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EFFECTS OF ACETYLCHOLINE AND PARASYMPATHETIC NERVE STIMULATION ON MEMBRANE POTENTIAL IN QUIESCENT GUINEA-PIG ATRIA

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SUMMARY

1. In quiescent preparations of guinea-pig right atria the action of ACh applied in the superfusion medium or released from parasympathetic nerve fibres was investigated by membrane potential measurements.

2. ACh-containing solutions induced hyperpolarizations which did not show desensitization.

3. The relationship between hyperpolarization amplitude (corrected for non-linear summation) and ACh concentration could formally be described by simple saturation kinetics with an apparent dissociation constant $K_{ACh} = 1{\cdot}3 \times 10^{-6}$ M.

4. Stimulation with impulses subthreshold for myocardial excitation induced a membrane hyperpolarization by releasing ACh from post-ganglionic parasympathetic nerve fibres.

5. The hyperpolarization reached maximum about 1 sec after the onset of stimulation. This slow development of hyperpolarization cannot be accounted for simply by diffusion from the site of release to the receptor site.

6. The hyperpolarization declined exponentially with a time constant of about 3 sec. In the presence of neostigmine (3×10^{-6} M) the hyperpolarization lasted for 2 min or more. It is concluded that the action of ACh is primarily terminated by enzymic hydrolysis.

INTRODUCTION

Repetitive vagal stimulation or application of acetylcholine (ACh) produces a hyperpolarization of the resting membrane in cardiac sinus and atrial cells. This leads to slowing or cessation of the spontaneous action potential discharge (for reviews see Anderson & Del Castillo, 1972; Hutter, 1961; Trautwein, 1963).

The primary effect of ACh on the resting potential has been described as an increase in the K permeability of the sarcolemma which results in a membrane hyperpolarization towards the K equilibrium potential. This hypothesis is based on the finding that the reversal potential of the ACh-induced membrane response is close to the potassium equilibrium potential (Trautwein & Dudel, 1958). It is also in line with an increase in ^{42}K efflux from tortoise and frog sinus venosus tissue (Harris & Hutter, 1956; Hutter, 1961) and from rabbit auricles (Rayner & Weatherall, 1959). In addition to this effect of ACh on the resting potential an inhibition of the

slow inward current which flows during the cardiac action potential has been described in frog atrial muscle (Giles & Noble, 1976). In the mammalian atrium, however, this inhibition of the slow inward current, which might contribute to the negative inotropic effect of ACh, has been reported to occur only at relatively high concentrations (Ten Eick, Nawrath, McDonald & Trautwein, 1976).

The inhibitory membrane response of frog heart muscle cells to vagal stimulation or ionophoretic ACh application is exceedingly slow in comparison to other synaptic potentials (Del Castillo & Katz, 1955; Purves, 1976). This has recently been considered as being a specific property of muscarinic cholinergic transmission (Purves, 1974, 1976; Bolton, 1976).

The present paper reports the action of ACh applied in the superfusion medium or released from parasympathetic nerve fibres in quiescent guinea-pig right atrial cells. The method allows membrane potential measurements of single atrial cells over long periods of time and, thus, the comparison of the effects of a variety of ACh concentrations and experimental conditions on ACh action. The technique of selective stimulation of intrinsic parasympathetic nerve fibres (Vincenzi & West, 1963) in conjunction with intracellular membrane potential recording permits an analysis of the time course of the action of small amounts of ACh released from these nerves on the auricular cell membrane.

The results to be described demonstrate that the muscarinic ACh receptor in the preparation studied does not display desensitization. Taking into account nonlinear summation of changes in membrane potential induced by changes in membrane conductance a dose response relationship for ACh is derived. The slow time course of the hyperpolarization elicited by ACh released from post-ganglionic parasympathetic nerve fibres is discussed. A preliminary report on some of the findings has been published (Pott, 1977).

METHODS

Material. Isolated apices of right atria from the guinea-pig heart were used (animal weight 200–400 g). To prevent noradrenergic effects which might occur after subthreshold stimulation of atrial tissue (Vincenzi & West, 1963) all animals were reserpinized about 15 hr before killing (Serpasil^{R}, 2·5 mg/kg I.P.).

Solutions. The Tyrode solution had the following composition (mM): NaCl 137, KCl 5·4, $CaCl_2$ 2·0, $MgCl_2$ 1·0, NaH_2PO_4 0·42, $NaHCO_3$ 11·9, and glucose 5. The solutions were bubbled with carbogen (95 % O_2, 5 % CO_2); pH 7·2–7·4. The temperature was 35 ± 1 °C. The K concentration of the bathing fluids was routinely measured by means of a flame photometer (Eppendorf).

Experimental procedure. Following preparation the atrial tissue was fixed in a perfusion chamber (volume about 0·5 ml.) through which the bathing medium flowed at 10 ml./min. At the beginning of an experiment the preparation was equilibrated for 1 hr in the perfusion chamber. If the muscle displayed spontaneous contractions after this time basal portions were cut off. Preparations which showed spontaneous contractions after another 30 min were discarded. After the equilibration period, solutions with different ACh concentration (exogenous ACh) or stimulation of cardiac vagal nerve fibres (ACh release) were tested for their effect on atrial membrane potential under a variety of experimental conditions.

