Effects of Acetylcholine on Electrophysiological Properties of Rabbit Cardiac Purkinje Fibers

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SUMMARY. The action of acetylcholine (10^-4 m) was investigated in isolated rabbit cardiac Purkinje fibers, using standard microelectrode recording of transmembrane potentials and two-microelectrode voltage clamp technique. In nonstimulated fibers, acetylcholine hyperpolarized the diastolic membrane potential and slowed or suppressed spontaneous activity. The hyperpolarization was more pronounced in low potassium solutions and in depolarized fibers; it was less marked in the presence of cesium (2 x 10^-2 m), and was suppressed by barium (3-5 x 10^-3 m). In stimulated fibers, acetylcholine shortened the action potential duration and shifted the plateau level to more negative values; this effect was influenced little by the stimulation frequency and not by chloride removal from the perfusing solution. In voltage-clamped preparations, acetylcholine shifted the holding current in the outward direction at potentials less negative than E_K, while it shifted the current in the inward direction at potentials more negative than E_K. The changes induced by acetylcholine were concentration-dependent (apparent K_M: 1.5 x 10^-7 m); they were mimicked by carbachol (10^-8-10^-5 m) and blocked by atropine (10^-8-10^-7 m). The time course of the effects was biphasic: a maximum was reached in the first minute after addition of acetylcholine, thereafter, the effect decayed to a steady value. On removal of acetylcholine, a transient inversion of the changes produced by acetylcholine was observed, the magnitude of which depended on the acetylcholine concentration used and on the duration of exposure to acetylcholine. This time course was not abolished by pretreatment with physostigmine (10^-5 m), manganese ions (2 x 10^-3 m), or with adrenoceptor blockers [propranolol (2 x 10^-7 m) and/or phentolamine (10^-7-10^-4 m)]. The results show that rabbit Purkinje fibers are as sensitive to acetylcholine as atrial preparations. The changes produced by acetylcholine are suggestive of an increase in an inward rectifying potassium ion conductance and are mediated by muscarinic receptor stimulation. The secondary decay in the effects of acetylcholine and their inversion on washout can be explained by a desensitization mechanism if it is assumed that the acetylcholine-sensitive channel is already functional in the absence of acetylcholine and is modulated in its conductance and/or open state probability by acetylcholine. (Circ Res 53: 740-751, 1983)
underlying mechanism. According to more recent observations by Bailey et al. (1979), lower concentrations of ACh are able to affect the action potential duration in dog Purkinje fibers if the preparations are pretreated with catecholamines.

In this paper, a description will be given of the effects of ACh on rabbit Purkinje fibers. These preparations are very sensitive to ACh and the effects resemble the changes in atrial preparations, i.e., hyperpolarization and marked action potential shortening. In addition, spontaneous activity, when present, is reduced or suppressed. A further reason for studying rabbit Purkinje fibers is that these preparations offer structural advantages for the analysis of ionic permeability mechanism by the voltage clamp technique; because of less packing of the cells, accumulation and depletion of ions are practically absent (Sommers and Johnson, 1968; Colatsky and Tsien, 1979). A preliminary report has already appeared (Mubagwa and Carmeliet, 1981).

**Methods**

**Preparation**

The experiments were performed on rabbit Purkinje fibers superfused with normal Tyrode's solution at 37°C. The rabbits were killed by a blow on the neck and bled through a section of the carotid artery. The heart was removed immediately and rinsed in K+- and glucose-rich, warm Tyrode's solution. Fibers were dissected from both ventricles and held to the bottom of the perfusion bath by a wire grid (0.8–2 mm spacing between wires). They remained exposed to the K+-rich glucose-rich solution for at least 30 minutes before being equilibrated in normal Tyrode's solution for 1 hour.

**Solutions**

The composition of the normal Tyrode's solution was as follows (in mM): NaCl, 126; KCl, 5.4; CaCl2, 1.8; MgCl2, 0.5; NaHCO3, 24; glucose, 5.5. For the dissection solution, KCl and glucose were increased to 10.8–27 mM and 25–50 mM, respectively, without any correction for the osmolality. Thus, the dissection solution was hypertonic, but remained exposed to the K+-rich glucose-rich solution for at least 30 minutes before being equilibrated in normal Tyrode's solution for 1 hour.

