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CHARACTERIZATION OF THE ACETYLCHOLINE-INDUCED POTASSIUM CURRENT IN RABBIT CARDIAC PURKINJE FIBRES

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SUMMARY

1. Acetylcholine (ACh) induces a K^+ current in rabbit cardiac Purkinje fibres. The question was studied whether ACh produces this effect by modifying the properties of K^+ channels pre-existing in the absence of the neurotransmitter or whether it induces the formation of a different type of K^+ channels.

2. The relaxation properties of the ACh-induced current and its blockade by Cs^+ and Ba^{2+} have been investigated using voltage clamp.

3. During hyperpolarizing or depolarizing voltage pulses of moderate amplitude, the ACh-induced current is time independent. For large voltage pulses, time-dependent changes of the ACh-induced current are observed. These latter changes can be explained by intercellular K^+ accumulation/depletion phenomena or by the effects of ACh on time-dependent currents (e.g. the late outward current, i_x).

4. Cs^+ and Ba^{2+} block the ACh-induced current. The block produced by 20 mM-Cs^+ is instantaneous and increases with hyperpolarization, i.e. it is voltage dependent. The block produced by Ba^{2+} at high concentrations (> 1 mM) is also instantaneous but complete at all potentials studied, and thus voltage independent. At these concentrations, either ion also blocks the background inward rectifier (i_{K1}) current in a similar way.

5. Low [Ba^{2+}] ($< 0{\cdot}1$ mM) cause a block of the ACh-induced current which is instantaneous and little voltage dependent. The block of i_{K1} in contrast is time and voltage dependent for the same concentrations. These results indicate that the ACh-induced K^+ current is different from the background i_{K1} current.

INTRODUCTION

In the preceding paper (Carmeliet & Mubagwa, 1986*a*) it is shown that acetylcholine (ACh) increases a K^+ conductance in rabbit cardiac Purkinje fibres. The question that is immediately raised is whether ACh produces such an effect by modifying the properties of K^+ channels pre-existing in the absence of the neurotransmitter, or whether it induces the formation of a new type of K^+ channel.

A major characteristic of the ACh-induced current is inward-going rectification: more inward current is obtained by a given hyperpolarization than outward current obtained by a depolarization of the same amplitude. Since the inward rectifier

constitutes an important fraction of the cardiac background current, among pre-existing K^+ currents, the most plausible candidate to be modified by ACh is the background inward-rectifying component i_{K1}. Results obtained in frog atrial preparations seem to be in favour of the hypothesis that ACh increases a K^+ current indistinguishable from i_{K1}: both currents appear time independent (Garnier, Nargeot, Ojeda & Rougier, 1978) and they show similar sensitivities to the blockade produced by Cs^+ (Ojeda, Rougier & Tourneur, 1981; Argibay, Dutey, Ildefonse, Ojeda, Rougier & Tourneur, 1983). On the contrary, experiments in sino-atrial and atrioventricular preparations suggest that the K^+ channels activated by ACh are different from i_{K1} channels. In both preparations, ACh produces a current which is time dependent following a voltage pulse, and in sino-atrial tissue ACh gives rise to a Lorentzian component with voltage-dependent corner frequency in the membrane noise spectrum (Noma & Trautwein, 1978; Noma, Peper & Trautwein, 1979). Single-channel recordings in the same preparations support the hypothesis that ACh-sensitive channels are different from i_{K1} channels, since the former channels have a mean open life time much shorter than that of i_{K1} channels (Sakmann, Noma & Trautwein, 1983).

The following experiments were carried out to test whether the ACh-induced current in rabbit cardiac Purkinje fibres may be distinguished from i_{K1}. For this purpose, we investigated the relaxation properties and the sensitivity to Cs^+ and Ba^{2+} of the ACh-induced current.

METHODS

The experiments were carried out on isolated cardiac Purkinje fibres and the two-micro-electrode voltage-clamp technique used in the present experiments is the same as the one used previously (Carmeliet & Ramon, 1980; see also Carmeliet & Mubagwa, 1986*a*).

For the experiments in which relaxation of the ACh-induced current was investigated, voltage pulses of 1–5 s to a given level were given repetitively (every 10–20 s). After a sufficient number of pulses in normal Tyrode solution, the preparation was exposed to ACh-containing Tyrode solution. In ACh, only currents recorded during pulses given after 10 min in the presence of the agonist were used. After enough recordings in ACh, a wash-out period was observed. The currents recorded after 10 min of ACh wash-out were used as a second control.

For the experiments in which the effects of Cs^+ or Ba^{2+} on the ACh-induced current were tested, membrane currents were first recorded in normal Tyrode solution, before and after application of ACh. The same measurements were repeated in the presence of 20 mM-Cs^+ or in the presence of various Ba^{2+} concentrations, again before and after application of ACh.

The current signals to be used for relaxation analysis were recorded on a magnetic tape (Hewlett-Packard 3964A). They were later sampled at 500 Hz on a computer (PDP-11). Averages of three to ten currents in the same conditions were made. The average current in normal Tyrode solution was subtracted from the average current in ACh to give the ACh-induced current.

RESULTS

Absence of relaxation of the ACh-induced current

Fig. 1 illustrates the results of an experiment in which relaxation of the ACh-induced current in rabbit Purkinje fibres was tested. In this experiment tetrodotoxin (TTX, 10^{-5} M) was present in the perfusing solution to reduce Na^+ currents. The holding potential was also kept negative to the activation potential of other time-dependent

currents. Average currents in the absence and in the presence of ACh are superimposed (Fig. 1, left). For voltage pulses of moderate amplitude, i.e. to potentials between −40 and −95 mV, ACh produces a parallel shift of membrane currents during the pulse. This is what is expected if ACh increases a time-independent current. The difference current (Fig. 1, right) changes instantaneously and remains relatively