Voltage recording. The membrane potential of the auricles was recorded intracellularly by conventional glass micro-electrodes (filled with 2 M-KCl; tip potential 10–20 mV; resistance 10–20 MΩ) with the usual technique. The potentials were displayed simultaneously on the screen of an oscilloscope (Tektronix 546B) and on a pen recorder (Philips PM 8240).

Stimulation. Parasympathetic nerve fibres were stimulated (Grass 58 stimulator) via a platinum wire which was insulated except at the tip (ϕ 100 μm). This wire (cathode) was gently

placed on the surface of the preparation. A coiled platinum wire as anode was fixed in the chamber a few millimetres from the preparation.

To test whether stimulation was successful the cathode was brought into a position in the immediate proximity (50–100 μm) of the intracellular micro-electrode. The preparation then was stimulated with impulse trains (50 Hz, 1 sec, stimulus duration 50 μsec) with increasing intensity until a distinct hyperpolarization was elicited. If threshold for myocardial excitation was reached before a hyperpolarization could be detected, the procedure was repeated at another site of the preparation.

Evaluation of results. Most of the measured changes in membrane potential were corrected by a factor first derived by Martin (1955) for correction of measured end-plate potentials. This will be described in detail under Results.

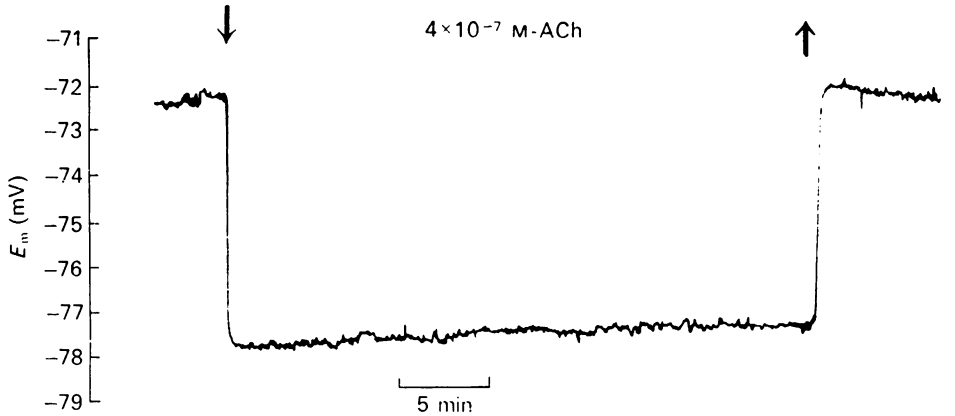

Fig. 1. Effect of ACh (4×10^{-7} M) on transmembrane potential (ordinate) of a quiescent guinea-pig right atrium. The arrows denote the period of ACh application.

RESULTS

The action of exogenous ACh on the membrane potential of quiescent atrial cells

Superfusion of quiescent preparations of guinea-pig right atria with ACh-containing solutions causes membrane hyperpolarizations up to 12 mV. Fig. 1 shows a recording of the alteration of the membrane potential when the superfusion medium was changed from normal Tyrode to a solution containing ACh (4×10^{-7} M). The resting potential which was -72 mV in this cell increased by 5·5 mV within 30 sec after the solution change. This hyperpolarization persisted for the whole period of ACh application which was 30 min in this experiment. In several experiments, however, a slow decrease of the hyperpolarization with time was observed, which was followed by a subsequent depolarization beyond the initial resting potential after readmission of the ACh-free solution. These effects are probably due to a loss of intracellular potassium and/or an accumulation of potassium at the outer face of the membrane rather than to desensitization of the muscarinic ACh receptor (Katz & Thesleff, 1957). During the first 2–5 min after the beginning of ACh superfusion, the extent of hyperpolarization remained fairly constant in all cells tested. Thus the dependence of the hyperpolarization on the concentration of ACh in the external solution could be studied by a simple superfusion technique.

Fig. 2 illustrates the hyperpolarizations induced by different ACh concentrations measured during a continuous micro-electrode impalement of a single auricular cell.

The superfusion time with ACh-containing solutions was 2 min in each case which was sufficient to reach a steady membrane potential. The interval between two measurements was 10 min. The lowest ACh concentration tested in this experiment was 2×10^{-8} M which did not induce a measurable hyperpolarization.

The dependence of the hyperpolarization amplitude on the ACh concentration from this experiment has been plotted in Fig. 3*A* (filled circles).