**Electrical Recording and Measurements**

The membrane potential was recorded differentially between an intracellular microelectrode and another microelectrode located just outside the fiber. Both microelectrodes were filled with 2.7 M KCl and had a resistance of 10–25 MΩ. A fine Ag-AgCl electrode was positioned on the surface of the fiber, or at one of the grid wires crushing the impaled fiber segment, and served as the stimulus electrode. Membrane potentials and their differentiated signals were recorded on an oscilloscope and on a pen recorder (Gould Brush 220). Action potential duration was evaluated at 90% or at 100% repolarization and is expressed in relative values, namely, in percent of the control duration, to allow comparison.

In other cases, two microelectrodes were introduced in short (0.8–1.0 mm) Purkinje segments for stimulation, for passing constant current pulses, or for performing voltage clamping. These short segments were electrically isolated from each other, as checked by introduction of electrodes in neighboring segments, and they showed a uniform membrane potential, as checked by introduction of two electrodes into the same segment.

**Drugs**

Drugs used include acetylcholine chloride (Roche, and Sterop), carbachol (Merck), atropine sulfate (Merck), propranolol hydrochloride (ICI), phentolamine hydrochloride (Ciba), physostigmine (Merck), adenosine (Sigma), adenosine 5'-triphosphate (ATP, Sigma), dibutyryl guanosine 3',5'-cyclic monophosphate (dBCGMP, Sigma).

**Results**

**Acetylcholine Hyperpolarizes the Membrane Potential and Inhibits Spontaneous Activity**

The effect of acetylcholine (ACh) on the resting potential and on spontaneous activity was investigated in nonstimulated (quiescent or spontaneously beating) fibers and is illustrated in Figure 1.
Figure 1A shows results obtained in a preparation superfused with 5.4 mM K+-Tyrode (A1) or 1.35 mM K+-Tyrode (A2). In 5.4 mM K+, the maximum diastolic potential was -83 mV and the frequency 48/min. On addition of 10⁻⁶ M ACh, the frequency first decreased and then pacemaker activity was completely suppressed concomitantly with a hyperpolarization of the membrane to -86 mV. During washout of ACh, the membrane depolarized again, and spontaneous activity resumed (not shown). When the same fiber was superfused with a Tyrode’s solution containing 1.35 mM K⁺, maximum diastolic potential increased to -90 mV; the frequency was lower (30/min) than in 5.4 mM K⁺, but this change was essentially due to a prolongation of the plateau, the rate of diastolic depolarization being increased when compared to the value in 5.4 mM K⁺. On addition of ACh, spontaneous activity was arrested before any important change in action potential duration. Following the arrest of the pacemaker activity, the membrane potential hyperpolarized within 20 seconds to -108 mV.

Figure 1, B and C, illustrates the effect of ACh in fibers depolarized in low or in high K⁺. In Figure 1B, the fiber, which had a maximum diastolic potential of -79 mV in normal Tyrode’s solution, presented oscillations between -26 mV and -62 mV when the K⁺ concentration in the perfusing solution was lowered to 0.54 mM. Exposure to 3 x 10⁻⁶ M ACh transiently hyperpolarized the membrane to -105 mV. At this level, the spontaneous activity disappeared. Then the resting potential slowly depolarized to -93 mV, from where a spontaneous action potential was generated. The fiber did not repolarize to the high level of diastolic potential but, instead, again showed oscillations (i.e., slow action potentials) between -30 mV and -62 mV, despite the continuous presence of ACh. After washout of ACh, the membrane potential depolarized above the control maximum diastolic potential in 60 seconds, before slowly returning to the control state.

In Figure 1C, another fiber was depolarized by 10.8 mM K⁺-Tyrode. ACh (10⁻⁶ M) initially decreased the spontaneous frequency from 36/min to 18/min (C1). In steady state, the frequency raised to 24/min (C2). The decrease in spontaneous activity by ACh was due to a slowing of diastolic depolarization and was not associated with a membrane hyperpolarization.

These examples indicate that ACh hyperpolarizes the membrane potential and decreases spontaneous activity in rabbit Purkinje fibers. In 36 different fibers with high (at least -70 mV) diastolic membrane potential, 10⁻⁶ M ACh and 2 x 10⁻⁶ M ACh hyperpolarized the potential from -77.2 ± 0.98 mV (mean ± SEM) to -82.5 ± 0.63 mV (23 fibers) and from -77.0 ± 1.25 mV to -83.0 ± 1.80 mV (13 fibers), respectively. Among these fibers, 28 (75%) showed spontaneous activity. In the last fibers, ACh (1-2 x 10⁻⁶ M) exposure always produced a decrease or a suppression of the spontaneous activity.

