 μM or higher), cessation of spontaneous activity occurs. The $I_{K,ACh}$ sensitivity reported here is consistent with that reported by Breitwieser and Szabo (*11*) in single atrial cells (0.16 μM) and is considerably greater than that reported in intact SA node tissue by Osterrieder, Noma, and Trautwein (*12*) (1.7 μM). Thus, the 20-fold difference in sensitivity we observe between the actions of ACh on I_f and $I_{K,ACh}$ is not due to an abnormal insensitivity of $I_{K,ACh}$ in these isolated SA node cells.

Our data provide an explanation of the results of Shibata *et al.* (*2*), who found that moderate vagal stimulation led to a slowing

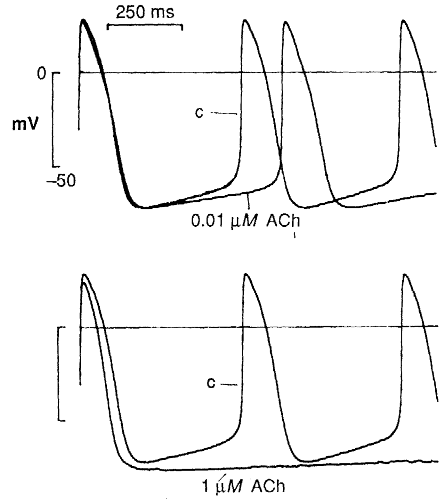

Fig. 3. Effects of different ACh concentrations on the rate of spontaneous activity in an SA node myocyte. Activity was recorded in control Tyrode solution (c) and during superfusion with ACh 0.01 μM (**top**) and 1 μM (**bottom**), as indicated. Similar results were obtained in three cells.

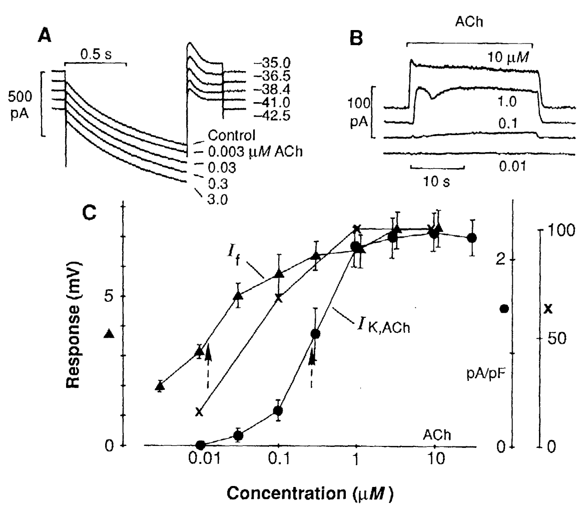

Fig. 2. Dose-response relations. (**A**) Typical protocol used to measure voltage shifts of the I_f activation curve as caused by ACh. A hyperpolarization to −90 mV followed by a depolarization to +5 mV was applied from a holding potential of −35 mV in the control solution every 3 s. Individual ACh concentrations were then applied, each followed by a return to control solution. All solutions contained $BaCl_2$ (1 mM) and $MnCl_2$ (2 mM). To estimate the voltage shift of the I_f activation curve caused by ACh, the holding potential was adjusted during each ACh superfusion until the ACh-induced decrease in I_f during the negative step was compensated and the control I_f size was fully restored. The panel shows current traces recorded at ACh concentrations in the range 0.003 to 3 μM, as indicated. The traces overlapped fully and have been displaced vertically for clarity. Values of the holding potential used are labeled near corresponding traces. (**B**) Protocol used to measure $I_{K,ACh}$ in normal Tyrode solution. The holding potential was set at −40 mV, a voltage at which interference from either I_f or the I_K is minimal, and ACh was applied at concentrations in the range 0.1 to 10 μM, as indicated. Distortion of the waveform at early times was sometimes seen at high doses, however $I_{K,ACh}$ values were always measured at the end of the 20-s pulse. (**C**) Dose-response relations for I_f inhibition (mV, ▲), $I_{K,ACh}$ activation normalized to cell capacity (pA/pF, ●), and the percent slowing of pacemaker rate (x, n = 3 cells), all normalized to the same amplitude. The I_f dose-response curve was obtained from two sets of nine cells, each superfused with four concentrations of ACh (either 0.003, 0.03, 0.3, and 3 μM or 0.01, 0.1, 1, and 10 μM). The $I_{K,ACh}$ dose-response curve was similarly obtained from two sets of six different cells, each superfused with four concentrations (0.01, 0.1, 1, 10 μM; 0.03, 0.3, 3, 30 μM). Concentrations were tested from low to high, with return to control solution after every dose. The arrows indicate half-maximal concentrations (0.013 and 0.26 μM), respectively, for the I_f and $I_{K,ACh}$ curves. The pacemaker rate curve was obtained by averaging periods of 10 s (or less) before and after ACh superfusion. Values (mean ± SEM) were 0.11 ± 0.01 and 0.68 ± 0.12 at 0.01 and 0.1 μM, respectively. Means and SEMs are displayed for the first two curves and only mean values are shown for the third curve.

of sinus rate without membrane hyperpolarization. At stronger vagal stimulation, membrane hyperpolarization appeared. Indeed, we show here that inhibition of I_f can account for the slowing observed at low doses of ACh. Obviously, effects of ACh on $I_{K,ACh}$ also contribute to rate slowing, as evidenced by the fact that the ACh dose-response relation for the effect on pacemaker rate is between the curves for I_f and $I_{K,ACh}$. However, this only applies to higher concentrations of ACh. In addition, effects of ACh on the slow inward current (I_{si}) also may occur (*8*). Although we did not perform a detailed study of the action of low doses of Ach on I_{si}, we observed that ACh at 0.01 μM did not affect I_{si} in nine out of nine cells, whereas ACh at concentrations of 0.1 and 1 μM decreased I_{si} in four out of seven and six out of eight cells, respectively. Furthermore, we did not observe any marked effect of low concentrations of ACh on action potential amplitude (Fig. 3, top). Because a moderate reduction of I_{si} would have a minor effect on the slope of diastolic depolarization (*13*), it seems unlikely that I_{si} plays a major role in underlying frequency changes at low ACh concentrations.

The presence of two distinct mechanisms of muscarinic action involving two different concentration ranges of ACh may be useful for regulating cardiac rhythm under different conditions. For example, in the resting heart, modulation of rate by vagal tone could arise from control of I_f availability. This would maintain slowing at less energy cost to the cell than by increasing K^+ permeability, which requires the recovery of K^+ ions extruded during activity. Under conditions of more marked vagal activity, $I_{K,ACh}$ also would be activated and I_{si} reduced, resulting in a greater bradycardia and a depression of excitability in the atrium as well as in the SA node.

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