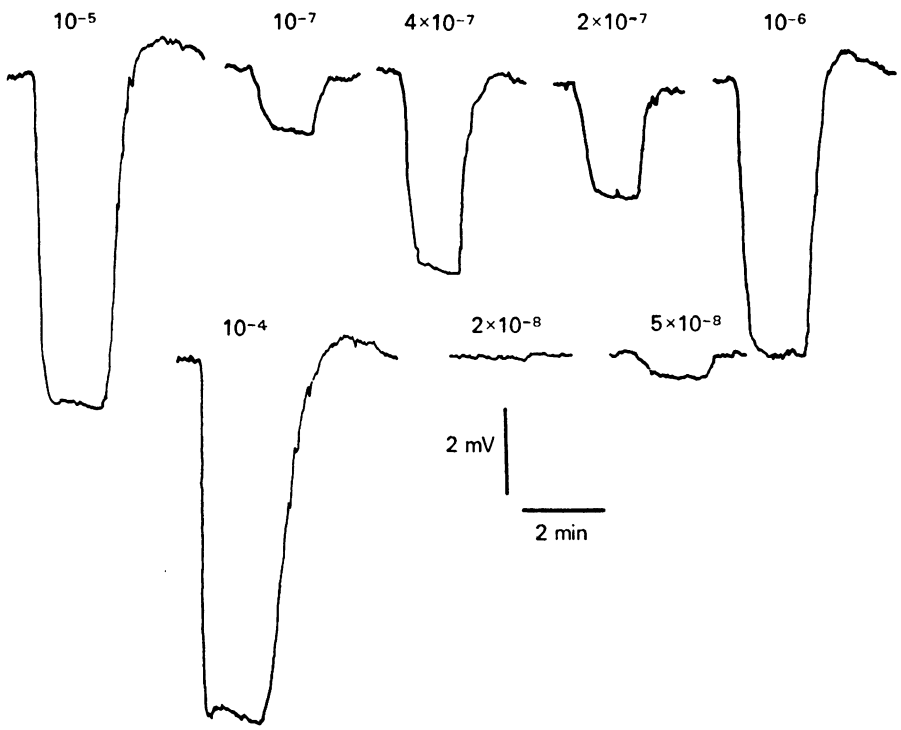

Fig. 2. Membrane hyperpolarization measured in one atrial cell during superfusion with solutions containing different ACh concentrations. The ACh concentrations (M) are given by the numbers above the traces. The order of the traces corresponds to the original experimental sequence. The time interval between ACh applications was 10 min. Resting potential was −72·5 mV at the beginning of the experiment.

As the hyperpolarization measured in atrial cells under the influence of ACh is brought about by an increase of the membrane permeability to potassium ions (Trautwein & Dudel, 1958) its magnitude is limited by the difference between the resting potential and the K-equilibrium potential ($E_m - E_K$). This driving force for the potassium outward current is reduced by the ACh-induced hyperpolarization itself so that the hyperpolarization is not linearly related to the underlying conductance change. In order to linearize the relationship between conductance change and hyperpolarization we applied the expression first derived by Martin (1955) for the motor end-plate potential. The corrected hyperpolarization is given by

$$\Delta V^* = \frac{\Delta V . (E_m - E_K)}{E_m - E_K - \Delta V}, \qquad (1)$$

where ΔV is the amplitude of the measured hyperpolarization. In the calculation of E_K the intracellular K concentration was assumed to be 135 mM (Glitsch, Pusch & Venetz, 1976). This corresponds to an E_K of $-85{\cdot}3$ mV at a $[K]_o = 5{\cdot}4$ mM and 35 °C.

The resting potential in the experiment shown in Figs. 2 and 3*A* was $-72{\cdot}5$ mV, and the $[K]_o$ was determined to be 5·1 mM corresponding to an E_K of $-86{\cdot}8$ mV. The open circles in Fig. 3*A* represent the hyperpolarization amplitudes obtained by correction for nonlinear summation according to eqn. (1). At low hyperpolarization