Table 1

<table>
<thead>
<tr>
<th>Experimental conditions (mM K⁺)</th>
<th>Maximum diastolic potential (mV)</th>
<th>Change in diastolic potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>86.9 ± 4.9*</td>
<td>10.4 ± 4.4</td>
</tr>
<tr>
<td>ACh 10⁻⁶ M</td>
<td>101.4 ± 4.4</td>
<td>14.4</td>
</tr>
<tr>
<td>2.7</td>
<td>83.0 ± 5.2</td>
<td>91.8 ± 1.6</td>
</tr>
<tr>
<td>5.4</td>
<td>77.25 ± 3.1</td>
<td>79.75 ± 2.7</td>
</tr>
<tr>
<td>10.8</td>
<td>56.6 ± 1.8</td>
<td>58.9 ± 2.5</td>
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</table>

* Values are mean ± SEM for four nonstimulated preparations.
the fact that the diastolic potential approaches the equilibrium potential for \( K^+ \) due to stimulation of the electrogenically active \( Na^+-K^+ \) pump during electrical stimulation.

Because a shortening of the action potential can result from changes in one or more of the various ionic currents underlying the action potential, it was interesting to examine the influence on the effect of ACh of various conditions (e.g., stimulation frequency, ionic substitution, channel blockers) known to affect membrane current components.

Figure 2A illustrates the effect of \( 2 \times 10^{-6} \) m ACh at three different stimulation frequencies. ACh was applied after the action potential duration had reached a steady value at the given frequency. In Figure 2 (A1), the fiber was paced at 0.2 Hz; addition of ACh initially shortened the action potential duration to 47.3% of the control (872 msec), but this was followed by a lengthening to 62.1% at steady state. During stimulation at 1 Hz (A2), exposure to ACh produced peaks and steady state shortenings to 52.9% and to 67.8% of the control (588 msec), respectively. At a higher frequency [2 Hz (A3)] the relative durations (control 405 msec) were 58.2% and 74.5% of the control at peak and steady state ACh effect, respectively. In three different preparations stimulated at these three frequencies, no significant difference was found between the effects of ACh at 0.2 and 1 Hz, whereas there was a significant (\( P < 0.01 \)) decrease of the relative shortening at 2 Hz.

In two other experiments, the action potential duration was measured after a 10-minute stimulation period at various frequencies (1–5 Hz) in normal Tyrode's solution. This was repeated in the presence of ACh. When action potential durations for similar frequencies were compared in normal Tyrode's solution and in ACh-containing medium, the effect of ACh was comparable at the various frequencies, although there was a certain tendency for decrease at 5 Hz.

The shortening of the action potential by ACh and its secondary lengthening were not affected by \( Cl^- \) removal from the perfusing solution (Fig. 2B). \( Cl^- \) removal, in itself, resulted in prolongation of the initial repolarization phase and of the plateau phase of the action potential (Carmeliet, 1961; Hutter and Noble, 1961; Kenyon and Gibbons, 1977).

In two experiments, the effects of ACh in normal Tyrode's solution were compared to the effects when the same fiber had been pretreated with 2 mM Mn++. At this concentration, Mn++ produced a decrease of the plateau level and a lengthening of the action potential duration, without a change in resting potential. The decrease in plateau level in Mn++ is explained by a decrease in the slow inward current, \( i_w \) (Rougier et al., 1969), whereas the lengthening of the action potential duration probably results from a decrease in the late outward currents, \( i_o \) (Kass and Tsien, 1975). The relative shortening of the action potential by ACh in both experiments was more pronounced in the presence of Mn++ (Fig. 2C).

In the experiments described above, ACh was used in a concentration between \( 10^{-6} \) and \( 10^{-5} \) m. Rabbit Purkinje fibers, however, are quite sensitive to ACh, and shortening of the action potential is observed at concentrations as low as \( 10^{-8} \) m ACh. When preparations were exposed to different ACh concentrations in succession (but with interpolation of a washout of at least 10 minutes), the following dose-response curve was obtained for the action potential shortening (Fig. 3). The relation has a sigmoid shape, with a half-maximum effect at \( 1.5 \times 10^{-7} \) m ACh. For the steady state effect, the curve tends to decrease again for concentrations higher than \( 10^{-5} \) m ACh. This is due to a larger secondary lengthening at these high concentrations (see Fig. 6).