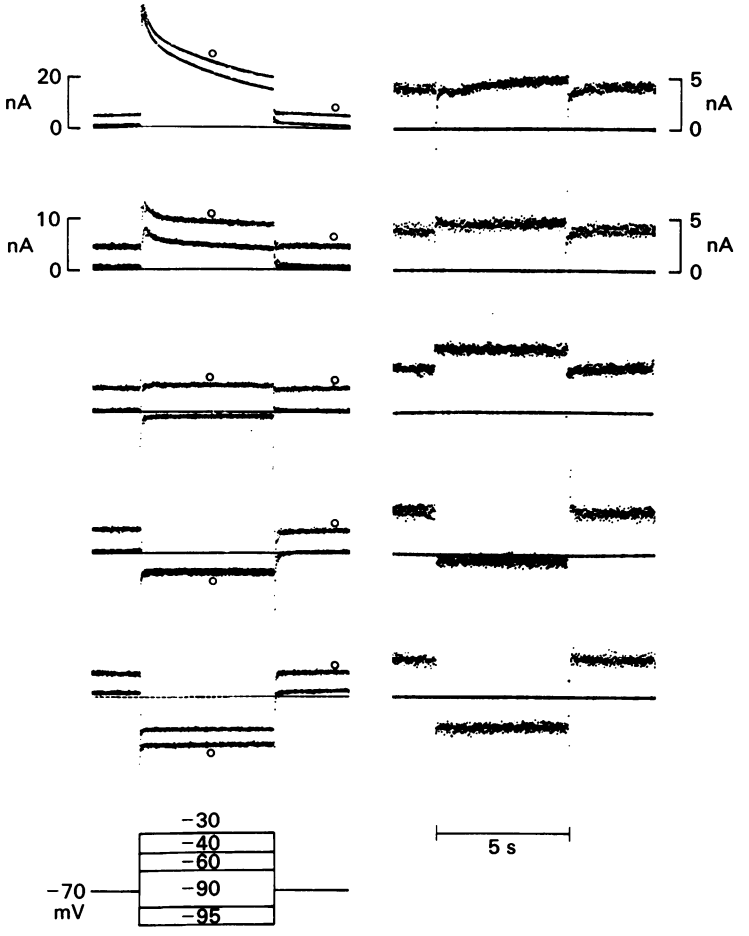

Fig. 1. Effect of ACh (2×10^{-6} M) on membrane currents. The holding and pulse potentials are indicated in the lower part of the Figure. Left: superimposed current traces before and during ACh exposure (indicated by an open circle). Each current trace is the average of five original current recordings. Right: ACh-induced current, obtained by subtracting the current in the absence from the current in the presence of ACh. The current calibrations are the same in the second and in the lower rows. The horizontal lines give zero current levels. Tetrodotoxin (10^{-5} M) was present.

constant with time. On return to the holding potential, it also instantaneously recovers to the pre-pulse value. The results suggest that in this range of potentials, the ACh-induced current is time independent.

During depolarizations to potentials beyond −40 mV or during hyperpolarizations to potentials more negative than −95 mV, the ACh-induced current frequently shows

time dependence. For hyperpolarizations beyond −95 mV, the amplitude of the inward ACh-induced current usually decreases with time. Only in exceptional cases does the ACh-induced current show an initial rapid increase. Such an increasing ACh-induced current is shown in Fig. 2. The ACh-induced current during hyperpolarization to −130 mV shows an initial increase followed by a slow decrease. The early increase in current during hyperpolarization is also obtained at −100 and

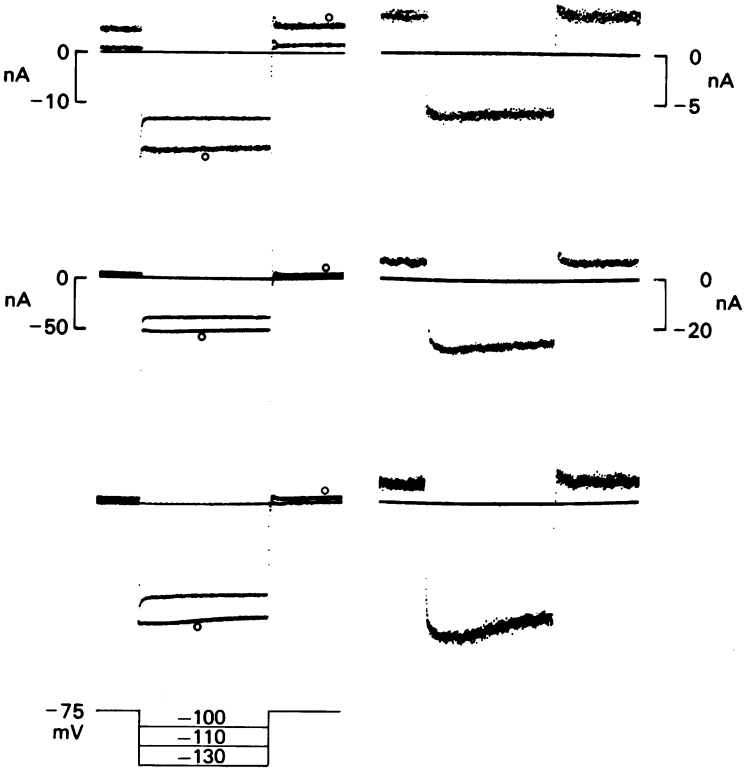

Fig. 2. Effect of ACh (2×10^{-6} M) on membrane currents during large hyperpolarizations. Same preparation and same conventions as in the preceding Figure. Notice time-dependent changes of the ACh-induced current. The current calibrations are the same in the second and in the third rows.

−110 mV and resembles the relaxation observed in sino-atrial cells (Sakmann *et al.* 1983). Such an increase in ACh-induced current during hyperpolarization is consistent with an increase of open probability of the ACh-sensitive channels. The secondary decrease of ACh-induced current observed at −130 mV may result either from an inactivation of the ACh-sensitive channels or from a depletion of K^+ in the intercellular spaces. Although rabbit Purkinje fibres usually possess wide intercellular spaces, it is possible that with a very large influx of K^+ such as the one produced by the hyperpolarizing step to −130 mV, some depletion occurs. On return to the holding potential, the initial current is usually higher than the control, i.e. an outward

tail is obtained. Outward tails probably result from the deactivation of ACh-sensitive channels, but they may result from intercellular K^+ depletion as well.

For large depolarizations, ACh-induced outward currents decreasing with time as well as currents increasing with time could be observed. The time-dependent increase (see Fig. 1) might be related to a K^+ accumulation process. Changes in time-dependent currents might also contribute to the effect of ACh. A decrease of the late outward current, i_x (see Carmeliet & Mubagwa, 1986*a*), would tend to superimpose on the effect of ACh on other currents. This will result in a total ACh-induced current decreasing with time during depolarizations to potentials positive to -50 mV and in an inward tail on return to the holding potential.

From the present results, it can be concluded that, except in rare cases, the ACh-induced K^+ current in rabbit Purkinje fibres does not show relaxation. The time dependence observed during very large voltage pulses can be accounted for by K^+ accumulation/depletion phenomena or by a change of time-dependent currents by ACh.

Blockade of the ACh-induced current by Cs^+ and Ba^{2+}

The permeation of K^+ through inward-rectifying channels is blocked by many alkali or earth–alkali cations in the various tissues where these channels have been identified (Hagiwara, Miyazaki & Rosenthal, 1976; Isenberg, 1976; Gay & Stanfield, 1977; Standen & Stanfield, 1978, 1980; Carmeliet, 1979; DiFrancesco, 1981; Van der Heyden, Vereecke & Carmeliet, 1983). In rabbit cardiac Purkinje fibres, the magnitude of the hyperpolarization produced by ACh is reduced by Cs^+ or Ba^{2+} (Mubagwa & Carmeliet, 1983), suggesting that these ions also block the ACh-sensitive channels. In the following results, the pattern of block of the ACh-induced current is compared to the block produced on i_{K1}. The effects of Cs^+ and Ba^{2+} on the ACh-sensitive channel were obtained by comparing the ACh-induced currents in the absence and in the presence of these ions.

Fig. 3*A* shows current–voltage relations obtained in the same preparation, in the absence or in the presence of Cs^+ and/or ACh. In the absence of Cs^+ (continuous lines) the current–voltage relation showed a clear inward rectification, whereas in 20 mM-Cs^+ (dashed lines) the relation became more linear. The effects of Cs^+ on membrane currents of rabbit Purkinje fibres are, therefore, similar to those observed in other cardiac fibres (for example, see Isenberg, 1976).

The ACh-induced currents in the absence and in the presence of Cs^+ are shown in Fig. 3*B*. It appears that Cs^+ tremendously reduced the ACh-induced current at very negative potentials but less at potentials positive to K^+ equilibrium potential, E_K, so that the ACh-induced current in Cs^+ became more or less linear.