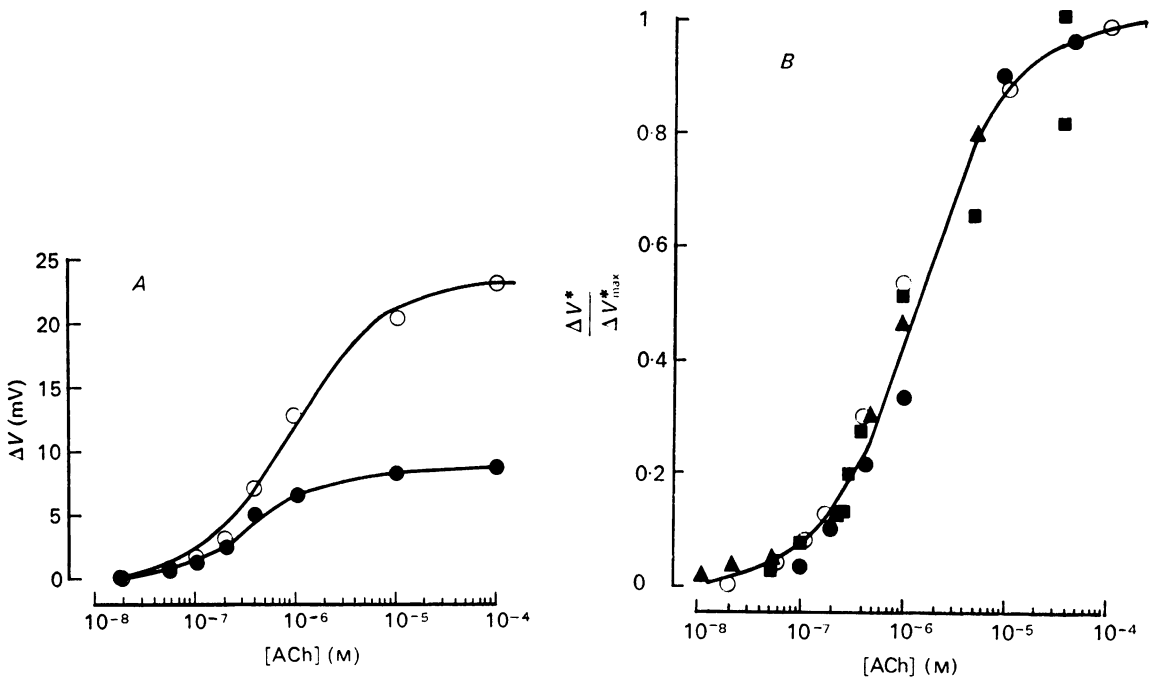

Fig. 3*A*, dose–response curve from the experiment shown in Fig. 2. Abscissa: ACh concentration (M, logarithmic scale). Ordinate: hyperpolarization amplitude (mV). The filled circles represent the measured hyperpolarization amplitude, the open circles were obtained by correction for non-linear summation (according to eqn. (1)). The lower curve was drawn by eye, the upper one was calculated by eqn. (2). *B*, dependence of the hyperpolarization amplitude (ΔV^*) on ACh concentration in the Tyrode solution. Abscissa: ACh concentration (M, logarithmic scale). Ordinate: normalized hyperpolarization (corrected, $\Delta V^*/\Delta V^*_{max}$). The curve was calculated according to eqn. (2). The symbols represent four different experiments.

amplitudes the difference between the measured and the corrected values is negligible. With progressing hyperpolarization, however, the correction becomes more and more important. The maximum of the measured hyerpolarization was about 8·8 mV, corresponding to a corrected value of 22·9 mV (ΔV^*_{max}). The curve connecting the open circles was calculated under the formal assumption of simple saturation kinetics:

$$\Delta V^* = \frac{\Delta V^*_{max} \cdot [ACh]}{K_{ACh} + [ACh]} \qquad (2)$$

with an apparent dissociation constant K_{ACh} of $9{\cdot}4 \times 10^{-7}$ M. This relation which implies a one to one ACh receptor interaction without co-operativity and a direct proportionality between receptor occupancy and conductance change yields the best approximation to the experimental results. Similar results were obtained in four other experiments where the determination of complete dose–response curves with single micro-electrode impalements was possible. They are plotted in Fig. 3*B* as normalized values with respect to ΔV^*_{max}. The curve was calculated according to eqn. (2) with $K_{ACh} = 1{\cdot}3 \times 10^{-6}$ M. However, the characteristics of the dose–response curves strongly depend on the assumptions made in the calculation of E_K and should be considered with some reservations.

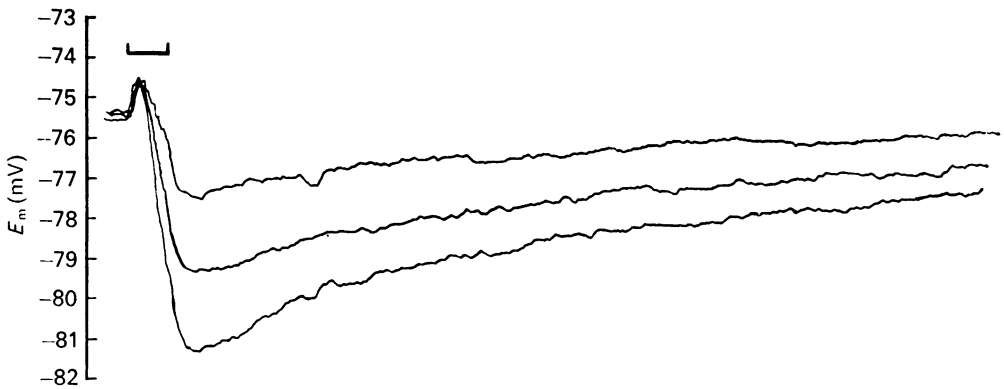

Fig. 4. Effect of direct subthreshold stimulation on transmembrane potential (ordinate) in quiescent guinea-pig right atrium. Stimulation was applied with 50 Hz for 1 sec (indicated by the horizontal bar). The lower trace was obtained during superfusion with normal Tyrode solution, the middle and upper one in the presence of 10^{-8} and 2×10^{-8} M-atropine, respectively. Neostigmine (3×10^{-6} M) was present throughout the experiment. The small initial hump in the depolarizing direction represents the stimulus train as recorded by the pen recorder (compare Fig. 7).

Hyperpolarization induced by ACh release from intracardiac parasympathetic nerve endings

Vincenzi & West (1963) described an ACh release from intracardiac parasympathetic nerve fibres by direct stimulation of the sinoatrial node of the rabbit heart with impulses subthreshold for excitation of the sinus cells. Stimulation with impulse trains caused negative chronotropic responses which were blocked by atropine and prolonged by inhibitors of enzymic ACh hydrolysis. Corresponding changes in membrane potential have been measured by a micro-electrode technique in the rabbit sinoatrial node (Toda & West, 1967*a*, *b*). This method, however, has not been applied so far in association with systematic membrane potential measurements in quiescent auricular preparations.