**Biphasic Time Course of the ACh Effects**

The effects of ACh on resting potential, spontaneous frequency, and action potential duration followed a biphasic time course. For the experiments...
FIGURE 3. Dose-response curve for the effect of ACh on the action potential duration. Relative decrease in action potential duration is plotted as a function of the ACh concentration used. Each fiber was stimulated at 1 Hz and submitted to different concentrations with 10-minute washout following each application. Circles and bars represent mean and SEM, respectively, for nine different preparations. Filled circles, maximal shortening; open circles, steady state shortening. The relationship between ACh concentration and maximal shortening is sigmoidal with an apparent $K_m$ of $3.5 \times 10^{-7}$ M ACh.

At ACh concentrations higher than $3 \times 10^{-5}$ M, the steady state shortening declined, as illustrated in Figure 1B and performed in 0.54 mM K+-Tyrode, attention was drawn on the fact that the hyperpolarization went through a maximum and subsided afterward. On washout, the mirror image was obtained with a transient overshoot in the depolarizing direction. In eight similar experiments on four fibers, ACh (3-5 × 10^{-6} M) initially hyperpolarized the fibers from $-51.6 \pm 2.56$ mV to $-92.9 \pm 2.75$ mV within the first 60 seconds of exposure. After a few minutes, the fibers depolarized again to $-52.5 \pm 3.14$ mV, despite the continuous presence of ACh. In 5.4 mM or higher K+ concentrations, the hyperpolarization also followed a biphasic time course. Since the magnitude of the potential changes was less pronounced, this aspect was not systematically investigated.

ACh induced similar biphasic changes in the frequency of spontaneous activity. In all the fibers where spontaneous activity was present, addition of ACh led to one of the following situations: decrease of the spontaneous frequency with later stabilization at an intermediary rate (e.g., Fig. 1C); transient standstill at the beginning followed by a slow spontaneous activity in the later period of ACh exposure (e.g., Fig. 1B); or permanent standstill throughout ACh application. The washout period was always associated with increased spontaneous frequency above control in already spontaneous fibers, or induction of spontaneous activity in quiescent fibers.

However, the action potential duration was the most sensitive parameter, and its evolution was studied in more detail. As illustrated by the example in Figure 4, the shortening of the action potential reached a maximum after 30-60 seconds, but was followed by a secondary lengthening, notwithstanding the continuous presence of ACh. This lengthening, however, was not progressive and a steady state was obtained in 5-10 minutes; the magnitude of the secondary lengthening was also smaller than that of the initial shortening. During washout the mirror picture was observed, i.e., the action potential quickly lengthened during the first 2 minutes to a value that was well above the control value.

The complete time course of action potential duration was studied during application and during washout of $10^{-6}$ M ACh in nine different preparations. The maximum effect led the action potential duration to 48.5 ± 5.0% (mean ± SEM) of the control in 48.9 ± 5.3 seconds. After this, the action potential lengthened to 73.2 ± 4.5% of the control at steady state (5-10 minutes). Washout of ACh in these fibers was associated with a transient lengthening to a maximum of 111.5 ± 2.0% of the control after 121.1 ± 9.0 seconds.

In order to obtain more insight in the mechanism underlying the biphasic changes, we tested the following possibilities for the secondary lengthening: (1) enzymatic breakdown of ACh by cholinesterase, (2) release of norepinephrine and activation of $\alpha$- or $\beta$-receptors, (3) decrease in the sensitivity or the number of active ACh receptors, a process often called desensitization or fade, and (4) production or release of an intracellular substance (second messenger).

Figure 4 compares the effect of ACh in normal...
Tyrode’s solution (filled circles) with the effect after the same fiber has been pretreated with 10^{-6} M physostigmine, a cholinesterase inhibitor (open circles). The initial and the steady state relative shortenings during ACh exposure, as well as the overshoot on washout, were more marked in the presence of physostigmine. Similar increases in the effect of ACh by physostigmine were observed in two other fibers. Since the secondary shortening of the action potential did not disappear, but was more pronounced in the presence of physostigmine, enzymatic breakdown of ACh cannot be made responsible for this effect. In confirmation of this result, it was found that carbachol, a muscarinic receptor agonist that is not broken down by cholinesterase, produced the same changes in membrane potential as ACh at similar molar concentrations (not shown).