The persistence of an ACh-sensitive component in the presence of Cs^+ may be attributed to an incomplete and voltage-dependent block by Cs^+ of ACh-sensitive inward-rectifying K^+ channels. The same result could, alternatively, be explained by assuming that ACh affects more than one channel: on the one hand an inward-rectifying current which is completely blocked by 20 mM-Cs^+ at all potentials, and on the other hand a Cs^+-insensitive component. Possible candidates for the Cs^+-insensitive component are the slow inward current (a steady-state component of which would be decreased by ACh) and the K^+ outward rectifier (which would be

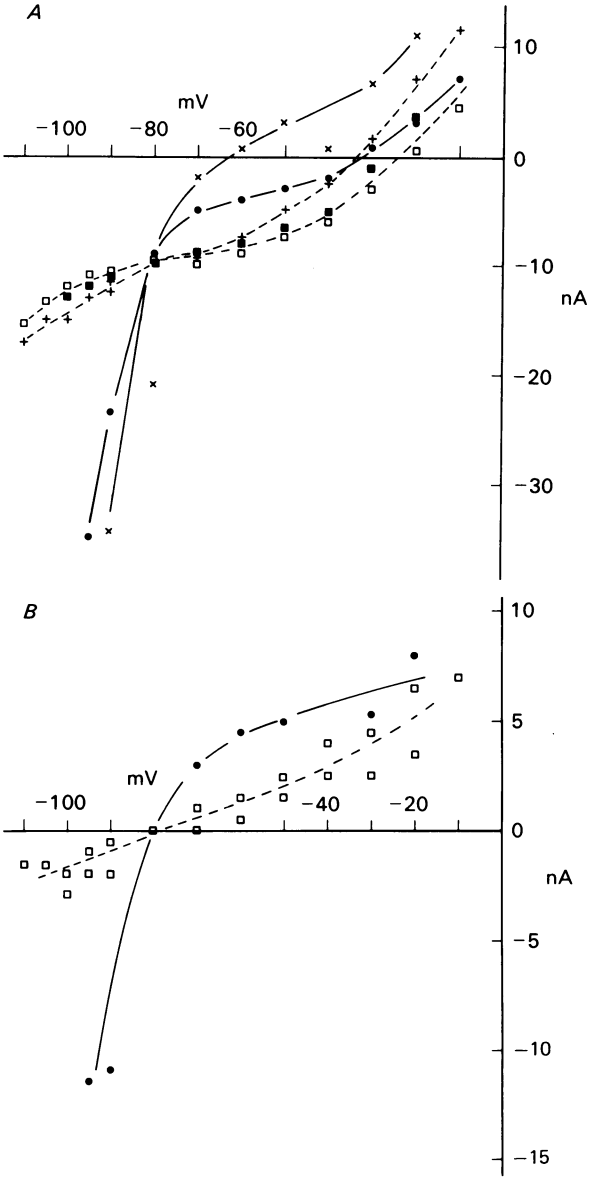

Fig. 3. Effect of 20 mM-Cs^+ on the ACh-induced current. *A*, the influence of Cs^+ was studied by measuring the change in 'steady-state' membrane current produced by ACh in the presence (dashed line) and after wash-out (continuous line) of Cs^+. (●): normal Tyrode solution (NTS), before exposure of ACh. (×): NTS + ACh. (■): Cs^+, before exposure of ACh. (+): Cs^+ + Ach. (□): Cs^+, after ACh wash-out. *B*, ACh-sensitive currents in the absence (●) and in the presence (□) of Cs^+. Holding potential: −60 mV.

increased by ACh). In Carmeliet & Mubagwa (1986*a*), it was shown that ACh does not change the slow inward current in the absence of catecholamines. It is therefore unlikely that a steady component of the slow inward current accounts for the Cs^+-insensitive ACh-induced component. The possibility that the ACh-sensitive current

persisting in Cs^+ is a K^+ outward rectifier also seems unlikely, since the ACh-induced current in normal Tyrode solution is usually flat at potentials very positive to E_K (see Figs. 2*B* and 4*B* in Carmeliet & Mubagwa, 1986*a*).

Due to continuous changes in currents probably by Cs^+ loading into the cells, the ACh-induced current in the presence of Cs^+ could not be precisely measured. This is especially true at very negative potentials, where the magnitude of the ACh-induced

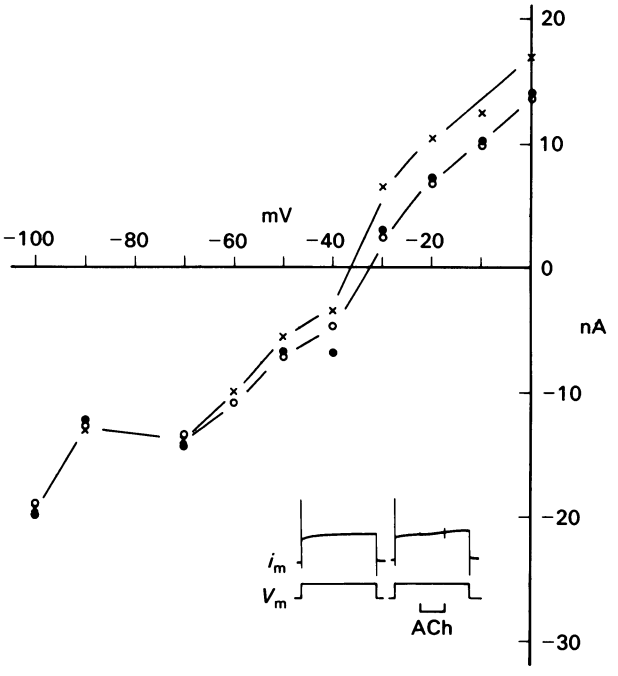

Fig. 4. Effect of ACh on membrane currents in the presence of 20 mM-Cs^+. Currents were measured at the end of three successive voltage pulses of equal magnitude. During the first (●) and the third (○) pulses, no ACh was applied. During the second pulse (×), ACh was applied for 5 s by pressure from an ACh (0·1 M)-filled electrode. Inset: original recordings showing the change in membrane current following the application of ACh. V_m, pulse potential. i_m, membrane clamp current.

current became too small. In order to verify whether a significant Cs^+-insensitive ACh-induced component persists at these negative potentials, the effect of ACh was also studied by pressure application of the drug on fibres clamped at different potentials in the presence of Cs^+. In the experiment of Fig. 4, the membrane potential was clamped at a given level for 15 s, and the current was measured at the end of the voltage pulse. Each voltage pulse was followed by a second pulse during which ACh was administered for 5 s. The application of ACh started 5 s after the beginning of the second pulse (Fig. 4, inset). For voltage pulses to potentials positive to −60 mV, ACh application resulted in current changes in the outward direction, whereas for pulses to potentials negative to −60 mV, ACh produced no effect. This means that no reversal for the ACh-sensitive current in Cs^+ was found in this experiment. It is more likely therefore that the effects of ACh which persist in the presence of Cs^+ are

due to a less efficient Cs^+ block of the ACh-induced inward rectifier than to the presence of an ACh-sensitive outward rectifier component which is not blocked by Cs^+. In this last case, a reversal of the ACh-induced current in Cs^+ should be present. The results suggest that Cs^+ blocks the ACh-sensitive channels and that this blockade by Cs^+ is strongly voltage dependent, being more pronounced with hyperpolarization.