Fig. 4 shows membrane potential recordings obtained during stimulation of a non-beating auricle. The stimulation (frequency 50 Hz, train duration 1 sec, impulse duration 50 μsec) was subthreshold for excitation of myocardial cells. The bottom record was obtained during superfusion with Tyrode solution, the middle and top ones in the presence of 10^{-8} M and 2×10^{-8} M-atropine, repectively (neostigmine 3×10^{-6} M was present throughout the experiment). In solutions containing atropine

at a concentration of 10^{-7} M (not shown) the hyperpolarization due to subthreshold stimulation was usually abolished. These results demonstrate that the hyperpolarization induced by subthreshold stimulation is in fact mediated by the parasympathetic transmitter ACh and confirm the findings reported by Vincenzi & West (1963) and Toda & West (1967*a*, *b*). The latter authors, however, could not establish substantial membrane potential changes in atrial cells following subthreshold excitation.

How is ACh release induced? As the post-ganglionic parasympathetic nerve fibres emerge from intramural ganglia in some sinus and auricular tissues (e.g. McMahan & Kuffler, 1971) it is not clear *a priori* whether excitation initially takes place in pre- or post-ganglionic fibres of the guinea-pig atrium. If ganglionic transmission is involved secondarily leading to excitation of the short post-ganglionic fibres, drugs known to block synaptic transmission in autonomic ganglia should inhibit stimulation-induced hyperpolarization. Hexamethonium which has long been known as a

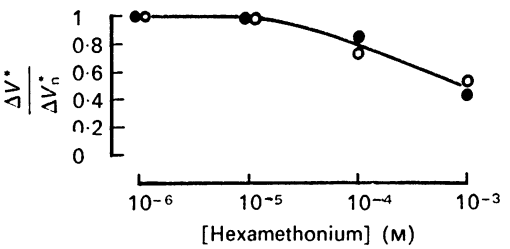

Fig. 5. Effect of hexamethonium on the hyperpolarization induced by subthreshold stimulation (●, 50 Hz, 1 sec) and by exogenous ACh (○, 5×10^{-7} M). Abscissa: hexamethonium concentration (M, logarithmic scale). Ordinate: normalized hyperpolarization with respect to the amplitude measured in hexamethonium-free solution. The curve was drawn by eye.

ganglionic blocking agent (Paton & Zaimis, 1951) has no effect on the hyperpolarization induced by subthreshold stimulation (50 Hz, 1 sec) in concentrations up to 10^{-5} M. In solutions containing higher concentrations the amplitude of the hyperpolarization was diminished. The diminution was about 55% in one experiment where 10^{-3} M hexamethonium was applied (correction for non-linear summation taken into account). It would be premature, however, to conclude from these results that preganglionic excitation is involved in the process which causes ACh release from post-ganglionic parasympathetic nerve endings. Control experiments carried out with exogenous ACh revealed that the hyperpolarizing response diminished to the same amount. The action of hexamethonium on the hyperpolarization induced by subthreshold stimulation (●) and by exogenous ACh (○) is illustrated in Fig. 5. The normalized hyperpolarization amplitudes measured during a single continuous micro-electrode impalement are plotted against the logarithm of the hexamethonium concentration. The reduction of the stimulus-evoked hyperpolarization by hexamethonium parallels the decrease in ACh sensitivity. Hence one has to consider the possibility that hexamethonium not only acts as an ACh antagonist in ganglionic, i.e. nicotinic cholinergic transmission but also inhibits the muscarinic ACh action. Thus, it can be concluded tentatively that excitation takes place in post-ganglionic fibres.

The time course of the hyperpolarization induced by endogenous ACh

To induce a measurable hyperpolarization a train of stimuli was generally required. In a few experiments, however, hyperpolarizations were recorded after stimulation with a single impulse. Membrane potential recordings of such an experiment are reproduced in Fig. 6. The traces show the hyperpolarizations induced by stimulation with 1, 2, 4, 10, and 25 pulses (from top to bottom; voltage gain was increased by

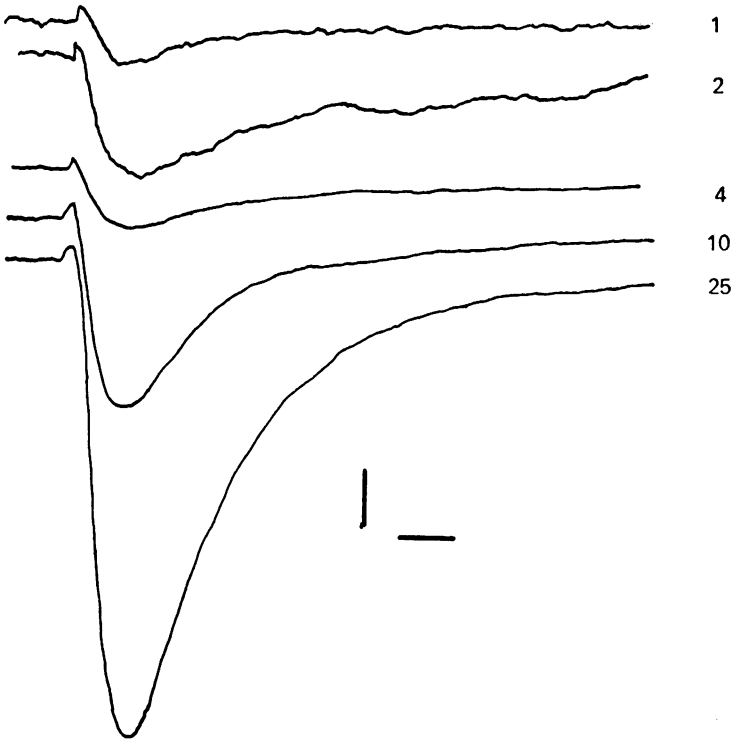

Fig. 6. Membrane hyperpolarization induced by ACh release. Post-ganglionic parasympathetic fibres were stimulated with 1, 2, 4, 10 and 25 stimuli (from top to bottom). The pulse interval during the stimulus trains was 0·02 sec. The interval between the stimulus trains was 5 min. Calibration bars: horizontal 1 sec; vertical 1 mV for the lower three traces, 0·2 mV for the upper two. Resting potential was −69 mV.