The second hypothesis was tested by measuring the effect of ACh also in the presence of phentolamine (10^{-7}–10^{-5} M) and propranolol (2 × 10^{-7} M). The aim of these experiments was to see whether the effect of ACh, especially with respect to its time course, was related to an ACh-induced release of norepinephrine, exerting an a- and/or b-adrenergic stimulation on the cardiac cells. Figure 5 illustrates the results of a typical experiment. In normal control Tyrode, ACh exerted its usual effect (Fig. 5A): initial pronounced shortening during the first minute was followed by a secondary shortening which became even so pronounced that the steady state shortening was obtained after 5 minutes. On washout of the drug, a transient overshoot in action potential duration above the control value was obtained.

The second hypothesis was tested by measuring the effect of ACh at similar molar concentrations (not shown). The action potential shortened in a biphasic way: a marked shortening during the first minute was followed by a secondary shortening until a steady state duration, shorter than control, was obtained after 5 minutes. On washout of the drug, a transient overshoot in action potential duration above the control value was obtained.

In four experiments of this type ACh (1–2 × 10^{-6} M) produced initial and steady state shortenings to 33.9 ± 5.1% and to 57.4 ± 4.4% in control conditions, whereas, after pretreatment with propranolol (2 × 10^{-7} M) and phentolamine (10^{-7} M), the same ACh concentration produced initial and steady state shortenings to 44.1 ± 6.1% and 60.9 ± 5.6% of the control, respectively. The overshoot of action potential shortening on ACh washout also persisted in the presence of the adrenergic blockers: 108.2 ± 7.5% of the control duration compared to 113.0 ± 2.5% in normal Tyrode’s solution. From such experiments, it can be concluded that the shortening of the action potential and the biphasic time course during shortening and recovery are not due to a- or b-adrenoceptor stimulation.

As a further possibility, to explain the secondary decrease in the effect of ACh, we considered the process of desensitization. Such a phenomenon is suggested by the results presented in Figure 6, which show the time course of the changes in action potential duration when a preparation was successively exposed to different ACh concentrations, with a washout of at least 10 minutes after each exposure. The higher the concentration of ACh, the greater and faster not only the initial shortening, but also the secondary lengthening. At the highest concentration of ACh (10^{-4} M), the secondary lengthening became even so pronounced that the steady state action potential duration was longer than at 10^{-5} M ACh (see also Fig. 3).
Observations on the shortening of the action potential when rising concentrations of ACh were applied without interpolated washout give further support to the desensitization hypothesis. In the experiment of Figure 7, three increasing concentrations of ACh were used. Addition of $2 \times 10^{-7}$ M ACh rapidly shortened the action potential; as usual, the shortening was followed by a secondary lengthening. Increasing the concentration of ACh to $10^{-6}$ M again caused an initial shortening, which, however, was less pronounced than with the lower concentration (filled circles). Even with $5 \times 10^{-6}$ M ACh, the maximal shortening remained below the level attained with $2 \times 10^{-7}$ M ACh. These effects are to be compared to the maximal shortenings observed for the same ACh concentrations when applied following a washout period of at least 10 minutes (superimposed open circles); as expected, the maximal shortenings under those conditions were more pronounced with higher ACh concentration.

On the other hand, the overshoot, i.e., the prolongation of the action potential above control during washout, seems to be inconsistent with a desensitization hypothesis as described for the nicotinic receptor. It was found that this overshoot above control was strongly related to the secondary lengthening during ACh superfusion: the greater the secondary lengthening of the action potential during exposure of ACh, the greater the overshoot during washout. This relation was verified either by using different concentrations of ACh (Fig. 8, A and B), or by reducing the duration of perfusion with a given ACh concentration (Fig. 9).

Figure 7 shows results of an experiment where three different ACh concentrations were used. The overshoot on washout increased from 12.4% (above control) to 20% with an ACh concentration increase from $2 \times 10^{-7}$ to $5 \times 10^{-6}$ M. Figure 8B represents the relation between overshoot and secondary prolongation obtained by varying ACh concentrations in four different fibers. Figure 9 shows that a similar relationship between the overshoot effect and the secondary lengthening can be demonstrated by varying the time of exposure to a given concentration of ACh ($2 \times 10^{-6}$ M). When the exposure time to ACh was decreased to 30 seconds, the maximal shortening effect was still the same as for a 5-minute ACh exposure, but the extra-prolongation of
Mechanisms of ACh Effect

Mediation of ACh Effects by Muscarinic Receptors

It was mentioned above that carbachol, an agonist of muscarinic receptors, produced effects similar to those of ACh. Choline (10 mM) also shortened the action potential, although its effect was moderate. Addition of atropine (10^{-6}-10^{-7} M) prevented or suppressed the effects of ACh or carbachol. Thus, the ACh effects are mediated by muscarinic receptor stimulation.