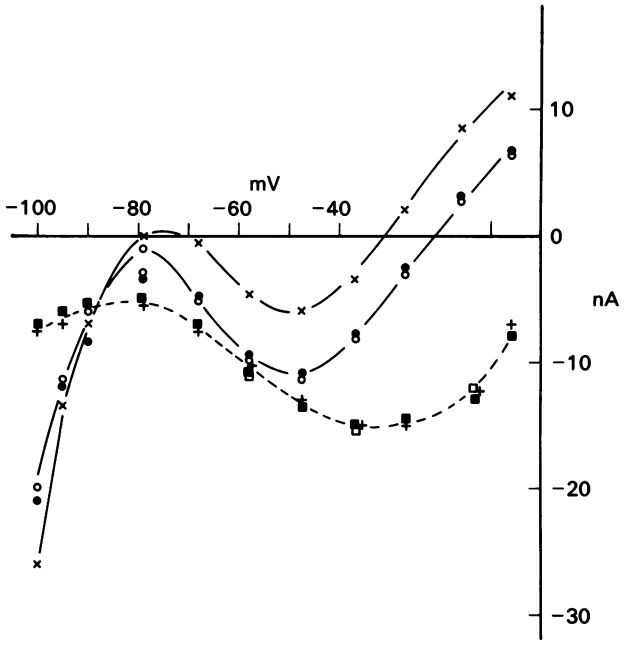

Fig. 5. Effect of 5 mM-Ba^{2+} on theACh-induced current. The influence of Ba^{2+} was obtained by studying the effects of ACh (2×10^{-6} M) on steady-state currents in the absence (continuous line) or in the presence (dashed line) of Ba^{2+}. (●): normal Tyrode solution (NTS), before exposure to ACh. (×): NTS + ACh. (○): NTS, after ACh wash-out: (■): Ba^{2+}, before exposure to ACh. (+): Ba^{2+} + ACh. (□): Ba^{2+}, after ACh wash-out. Holding potential: −79 mV.

Similar results have been obtained in frog atrium and were taken as evidence in favour of the hypothesis that ACh modulates the properties of i_{K1} channels (Ojeda *et al.* 1981; Argibay *et al.* 1983). Such results can, however, be obtained if ACh increases an inward-rectifying current which is different from i_{K1} but shows a similar sensitivity for Cs^+ (see below).

Other blockers of inward-rectifying channels were tested. In the experiment illustrated in Fig. 5, the effects of ACh in the absence (continuous lines) or in the presence of 5 mM-Ba^{2+} (dashed lines) are compared. At the concentration used, Ba^{2+} produced a large reduction in the current flowing during hyperpolarizing pulses. Positive to −60 mV, the current–voltage relation in Ba^{2+} is markedly shifted in the inward direction, indicating that apart from blocking outward K^+ currents, this ion also increases the slow inward current (Osterrieder, Yang & Trautwein, 1982; Cavalié, Ochi, Pelzer & Trautwein, 1983). Addition of ACh (2×10^{-6} M; crosses) in the presence

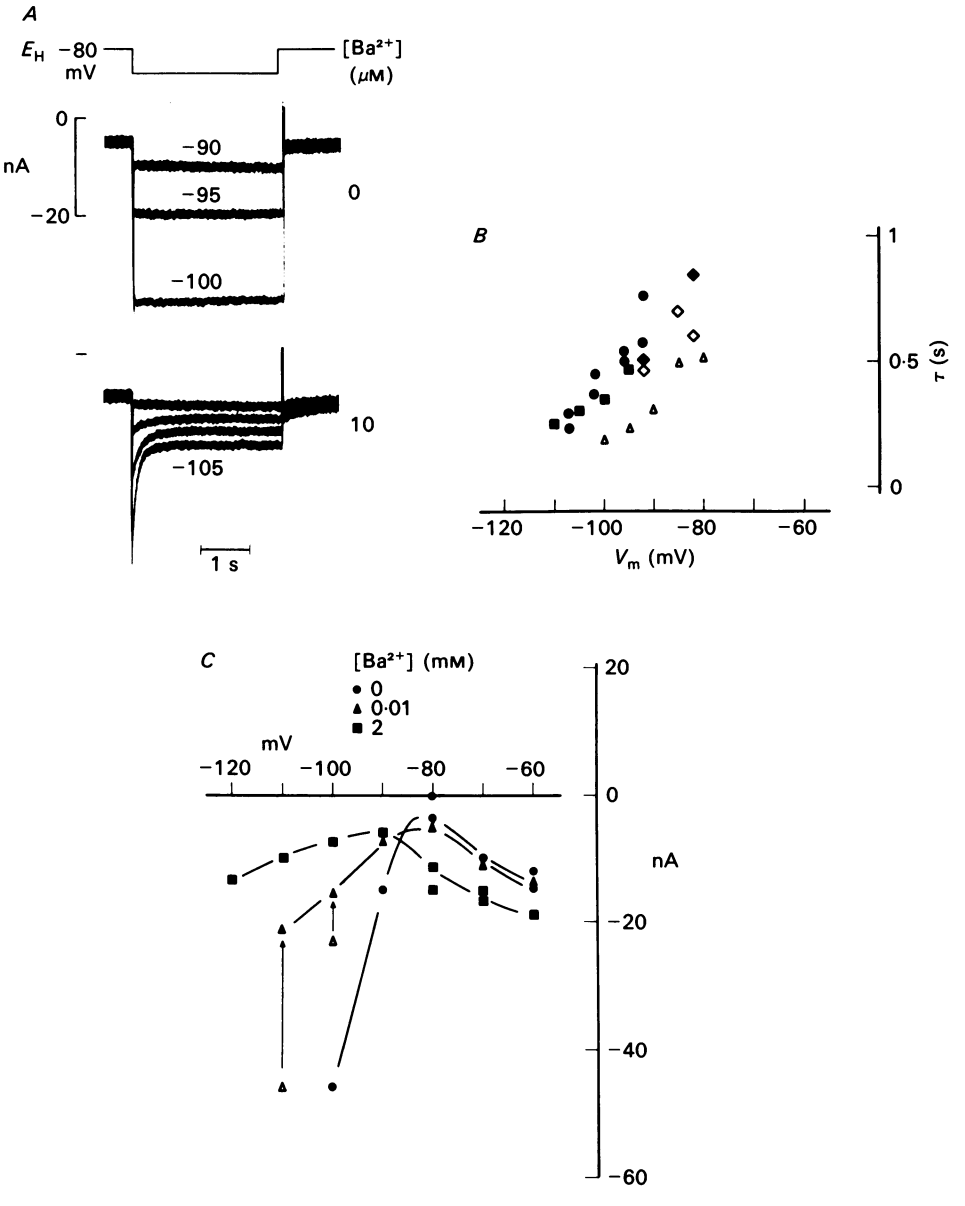

Fig. 6. Effect of Ba^{2+} on membrane currents. *A*, inward currents in the absence (upper panel) and in the presence (bottom panel) of 10^{-5} M-Ba^{2+} during hyperpolarizations from −80 mV. The pulse potentials are indicated near the current traces and are the same in control as well as in Ba^{2+}, except for the lowest current trace, obtained at −105 mV in Ba^{2+}. *B*, time constants (τ) of decay in membrane current during hyperpolarization in the presence of 5×10^{-6} M-Ba^{2+}. Different symbols correspond to different preparations. Filled symbols (●, ■, ◆): 5·4 mM-K^+ Tyrode solution. Open symbols (◇, △): 10·8 mM-K^+ Tyrode solution. *C*, steady-state current–voltage relations in the absence of Ba^{2+} (●), in 10^{-5} M-Ba^{2+} (▲) and in 2×10^{-3} M-Ba^{2+}. (■). Instantaneous currents (△) in 10^{-5} M. The arrows show the direction of change in current during hyperpolarizing pulses in 10^{-5} M-Ba^{2+}. Holding potential: −80 mV.

of Ba^{2+} did not produce any effect on membrane currents in the whole range of potentials studied, while there was a clear effect of ACh before Ba^{2+} application. The lack of ACh effect even at potentials positive to −50 mV further supports the idea that ACh does not change i_{si} in the absence of catecholamines (see Carmeliet & Mubagwa, 1986*a*).