a factor of 5 for the upper two traces). Stimulation frequency was 50 Hz for the impulse trains. The peak of the hyperpolarization elicited by the single pulse was 0·21 mV in this experiment. It is interesting to note from these records that the slow development of the hyperpolarization (compare Fig. 4) is not due to the application of impulse trains but is also present following application of a single stimulus. In the example shown the time to peak hyperpolarization after a single stimulus was about 1 sec. The shortest time to peak intervals observed in this study were about 600 msec which is still several orders of magnitude slower than normally found for synaptic potentials. The temporal relation between stimulation and the development of the hyperpolarization is shown in Fig. 7 on a faster time scale. The auricle was stimulated with 4 impulses at 50 Hz recognizable by the stimulus artifacts. The mem-

brane potential begins to rise only after the end of the stimulus train and reaches its maximum within about 600 msec in the experiment shown. The delay in onset of the hyperpolarization is estimated to be about 80 msec from the beginning of the stimulation.

Also the decay of the hyperpolarization induced by endogenous ACh is very slow. A semilogarithmic plot of this phase of a hyperpolarization elicited by subthreshold stimulation (50 Hz, 1 sec) is shown in Fig. 8*A*. The open circles represent the measured time course, whereas the filled circles were obtained by correction for non-linear summation. The corrected time course is exponential with a time constant of about 3 sec ($3{\cdot}12 \pm 0{\cdot}14$ sec; mean $\pm$ S.E. of the mean). Fig. 8*B* shows the decay phase of the hyperpolarization measured in the same atrial cell after 10 min superfusion with

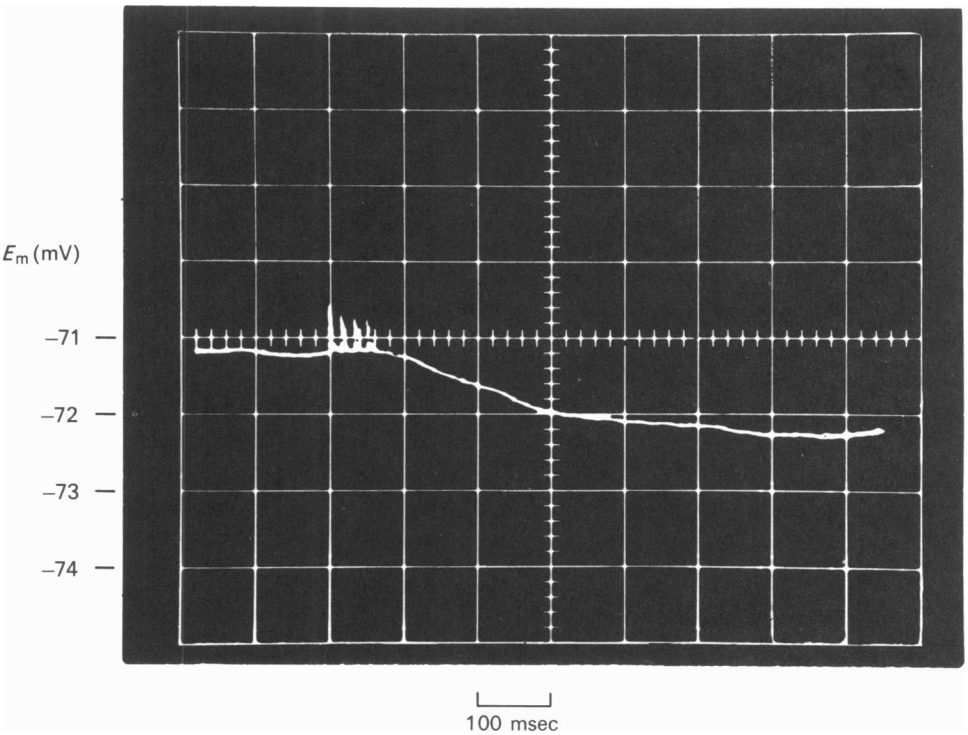

Fig. 7. Slow development of the hyperpolarization induced by ACh release. Four stimuli were applied with 20 msec intervals (recognizable by the stimulus artifact). The membrane potential is given by the ordinate, time mark is 100 msec. Note the latency between the beginning of the stimulus train and the development of the hyperpolarization.

a solution containing neostigmine (3×10^{-6} M.) If the enzymic hydrolysis of the released ACh is inhibited the time course of the potential decay is no longer exponential, and the hyperpolarization lasts for more than 2 min. In some experiments after stimulation in a neostigmine-containing solution the membrane potential remained more negative than before stimulation. Beyond this there was mostly a small increase in membrane potential after switching to the neostigmine–Tyrode solution. The hyperpolarization developed within 8–15 min after the solution change. This is