Conductance Mechanisms

The dependence of the hyperpolarization produced by ACh on K_{eq} and its sensitivity to Cs^{+} and Ba^{2+} suggest that an increase of the K^{+} conductance is the main mechanism for ACh action on the diastolic potential. This hypothesis is further substantiated in Figure 10A showing the ACh effect on fibers whose resting potential was varied, using constant current injection (K_{eq} concentration: 5.4 mM). ACh (2 \times 10^{-5} M) hyperpolarized the resting membrane potential from −75 mV to −80 mV when no current was injected. However, when the resting potential was artificially shifted to −90 mV, the same ACh concentration was without clear effect, whereas it produced a depolarization when the “resting” potential was maintained at −102 mV, i.e., negative to the K^{+} equilibrium potential.

In Figure 10B (different preparation from that of Figure 10A), the membrane potential was maintained at a constant level by voltage clamp while changing the perfusing medium from drug-free to ACh-containing Tyrode and vice versa. ACh (2 \times 10^{-6} M) induced an outward current at −77 mV and an inward current at −95 mV. Almost no effect was produced at −87 mV. Experiments similar to that of Figure 10B were carried out in two other fibers; ACh (10^{-7}-10^{-4} M) induced outward currents at potentials between −20 mV and −70 mV and an inward current at −90 mV.

The existence of a reversal potential near E_{K} for the ACh-induced polarization (under constant current), as well as for the ACh-induced current (under voltage clamp), is consistent with the hypothesis that ACh increases K^{+} conductance. Figure 10B further shows that the biphasic time course described for the resting potential, spontaneous activity, and the overshoot in action potential duration on washout and duration of exposure to a given ACh concentration. Exposure of 2 \times 10^{-6} M ACh for different durations (15 seconds, 30 seconds, and 5 minutes) during continuous impalement of one fiber.

The fourth possibility we investigated to explain the biphasic time course of the ACh action was to assume that ACh is responsible for the production or the release of a substance which prolongs the action potential. Possible candidates for such a substance are adenosine, ATP, and cGMP. We have tested the effect of adenosine and ATP in concentrations of 10^{-6} to 10^{-3} M, and of dibutyryl-cGMP in a concentration of 5 \times 10^{-4} M. Adenosine practically did not change the action potential of rabbit Purkinje fibers (three experiments), except for a slight shortening at the highest concentration. ATP had no effect at 10^{-6} M, but markedly shortened the action potential at 10^{-5} and 10^{-4} M. At 10^{-4} and 10^{-3} M, it caused a decrease in maximum resting potential and appearance of spontaneous oscillations (two experiments). Dibutyryl-cGMP (5 \times 10^{-4} M) had practically no effect for 15 minutes on the action potential duration (two experiments). These experiments do not entirely exclude the possible role of cGMP [which has been shown to prolong ventricular action potential duration (Trautwein et al., 1982)], and intracellular application of cGMP in single isolated cells should be performed to verify the eventual role of this compound.
action potential duration also is characteristic for the change in K⁺ conductance. The current shift induced by ACh was not maintained; it decreased with time and showed an overshoot on ACh washout.

The decrease with time of the ACh-induced current was not due to a change in K⁺ driving force due to accumulation (for potentials positive to Eₖ) or to depletion (for potentials negative to Eₖ). This is evident from Figure 11, which shows the currents when the membrane was repetitively clamped from −75 mV (i.e., positive to the calculated Eₖ of −85 mV) to −95 mV (i.e., negative to Eₖ) for 1 second. ACh shifted the currents in the outward direction at −75 mV and in the inward direction at −95 mV. The shifts in current at both potentials decreased with time. The biphasic time course therefore cannot be due to changes in the ionic gradient, as accumulation and depletion cannot be present at the same time.

**Discussion**

**High Efficacy of ACh in Rabbit Cardiac Purkinje Fibers**

The experiments described in this work provide evidence for important changes by ACh in electrophysiological characteristics of the rabbit cardiac Purkinje fibers, i.e., hyperpolarization of the diastolic membrane potential, shortening of the action potential, and decrease of pacemaker activity.