In one experiment, Sr^{2+}, another inward rectifier blocker (Ohmori, 1978; Standen & Stanfield, 1978) also decreased the ACh-induced current, although it seemed less potent than Cs^+ or Ba^{2+}. Its effect was also voltage dependent (not shown).

Separation of the ACh-sensitive current from i_{K1}

In the experiments described above, the effects of ACh were examined during superfusion with relatively high concentrations of Cs^+ or Ba^{2+}. For 5 mM-Ba^{2+} the blockade of ACh-induced current was complete at all potentials tested, whereas for Cs^+ the degree of block was strongly voltage dependent. The blockade of the ACh-sensitive current in these conditions was instantaneous. However, as will now be shown, when low concentrations of Ba^{2+} were used, the block of i_{K1} was incomplete and its kinetics were slow. It was then possible to test whether the ACh-induced current behaves in the same way as i_{K1} during build-up of block.

The effect of low concentrations of Ba^{2+} in the absence of ACh is illustrated in Fig. 6*A*. When the membrane potential was hyperpolarized in the absence of Ba^{2+}, inward currents were obtained which remained roughly constant with time. If the experiment was repeated in the presence of micromolar concentrations of Ba^{2+}, the inward currents became smaller than in control and they relaxed exponentially to a new steady-state value. The rate of development and the extent of the Ba^{2+}-induced decay in current were increased with hyperpolarization. Fig. 6*B* gives the time constants obtained from five different experiments in which the effect of 5×10^{-6} M-Ba^{2+} on membrane currents was tested. The time constants of decay in current decreased from about 700 to 150 ms between −80 and −110 mV. The kinetics and extent of block also depend on the $[Ba^{2+}]$. At low $[Ba^{2+}]$, the block was clearly time dependent, while at higher $[Ba^{2+}]$ it developed instantaneously (see Figs. 8 and 9). In Fig. 6*C*, current–voltage relations obtained in control and in the presence of two different $[Ba^{2+}]$ are shown. The relations cross each other near E_K, indicating that the current blocked by Ba^{2+} is a K^+ current, presumably i_{K1}.

The above results are consistent with models in which open inward-rectifying K^+ channels bind Ba^{2+} and are converted into non-conducting (blocked) channels (for example, see Standen & Stanfield, 1978). The decrease in current produced by Ba^{2+} may be assumed to be directly related to the binding of the cation on a site located inside the channel. In this case the decrease in current produced by Ba^{2+}, Δi, will be related to the Ba^{2+} concentration by a Hill–Michaelis–Menten-type equation:

$$\Delta i = \frac{i}{(1 + K_D/[Ba^{2+}])}, \quad (1)$$

where i is the current in the absence of Ba^{2+} and K_D is the apparent affinity coefficient for the binding of Ba^{2+} to its site.

The access of Ba^{2+} to its binding site (and consequently, the concentration of Ba^{2+} near this site) being influenced by the presence of an electrical field in the membrane, K_D will depend on the membrane potential (Woodhull, 1973; see also Argibay *et al.* 1983):

$$K_D = A \exp[-(\Delta G - z\delta F V_m)/RT], \quad (2)$$

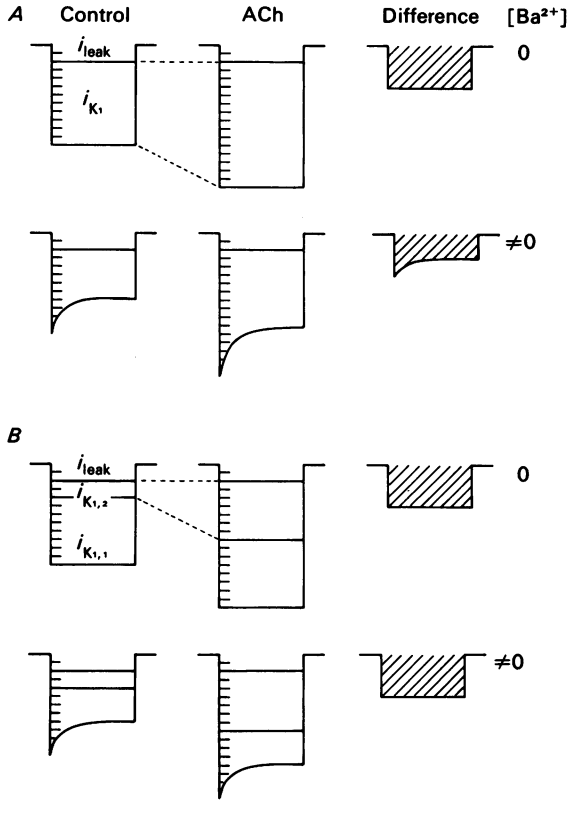

Fig. 7. Model predicting different ACh-induced currents in the presence of Ba^{2+} depending on whether ACh is assumed to produce its effects by increasing i_{K1} (*A*) or by increasing a current ($i_{K1,2}$) different from i_{K1} (also called $i_{K1,1}$ in the present context; *B*). In *A* and *B*, the upper panel shows currents in the absence of Ba^{2+} while the lower panel shows currents in the presence of low concentrations of Ba^{2+}. Currents in Ba^{2+} are drawn so that a block of four current units is obtained in steady state. Left: currents in the absence of ACh. Middle: currents during exposure to ACh. Currents in ACh are drawn so that a difference current of five units (compared to control) is obtained instantaneously upon hyperpolarization. Right: differences between currents in the presence and in the absence of ACh. In the absence of Ba^{2+}, the ACh-induced current is always time independent. In the presence of Ba^{2+}, the ACh-induced current is time dependent if ACh increases i_{K1}, whereas it is time independent if ACh increases a current different from common i_{K1}. The holding potential is supposed to be at E_K.

where A is a proportionality constant, ΔG is the change in free energy on binding of Ba^{2+} to a specific site in the channel, z is the ionic valency of the blocking ion, F is the Faraday number, V_m is the membrane potential, δ is the fraction of the membrane potential that is sensed at the binding site, R the gas constant and T the absolute temperature.