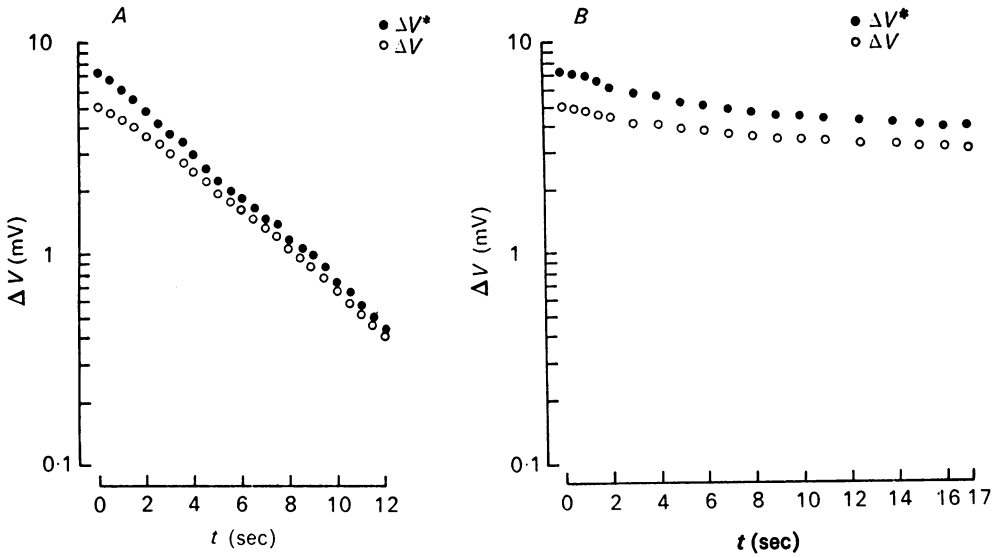

Fig. 8. Time course of the declining phase of the hyperpolarization induced by ACh release (stimulation: 50 Hz, 1 sec). Abscissa: time (sec); ordinate: hyperpolarization beyond the initial resting potential (mV, logarithmic scale). The open circles represent the measured values, the filled circles were obtained by correction for non-linear summation. *A*, control (normal Tyrode solution). *B*, in the presence of neostigmine (3×10^{-6} M).

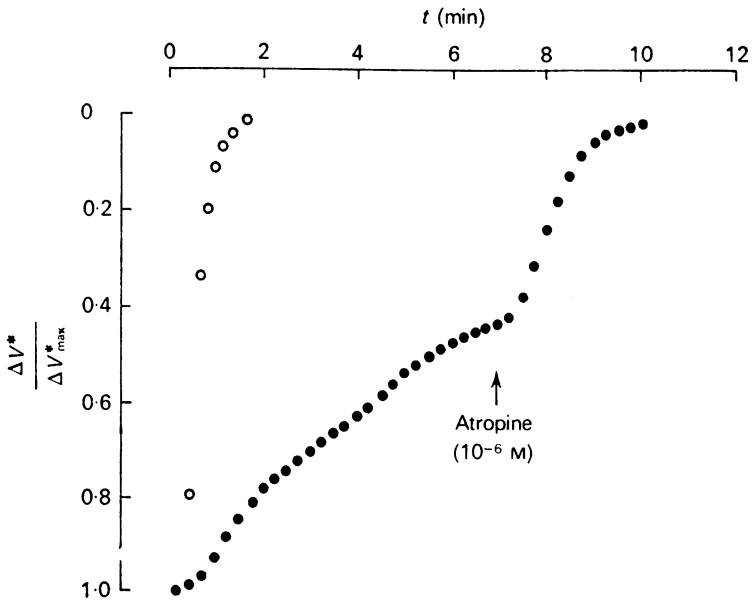

Fig. 9. Decay of the hyperpolarization induced by exogenous ACh (5×10^{-7} M). Abscissa: time (min); ordinate: normalized hyperpolarization with respect to the maximum ($\Delta V^*/\Delta V^*_{max}$, corrected values). At $t = 0$ solution was changed from ACh-containing to ACh-free Tyrode. Open circles, control; filled circles, in the presence of neostigmine (3×10^{-6} M). At the time indicated by arrow atropine (10^{-6} M) was added.

much slower than the response usually observed when exogenous ACh is applied. This slowly developing hyperpolarization is probably brought about by ACh released spontaneously from intracardiac parasympathetic nerve fibres (cf. Trautwein, Whalen & Grosse-Schulte, 1960).

The considerable prolongation of the stimulation-induced hyperpolarization demonstrates that under normal conditions hydrolysis by the ACh esterase is the only important physiological process terminating the action of ACh released from parasympathetic nerves, a contribution of diffusion to the limitation of the transmitter action is negligible. In the absence of enzymic destruction the ACh bound to the membrane is apparently not freely diffusible.

A corresponding result is obtained if exogenous ACh is washed out in the presence and absence of neostigmine. In Fig. 9 the potential decline after changing from ACh-containing (5×10^{-7} M) to ACh-free Tyrode solution is plotted. The open circles represent the normalized values with respect to the hyperpolarization maximum (non-linear summation taken into account) without neostigmine. The ACh action is reduced to 50% within 13 sec. If an identical ACh pulse is repeated after ACh esterase has been blocked by 3×10^{-6} M neostigmine the half-time of the decay is prolonged to 5 min (filled circles). At the moment indicated by the arrow atropine (10^{-6} M) was added. This reduces the half time to about 40 sec, wherein diffusion of the antagonist may still be rate limiting.