Compared to results in other Purkinje fibers, the efficacy of ACh in rabbit Purkinje fibers is quite large. An important shortening of the action potential (35% at peak) occurs at an ACh concentration of 3 × 10⁻⁷ M and increases to 60% at 10⁻⁵ M ACh. The dog Purkinje fiber, in contrast, is much less sensitive, and, at 10⁻⁵ M ACh, the shortening of the action potential is only 10–15% (Gadsby et al., 1978). The only other Purkinje fiber preparation which is as sensitive is the sheep Purkinje fiber; however, the changes in membrane potential by ACh in this preparation are quite different—i.e., lengthening of the action potential and increase in rate of diastolic depolarization (Carmeliet and Ramon, 1980a; Lipsius and Gibbons, 1980).

Rabbit Purkinje fibers strikingly resemble atrial fibers of different species in their qualitative and quantitative response to cholinergic stimulation. In the atrium, vagal stimulation or ACh application produces a hyperpolarization, a decrease of the action potential duration, and an inhibition of spontaneous activity (Burgen and Terroux, 1953; Hoffman and Suckling, 1953; Hutter and Trautwein, 1956; see Higgins et al., 1973, for review).

**Mechanism of ACh Action**

The action of ACh is mediated by stimulation of muscarinic receptors; the effects of ACh are inhibited by atropine, mimicked by other muscarinic agonists (e.g., carbachol, choline) and not abolished by α- or β-adrenergic receptor blockers.

In sinoatrial and atrial muscle fibers, the electrophysiological effects are explained by an increase of the K⁺ conductance (Harris and Hutter, 1956; Trautwein and Düdel, 1958; Ten Eick et al., 1976; Garnier et al., 1978; Noma and Trautwein, 1978) and/or by a decrease of the slow inward current; this last mechanism seems to play a major role in the frog (Giles and Noble, 1976; Ikemoto and Goto, 1977). The present experimental results suggest that the most important change in rabbit Purkinje fibers is an increase in a K⁺ conductance with inward rectifying properties. One of the main arguments is the effect of ACh on the resting or diastolic potential in different K⁺ concentrations. The extent of hyperpolarization was variable, and dependent on the difference between the actual membrane potential and the equilibrium potential for K⁺, Eₖ (see Fig. 1 and Table 1). When the membrane at a given K⁺ concentration was much lower than Eₖ, the addition of ACh resulted in a marked hyperpolarization. Conversely, when the resting potential of a preparation approached Eₖ, addition of ACh resulted in only a small hyperpolarization. The extent of hyperpolarization also was dependent on the existence of open K⁺ channels. When 20 mM Cs⁺, known to block inward rectifying K⁺ channels (Isenberg, 1976; Vereecke et al., 1980), was added to 5.4 mM K⁺ Tyrode, the fiber depolarized to −41 mV and ACh hyperpolarized the membrane temporarily only to −50 mV. The fact that the hyperpolarization was not completely suppressed in the presence of Cs⁺ could be due to some functional K⁺ channels not blocked by Cs⁺ at depolarized levels [Cs⁺ exert a potential-dependent block of the inward rectifier...
(Carmeliet, 1979)). In favor of this last explanation, we found that ACh was without effect when the inward rectifying K⁺ channels were completely blocked with 3–5 mM Ba⁺⁺ (DiFrancesco, 1981). A similar interference of Cs⁺ and Ba⁺⁺ with the ACh-induced increase in K⁺ conductance has been observed in the frog atrium (Argibay et al., 1981; Ojeda et al., 1981) and in the tortoise sinus venosus (Hutter and Stekar, 1982).

Stronger arguments in favor of the K⁺ hypothesis are provided by the experiments in which the membrane potential was changed by constant current or by voltage clamp (Fig. 10). When the membrane potential was at a level positive to the presumed K⁺ equilibrium potential, addition of ACh resulted in a hyperpolarization (constant current) or an outward current (constant voltage). Opposite changes, i.e., a depolarization or an inward current, were observed when the membrane potential was kept at a level negative to E_K.

It cannot be decided from the present data whether ACh increases the current through the background i_K channel (Garnier et al., 1978; Ojeda et al., 1981), or affects another type of K⁺ channel (Noma and Trautwein, 1978). The biphasic nature of the ACh effect and the overshoot during washout, however, strongly suggest that the ACh-sensitive K⁺ channel is not newly formed but only changed in its conductance and/or open state probability. An overshooting effect, i.e., a decrease of overall K⁺ conductance below control, would be impossible in the case of a newly formed K⁺ channel (see next section).