In the experiments to be described below, the effects of ACh were tested in the presence of low concentrations of Ba^{2+}. From a hypothetical point of view, two kinds of results can be expected, as sketched in Fig. 7. In the first case (Fig. 7*A*), ACh is supposed to modulate the properties of i_{K1} channels without changing their sensitivity for Ba^{2+}. Before application of ACh (Fig. 7*A*, left), i_{K1} is time independent in the

absence of Ba^{2+}, but becomes time dependent in the presence of these ions. After addition of ACh (Fig. 7*A*, middle), the magnitude of i_{K1} will be scaled by a factor which remains constant with time. In particular, the magnitude of the i_{K1} relaxation induced by Ba^{2+} will be increased in the presence of ACh by the same factor as the one with which the total current has been increased. Therefore, the ACh-induced current (Fig. 7*A*, right) will be different in the absence or in the presence of Ba^{2+},

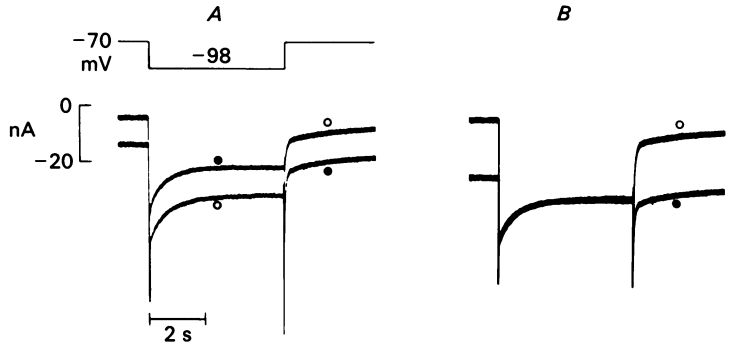

Fig. 8. Effect of ACh (2×10^{-6} M) in the presence of 3×10^{-6} M-Ba^{2+}. *A*, current recordings in the absence (●) and in the presence (○) of ACh are superimposed. Same zero level. *B*, the same recordings, but with the zero level for the current in the absence of ACh shifted downward until the relaxing components of the two recordings superimposed. Notice that neither the amplitude nor the time course of the Ba^{2+}-induced relaxation is changed by ACh.

since ACh will increase the magnitude of a time-independent current in the former case, and that of a time-dependent current in the later case. The ACh-induced current will be time-independent in the absence of Ba^{2+}. In the presence of Ba^{2+}, it will be time dependent. In the second case (Fig. 7*B*), ACh is supposed to modulate the properties of a population of channels different from i_{K1}. The current (called $i_{K1,2}$; see below) carried by these channels is supposed to be time independent. It is also assumed that this current is blocked by Ba^{2+} in a time-independent way, contrary to the standard i_{K1} (called $i_{K1,1}$) which is blocked in a time-dependent way. In this case, the ACh-induced current will always be time independent in the absence or in the presence of Ba^{2+}. The magnitude of the Ba^{2+}-induced relaxation will not be changed, since it is due to $i_{K1,1}$, which is assumed to be insensitive to ACh.

The results obtained when ACh was added in the presence of low [Ba^{2+}] are illustrated in Figs. 8 and 9. (1) Fig. 8*A* shows superimposed currents obtained upon hyperpolarization, before and after application of ACh in Ba^{2+}-containing Tyrode solution. ACh produced an increase in outward current positive to E_K and an increase in inward current negative to E_K, i.e. qualitatively the same effects as in normal Tyrode solution. Fig. 8*B* shows that the time-dependent components of the same two records in the absence and the presence of ACh can be superimposed. This makes it clear that, despite the fact that the magnitude of total current was doubled in the presence of ACh, the Ba^{2+}-induced time-dependent component of i_{K1} was not modified in its magnitude nor in its time course. The ACh-induced change in

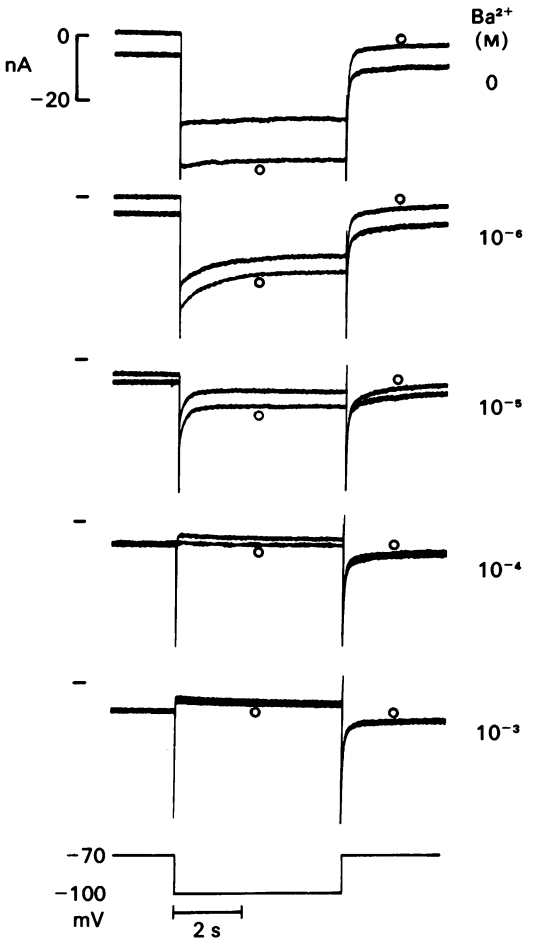

Fig. 9. Effect of increasing [Ba^{2+}] on the ACh-induced current. Current traces before and during exposure (marked by an open circle) of ACh are superimposed. [ACh]: 10^{-6} M.

membrane current must therefore be the result of an increase of a time-independent component. (3) Fig. 9 illustrates the results of an experiment in which the effect of a constant concentration of ACh was tested in the presence of varying [Ba^{2+}]. The changes produced by ACh depended on [Ba^{2+}], the ACh-induced current being decreased with increasing Ba^{2+} concentrations. At 10^{-3} M-Ba^{2+}, the effects of ACh were completely suppressed. (4) Despite the decrease in magnitude of the ACh-induced current by Ba^{2+}, this current never became time-dependent in the presence of various [Ba^{2+}], i.e. its decrease by Ba^{2+} was always instantaneous.

These results indicate that the ACh-sensitive current is different from the common i_{K1}, which is blocked by Ba^{2+} in a time-dependent way. Accordingly, the net current flowing during hyperpolarization may be grossly divided into the following components (see also Fig. 7): (1) a leak current (i_{leak}), defined as the current which remains in the presence of high ($> 10^{-3}$ M) [Ba^{2+}], (2) a current which is blocked by Ba^{2+} in a time-dependent way ($i_{K1,1}$) and (3) a current which is blocked by Ba^{2+}

in a time-independent way ($i_{K1,2}$). The basic name i_{K1} is also used for this last component since it is possible that some ACh-sensitive channels may be open in the absence of agonist (see following paper: Carmeliet & Mubagwa, 1986*b*). The subscript 2 is used to distinguish it from the ACh-insensitive component.