DISCUSSION

In quiescent guinea-pig atrial muscle cells considerable hyperpolarizations can be measured during superfusion with ACh-containing solutions or following stimulation of intracardiac parasympathetic nerves. Experiments carried out by Toda & West (1967*a*) on spontaneously beating rabbit atria with intact vagal innervation did not reveal substantial hyperpolarizations in auricular cells, whereas the diastolic potential in the sinoatrial node was distinctly increased during vagal stimulation. As the authors cited did not carry out measurements on ACh-sensitivity of nodal and atrial cells with exogenous ACh the question of whether rabbit atrial cells are less sensitive to ACh or whether this difference might be due to a less dense cholinergic innervation of the rabbit atrium cannot be answered here. It has to be mentioned, however, that also in the present experiments distinct hyperpolarizations could not be induced by the stimulation technique in all cells penetrated.

As the hyperpolarization is caused by an increase of the membrane permeability to potassium ions the diminution of the driving force for the K outward current during ACh application has to be taken into account when effects of different ACh concentrations are to be compared. If the measured hyperpolarizations are corrected for this non-linear summation the dependence of ACh action on the concentration applied can be described by a simple saturation relationship with an apparent dissociation constant for the 'ACh receptor complex' of about 10^{-6} M. This finding might be in contrast to results found in other cholinergic and non-cholingergic synapses where a co-operative action of transmitter molecules on the chemosensitive membrane has been described (Dudel, 1977; Peper, Dreyer & Müller, 1975). Our formal treatment of the dose–response curve implies a direct proportionality between

the fraction of ACh receptors occupied and the change in G_K. In nicotinic cholinergic transmission well founded models of ACh receptor interaction and induction of ionic channels have been proposed. However, little is known about the mechanisms involved in the ACh-dependent membrane conductance changes in muscarinic cholinergic transmission. In the slowest nicotinic synapses studied, the synaptic potential lasts no more than 30–100 msec (Blackman & Purves, 1969; Tosaka, Chichibu & Libet, 1968). The duration of muscarinic responses on the other hand is at least of the order of 0·5 sec (Del Castillo & Katz, 1955; Bennett, 1966; Creed & McDonald, 1975).

The slow time course of the stimulation-induced hyperpolarizations observed in this study is comparable to the inhibitory potential measured in the arrested frog's sinus after stimulation of the vagus nerve (Del Castillo & Katz, 1955) or after ionophoretic ACh application to the interatrial septum of the frog's heart (Purves, 1976). The conclusion of the latter report that the slow time course of the cholinergic response cannot be attributed solely to diffusion of ACh to the receptor sites appears to be obvious although non-linear summation has not been accounted for. It is in good agreement with our result, that the time to peak hyperpolarization is reached 0·6–1·2 sec after stimulation of post-ganglionic parasympathetic fibres (Figs. 4, 6, 7). Assuming diffusion from a point source the diffusion distance (z) would be given by:

$$z = \sqrt{[(6-4c)Dt_p]} \tag{3}$$

(Peper *et al.* 1975), where c is a constant depending on the geometrical arrangement of the receptors being 1 for receptors arranged in a plane. D is the diffusion constant and t_p denotes the time-to-peak of the response. Supposing a diffusion constant of $14{\cdot}8 \times 10^{-6}$ cm^2/sec at 36·5 °C (Krnjević & Mitchell, 1960) a time to peak of for example 1 sec would correspond to a diffusion distance of 54 μm. Although at present there is no substantiated hypothesis concerning the morphological site of ACh release from post-ganglionic parasympathetic nerves in heart, such a separation between the site of transmitter release and transmitter-sensitive membrane seems to be unlikely. Thus, the ACh receptor interaction or some step behind ACh binding to the receptor might be rate limiting for the development of the ACh-induced permeability change. This has recently been discussed to be a specific property of muscarinic cholinergic transmission (Bolton, 1976; Purves, 1974, 1976). In this context the participation of an intracellular second messenger (c-GMP) is considered in the muscarinic cholinergic ACh action (George, Polson, O'Toole & Goldberg, 1970).

The duration of the membrane response to ACh is affected by its inactivation by the ACh esterase (Figs. 8, 9). From the present data, however, it cannot be excluded that some reaction other than ACh cleavage might be rate limiting in the decay of the permeability change.

When ACh hydrolysis has been stopped the hyperpolarization following stimulation of intrinsic parasympathetic nerves is prolonged to 2 min or more. Even with enzymic ACh hydrolysis intact the response to stimulation of intracardiac parasympathetic fibres lasts for several seconds (see Figs. 6, 8*A*). This is much longer than usually observed in synaptic transmission, and is even longer than potential changes induced by muscarinic ACh action in other preparations studied (Bennett, 1966; Creed & McDonald, 1975; Gillespie, 1962; Ursillo, 1961). However, similar observations have recently been described in the heart of *necturus maculosus* (Hart-

zell, Kuffler, Stickgold & Yoshikami, 1977). This long-lasting, non-desensitizing response to small doses of the parasympathetic transmitter permits a continuous and graded regulation of cardiac function.

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