The present experiments, furthermore, strongly suggest that other currents, such as the fast inward current (i_h), the slow inward current (i_o), the transient outward current (i_o'), or the late outward current (i_o''), do not play an important role in the ACh effects. Maximal rate of depolarization during the upstroke was not changed. The rate of repolarization during phase 1 was not changed consistently in one or another direction, and Cl⁻ removal did not modify the ACh effect in a fundamental way. In the presence of 2 mM Mn⁺⁺ [known to block the slow inward current (Rougier et al., 1969; Vitek and Trautwein, 1971), the late outward current (Kass and Tsien, 1975) and the Ca⁺⁺-activated early outward current (Siegelbaum et al., 1977; Coraboeuf and Carmeliet, 1982)], ACh still shortened the action potential duration. The smaller effect of ACh on the action potential duration at high stimulation frequencies does not necessarily implicate an effect of ACh on time-dependent currents. It probably is a consequence of the important shortening in the control action potential at these frequencies, which implies a larger repolarizing current; a given ACh-induced change in membrane current will then result in a relatively smaller effect on the action potential duration. A similar explanation can be given for the decrease of the relative shortening after treatment with adrenergic receptor blockers.

Biphasic Time Course of the ACh Effects

A peculiarity of the ACh effect in rabbit Purkinje fibers is its biphasic time course during ACh exposure, as well as during washout. On exposure to a given concentration of ACh, the resting potential hyperpolarizes, the action potential shortens, and spontaneous activity decreases. The amplitude of these effects shows a maximum after about 1 minute, but decreases afterward. During washout, the opposite changes are observed, showing a definite overshoot. Different mechanisms can be considered to explain this biphasic time course during ACh exposure and the overshoot during washout. We will discuss the following possibilities successively: (1) a decrease in ACh concentration by increased cholinesterase activity, (2) a release of norepinephrine and secondary stimulation of α- or β-receptors, resulting, for instance, in an increased slow inward current, (3) muscarinic stimulation affecting more than one current system, i.e., a secondary stimulation of an inward current or inhibition of an outward current, and (4) a secondary decrease in K⁺ current after its initial increase.

The first two mechanisms were ruled out by our results obtained with physostigmine (Fig. 4) or carbachol, and with adrenergic receptor-blocking agents (Fig. 5). According to the second possibility, ACh would first increase the K⁺ conductance as primary change, resulting in hyperpolarization, decrease in spontaneous activity, and shortening of the action potential duration. In a second stage, an inward current (e.g., i_i) would be increased or an outward current (e.g., i_o) would be inhibited, resulting in the secondary action potential lengthening. In the previous section we have already stressed that the results obtained in the presence of 2 mM Mn⁺⁺ rule out an increase of slow inward current, or a decrease in late outward current. It is also difficult to see how a change in i_o or in i_i could be made responsible for the secondary changes in maximum diastolic potential. Furthermore, the hypothesis proposes an increase in i_o by ACh, whereas literature data rather suggest a decrease (Giles and Noble, 1976; Ikemoto and Goto, 1977; Hino and Ochi, 1980).

As a fourth possibility, we proposed a biphasic change in an inward rectifying K⁺ current, as suggested by the result of Figures 10 and 11. Such a change can result (1) from a decrease in K⁺ driving force following alteration in K⁺ concentration, or (2) from a decrease in membrane conductance following an ACh-induced change in concentration of some intracellular substance, or transformation of the ACh-activated channel in a state of a lower conductance and/or lower open probability. An increase of K⁺ concentration near the cell membrane with secondary changes in current due to a change in equilibrium potential, has been invoked to explain the slow decrease of the ACh-induced hyperpolarization of guinea pig atrium and the depolarization...
from that in nicotinic receptors, since washout of ACh is accompanied by a decrease of the K⁺ conductance below control level in rabbit Purkinje fibers. To explain this difference, we tentatively propose that the K⁺ channel is characterized by three different states: an open state in the absence of any neurotransmitter (state 1) and two muscarinic receptor-activated states, one with a higher (state 2) and one with a lower (even zero) than normal conductance and/or open state probability (state 3). During ACh activation, a fraction of channels in state 1 move into state 2, followed by a slow transformation into state 3: the membrane K⁺ conductance thus will rapidly increase, but the initial increase will be followed by a decrease to an intermediate level. On washout, channels in state 2 will rapidly return to state 1, but the transformation from state 3 to 1 will be much slower. Temporarily, therefore, the membrane conductance will be smaller than normal. This model resembles those used to explain desensitization at the nicotinic receptor, with the difference that the channel is functional in the absence of ACh.

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