A priori, the total change in current following exposure to ACh, Δi, is given by the sum of ACh-induced changes in the various components:

$$\Delta i = \Delta i_{leak} + \Delta i_{K1,1} + \Delta i_{K1,2}.$$

From the experimental results, neither the leak current nor the current giving the time-dependent component are changed by ACh, i.e. $\Delta i_{leak} = 0$ and $\Delta i_{K1,1} = 0$. Thus, only changes of $i_{K1,2}$ are responsible for the ACh-induced current:

$$\Delta i = \Delta i_{K1,2}.$$

Therefore, for obtaining the sensitivity of $i_{K1,2}$ to Ba^{2+}, the magnitude of the ACh-induced current has been measured in function of $[Ba^{2+}]$. Fig. 10*A* compares the relative blocks produced by Ba^{2+} on the ACh-induced current at holding (−70 mV) and pulse potentials (−100 mV). The ACh-induced difference current was measured at both potentials in solutions containing various $[Ba^{2+}]$. The magnitude of the ACh-sensitive component decreased with increasing $[Ba^{2+}]$ (see also Fig. 9). The apparent K_D for binding of Ba^{2+} on ACh-sensitive channels (given by the concentrations of Ba^{2+} necessary to decrease the ACh-induced current to 50 % of the maximal value) are close to each other at both potentials (about $4{\cdot}2 \times 10^{-6}$ M at −70 mV and $2{\cdot}8 \times 10^{-6}$ M at −100 mV), which suggests that the blockade of the ACh-sensitive channel by Ba^{2+} is little voltage dependent in this range of potentials. In another experiment, the apparent K_D changed from $1{\cdot}8 \times 10^{-5}$ M at −70 mV to $4{\cdot}8 \times 10^{-6}$ M at −100 mV. The average value of δ for the block of the ACh-sensitive channel, obtained by plotting log K_D as a function of the membrane potential, is 0·4 from the two experiments. The weak voltage dependence of the block of ACh-sensitive channels by Ba^{2+} was confirmed in two other experiments in which the amplitude of the ACh-induced current was measured at various potentials (between −10 and −100 mV) in the absence and in the presence of 10^{-5} M-Ba^{2+}. When the ACh-induced current in Ba^{2+} was expressed, for each potential, relative to the ACh-induced current in the absence of Ba^{2+}, the ratio between the two currents changed little (values range: 25–55 %) for the whole range of potentials (not shown).

The lack of significant voltage dependence of the Ba^{2+}-induced blockade of ACh-sensitive channels is in contrast with the large voltage dependence expected for $i_{K1,1}$ from its relaxation in Ba^{2+}. In order to obtain the voltage dependence of the block produced by Ba^{2+} on $i_{K1,1}$, a way for measuring this current had to be found. The Ba^{2+}-sensitive current which exists before the addition of ACh was taken as an approximate measure of $i_{K1,1}$. By this method, we actually obtain a measure of the ACh-insensitive K^+ current (i.e. $i_{K1,1}$) plus the pre-existing part of the ACh-sensitive component (i.e. $i_{K1,2}$ at [ACh] = 0). If this last component is assumed to be small in comparison to the ACh-insensitive component, the behaviour of the sum will approximate to that of the ACh-insensitive component ($i_{K1,1}$). The Ba^{2+}-sensitive current was measured at holding and pulse potentials, in the same two experiments in which the Ba^{2+}-induced block of $i_{K1,2}$ was studied. In normal Tyrode solution, only

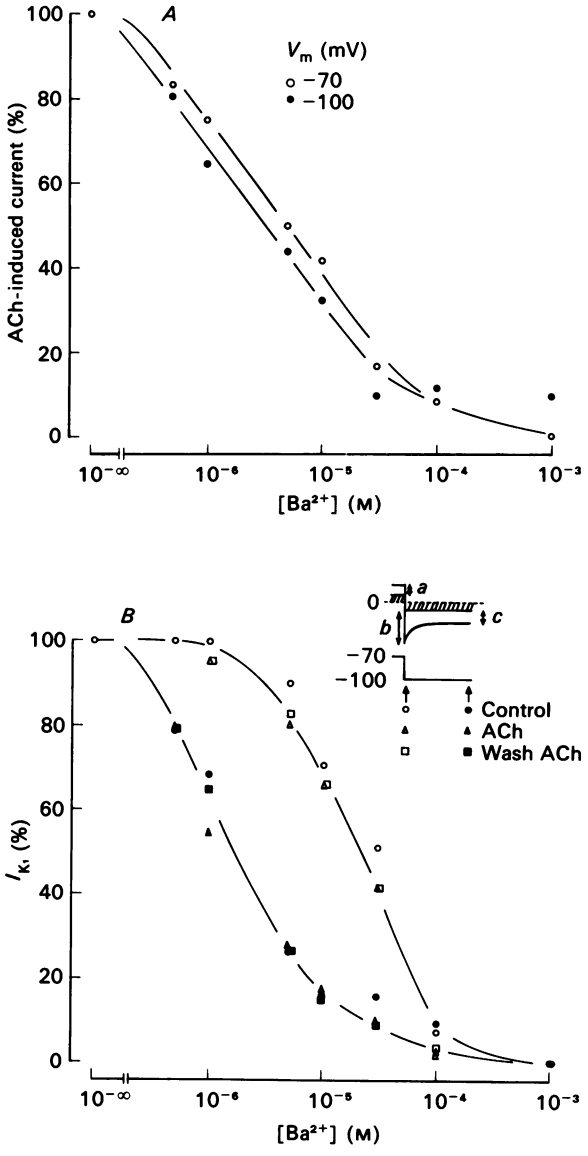

Fig. 10. Effect of increasing [Ba^{2+}] on the ACh-induced current and on i_{K1}. *A*, decrease of the ACh-induced current ($\Delta i_{K1,2}$; see text). This last current was measured as the difference current produced by ACh. (○): currents at holding potential (−70 mV). (●): currents at pulse potential (−100 mV). *B*, decrease of i_{K1} (which may approximate $i_{K1,1}$; see text). All currents were measured at −100 mV. Open symbols: instantaneous currents before exposure to ACh (○), during exposure to ACh (△), and after ACh wash-out (□). Filled symbols: steady-state currents before exposure to ACh (●), during exposure to ACh (▲), and after ACh wash-out (■). Inset: method for measuring i_{K1}. This last current was obtained by subtracting the Ba^{2+}-insensitive current (hatched area) from the net current. *a*: i_{K1} at holding potential; *b*: instantaneous i_{K1} at pulse potential; *c*: steady-state i_{K1} at pulse potential. The curves in *A* and *B* were drawn by eye.

i_{leak} was subtracted from the net current (Fig. 10*B*: inset), but the same results were obtained in the presence of ACh if both the i_{leak} and the ACh-induced current ($\Delta i_{K1,2}$) were subtracted. The approximate amplitude of $i_{K1,1}$, measured at the beginning or at the end of the voltage pulse is plotted as a function of $[Ba^{2+}]$ in Fig. 10*B*. The current at the beginning of the voltage pulse is proportional to the open probability at the holding potential (−70 mV), whereas the current at the end of the pulse is proportional to the open probability at the pulse potential (−100 mV). In this experiment, measurement of instantaneous $i_{K1,1}$ upon hyperpolarization was used to obtain the steady-state block at holding potential since it was difficult to obtain the magnitude of this current with satisfactory precision, due to the relatively large magnitude of the leak current compared to the net current at this level. In a second fibre, in which it was possible to measure $i_{K1,1}$ at holding potential (−70 mV), the $[Ba^{2+}]$–response curve at this potential was superimposable to that obtained using instantaneous currents following hyperpolarization to −100 mV. In the example shown, the amplitude of $i_{K1,1}$ decreases with increasing Ba^{2+} concentrations at both potentials. Interestingly, the concentration necessary to produce 50 % block (apparent K_D) is lower by one order of magnitude at the more negative potential $1{\cdot}3 \times 10^{-5}$ M at −70 mV, $1{\cdot}3 \times 10^{-6}$ M at −100 mV), reflecting a significant increase in affinity for Ba^{2+} by hyperpolarization. Similar results were obtained in another fibre (K_D: $2{\cdot}5 \times 10^{-5}$ M at −70 mV, $2{\cdot}1 \times 10^{-6}$ at −100 mV). This change in apparent K_D corresponds to a δ of about 0·9, indicating that the Ba^{2+}-binding site in ACh-insensitive channels senses a major part of the membrane potential, i.e. it is located close to the channel inner mouth.

From the assumption that only one Ba^{2+} is bound per channel (see eqn. (1) above), a Hill coefficient (n_H) of 1 is expected. In the above two preparations, Hill plots of the data for $i_{K1,1}$ give a n_H of $1{\cdot}01 \pm 0{\cdot}003$ (n = number of measurements = 6) and of $1{\cdot}045 \pm 0{\cdot}13$ (n = 6) at −70 mV and at −100 mV, respectively. For the ACh-sensitive current ($i_{K1,2}$), n_H is 0·57 (n = 1) and 0·76 (n = 1) at the same potentials, in the experiment shown in Fig. 10*A*. Such low Hill coefficients might be due to measurement errors caused by the small magnitude of ACh-induced currents. In the second experiment n_H was 0·61 at −70 mV (n = 1), but $1{\cdot}05 \pm 0{\cdot}02$ at −100 mV (n = 2).

DISCUSSION

After having shown that ACh increases a K^+ conductance (Carmeliet & Mubagwa, 1986*a*), it was interesting to investigate the characteristics of the ACh-sensitive K^+ current. An important question to answer was whether the properties of this conductance are superimposable on those of the pre-existing inward rectifier i_{K1} (Garnier *et al.* 1978; Ojeda *et al.* 1981; Argibay *et al.* 1983), or whether the ACh-sensitive current presents characteristics which suggest that it is different from i_{K1} (Noma & Trautwein, 1978; Sakmann *et al.* 1983).

The ACh-induced current is time independent during voltage pulses of moderate amplitudes (Fig. 1). For large voltage pulses, the ACh-induced current is frequently time dependent. The observed time dependence is, however, not always consistent with a voltage-dependent gating (i.e. activation or inactivation) of the ACh-sensitive channels. For depolarizing potentials, increasing as well as decreasing ACh-sensitive

currents are observed. This can be accounted for by an accumulation process or by superimposed changes in time-dependent currents. For large hyperpolarizing pulses, decreasing ACh-sensitive currents are usually obtained, probably following intercellular K^+ depletion. Exceptionally there may be an initial increase in ACh-induced current (Fig. 2) which suggests an increase with hyperpolarization of the open probability of ACh-sensitive channels. Only in this last case do the ACh-sensitive channels of rabbit cardiac Purkinje fibres behave like those of sino-atrial and of atrioventricular nodes (Noma & Trautwein, 1978; Sakmann *et al.* 1983).

Experiments with high concentrations of Cs^+ or Ba^{2+} (Figs. 3–5) do not allow a distinction to be made between common i_{K1} channels and ACh-sensitive channels. Such a distinction, however, is possible in experiments with low concentrations of Ba^{2+} (Figs. 6–10). These experiments reveal different sensitivities of the two types of channels to Ba^{2+}, thus allowing the separation of the inward rectifier in the presence of ACh into two components ($i_{K1,1}$ and $i_{K1,2}$).

When the perfusing solution contains low [Ba^{2+}] a time-dependent decrease in current is obtained upon hyperpolarization. This time-dependent decrease resembles the time-dependent block produced by Ba^{2+} in skeletal muscle (Standen & Stanfield, 1978) and results from a voltage-dependent binding of Ba^{2+} to a site in $i_{K1,1}$ channels. The binding of Ba^{2+} probably causes a decrease in the open-channel probability of the channels without changing the single-channel conductance (Bechem, Glitsch & Pott, 1983; Sakmann & Trube, 1984). In the presence of a relaxing i_{K1} (or $i_{K1,1}$), the ACh-induced current remains time independent, suggesting that ACh-sensitive channels are different from i_{K1} (or $i_{K1,1}$) channels. If ACh were to increase the K^+ conductance by affecting the same population of channels as the one blocked by Ba^{2+} in a time-dependent way, the following results would have been obtained: (1) a constant ratio between K^+ currents in the absence or in the presence of ACh, when measured at the beginning or at the end of a hyperpolarizing pulse, this ratio being given by the scaling factor of the exponential components measured in the two experimental conditions; (2) a time-dependent ACh-induced current during a hyperpolarizing pulse (Fig. 7*A*).

The two expectations are opposite to the experimental findings. The total magnitude of current is increased in ACh, but the exponential component (which is part of $i_{K1,1}$) remains unchanged and the difference current remains constant with time (Figs. 8–9). These results show that ACh affects a K^+ conductance different from the one blocked by Ba^{2+} in a time-dependent way.

It could be imagined that there is only one type of inward-rectifying channels (i_{K1}) and that ACh exerts its effects by modifying the properties of these channels in such a way that the block produced by Ba^{2+} on the ACh-activated channels becomes instantaneous and little voltage dependent. This possibility seems, however, unlikely since the magnitude of the Ba^{2+}-induced relaxation did not change with ACh. Even in the presence of increasing ACh concentrations (10^{-7}–10^{-4} M), the relaxing component was not changed (not shown). If ACh were to modify i_{K1} properties, increasing ACh would have produced a decrease in the relaxing component, since more i_{K1} channels would have been transformed into channels with different properties.

Finally, it is tempting to conclude from the difference between common i_{K1} channels and ACh-sensitive channels, that ACh induces the formation of a new type

of channels, as suggested for the sino-atrial and the atrioventricular nodes (Noma & Trautwein, 1978; Sakmann *et al.* 1983). However, the same results can be obtained if ACh-sensitive channels already exist in the absence of ACh, i.e. if the inward rectifier K^+ current is flowing through two types of channels ($i_{K1,1}$ and $i_{K1,2}$). Both types are sensitive to Ba^{2+}. One type of channel is insensitive to ACh, and its block by Ba^{2+} is strongly voltage dependent and of slow kinetics. The other type is sensitive to ACh, and the block produced by Ba^{2+} is less voltage dependent and time independent. While other experimental techniques (e.g. noise analysis or single-channel recording) are needed to test this hypothesis, there exists some evidence that the ACh-sensitive channel pre-exists in functional state in the absence of the agonist. With single-channel recording in nodal tissues, Sakmann *et al.* (1983) observed a channel activity in the absence of ACh, which had the same unit channel conductance as the one in ACh-treated membrane patches. Similar results have been obtained by Soejima & Noma (1984), who have been able to record the channel activity of a patch before and during its perfusion with ACh. They observed that muscarinic agonists increase the open probability of the basal activity, whereas muscarinic antagonists produce the opposite effect. In rabbit cardiac Purkinje fibres, the K^+ conductance decreases below the normal value on wash-out of ACh, as will be shown in the following paper (Carmeliet & Mubagwa, 1986*b*). This result is explained by assuming that desensitized channels are temporarily excluded from participating in the normal membrane conductance.

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