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The time course of muscarinic depolarization of guinea-pig myenteric neurones

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1 Intracellular recordings were made from neurones in the myenteric plexus of the guinea-pig ileum *in vitro*. Muscarinic depolarizations were evoked by brief (1–500 ms) ionophoretic applications of acetylcholine (ACh) or other agonists. Nicotinic responses to ACh evoked by the same ionophoretic pulse had short latencies and rapid rise times, indicating close proximity of the ionophoretic pipette to the neurone membrane.

2 The time course (duration several seconds) of the muscarinic depolarization was independent of the identity of the agonists applied (ACh, methacholine, carbachol, oxotremorine).

3 Hyoscine and barium were ejected onto the neurones by brief (30 ms–1 s) pressure pulses applied to micropipettes. Hyoscine applied immediately after ACh, during the latency and rising phase of the muscarinic depolarization, did not antagonize the response to ACh. The same application of hyoscine immediately prior to ACh caused complete antagonism.

4 Muscarinic depolarizations evoked by continuous application of ACh (by repeated ionophoresis or perfusion) were reversed by hyoscine. The time course of this reversal was similar to the decline of the muscarinic response following a single brief application of ACh.

5 Barium caused a depolarization similar to that produced by muscarinic agonists in its latency, time course and temperature sensitivity, and having the same reversal potential (−90 mV). These barium potentials were not affected by hyoscine.

6 It is suggested that neither diffusion of ACh to the receptors nor the kinetics of the agonist-receptor interaction contributes significantly to the latency and prolonged time course of the muscarinic depolarization.

Introduction

Muscarinic actions of acetylcholine (ACh) in various tissues occur after a significant latency (100–500 ms) and have a relatively slow time course (several seconds in duration) (Bolton, 1976; 1979; Hartzell *et al.*, 1977; Hill-Smith & Purves, 1978). These characteristics of muscarinic responses in other tissues have been variously suggested to result from (i) diffusion of ACh to the receptor, (ii) slow association and dissociation of ACh at the receptor, (iii) changing levels of an intracellular intermediate substance and/or (iv) inherent slowness of the ion channel affected (see Hartzell, 1981). In all the tissues in which careful studies of the time course have been made the response was an increase in membrane conductance, usually to potassium ions. However, activation of muscarinic receptors on S type (Type 1) neurones of the myenteric plexus leads to a long latency decrease in potassium conductance (Morita *et al.*, 1982; North & Tokimasa, 1982). It seemed of interest to examine the contribution of the above four factors to the time course of a muscarinic response mediated by a conductance decrease. Two main approaches were used. First, both agonists and antagonist were applied for periods of time which were brief compared to the muscarinic response itself. Second, the muscarinic response was mimicked by a brief application of barium ions, thereby bypassing the ACh-receptor interaction. Preliminary results have been reported (North & Tokimasa, 1983*a*).

Methods

Adult guinea-pigs (250–350 g) were heavily stunned and bled from the neck. The ileum was rapidly removed and placed in Krebs solution of the following

composition (mM): NaCl 117, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11; gassed with 5% CO_2 and 95%$_2$. When $BaCl_2$ was used $MgSO_4$ was substituted by $MgCl_2$. The myenteric plexus-longitudinal muscle preparation used was prepared as described by Nishi & North (1973). Single myenteric ganglia were immobilized by means of pinning through the closely adjacent muscle using stainless steel pins of 10 μm diameter. The ganglion was visualized by differential interference contrast optics (total magnification × 250). Intracellular recordings were made with glass microelectrodes containing 2 M KCl (d.c. resistance 30–100 MΩ), using a pre-amplifier (WP Instrument, M701) which permitted current injection. Values of resting potential were determined by sudden withdrawal of the microelectrode. A glass microelectrode (tip diameter 10–30 μm) filled with Krebs solution was used to stimulate neuronal elements within the ganglion. The tip of this electrode was positioned on the surface of the ganglion and moved to different positions around the impaled neurone under visual control. Single or repeated cathodal current pulses (100–500 μs duration) were passed through this electrode (focal stimulation).

Myenteric neurones were classified according to the criteria originally proposed by Nishi & North (1973) and Hirst *et al.* (1974). If the neurone responded to focal stimulation with a fast excitatory postsynaptic potential (e.p.s.p.) it was called an S (type 1) neurone. Those neurones which had no fast e.p.s.p. and which showed a prolonged afterhyperpolarization following a single soma action potential were called AH (type 2) neurones. The preparation was perfused (1–4 ml min^{-1}) with a preheated Krebs solution so that the temperature at the recording site was kept at 36°–37°C. Different solutions were applied to the preparation by means of a tap which changed the inflow to the tissue. The bath volume was 1–2 ml. Solutions of different ionic composition were maintained iso-osmotic by adjusting the sodium chloride concentration.

ACh, methacholine, carbachol and oxotremorine were applied by ionophoresis. The ionophoresis electrode contained ACh chloride (100 mM–1 M), methacholine bromide (1 M), carbachol (100 mM–1 M) or oxotremorine sesquifumarate (100 mM). The tip of such an electrode was positioned within 2–5 μm of the point of impalement and outward current pulses were used to eject the drugs. A small (2–5 nA) inward current was routinely applied to reduce diffusion.

ACh, hyoscine, barium chloride and neostigmine were applied by pressure ejection (puff) from an electrode of 10 μm tip diameter. The electrode contained either ACh chloride (10 mM), hyoscine hydrochloride (10 μM), barium chloride (4 mM) or neostigmine (10 mM). The tip of this electrode was positioned 20–100 μm (typically more than 50 μm) away from the point of impalement. Pressure pulses (30–70 kPa) were applied to this electrode by a stimulator controlled solenoid. Pulse durations were 20 ms to 1 s.

Temperature was monitored with a needle thermistor. Reduction in temperature by 10°C occurred within 5 min, and observations were made during the next 10 min period. Drugs used were ACh chloride (Sigma), oxotremorine sesquifumarate (Aldrich), hexamethonium bromide (Sigma), hyoscine hydrochloride (Sigma), methacholine bromide (Calbiochem), carbachol (Sigma) and tetrodotoxin (Sankyo).

Results

Time course of the muscarinic response

Passage of brief current pulses through the ACh ionophoresis pipette evoked rapid and slow depolarizations in S Type (Type 1) myenteric neurones. As we have previously described (Morita *et al.*, 1982; North & Tokimasa, 1982), the rapid depolarization was selectively blocked by hexamethonium and had a reversal potential near −10 mV, whereas the slow depolarization was selectively blocked by hyoscine and reversed polarity near −90 mV. We termed these responses nicotinic and muscarinic. When the ACh ionophoresis pipette was optimally positioned, the time course of the nicotinic response was similar to that of the fast e.p.s.p. (total duration 30–50 ms); even in these cases the muscarinic response had a latency of at least 100 ms.

This muscarinic response latency was not significantly reduced by increasing the amount of ACh applied or by changing the position of the tip of the iontophoresis pipette. The amplitude of the muscarinic response, and underlying conductance decrease, were dependent on the amount (current and/or duration) of ionophoretic application of ACh. However, the time constant of the rising phase (τ_1) and of the falling phase (τ_2) were not significantly dependent on the amount of ACh used to evoke the response. Carbachol, methacholine and oxotremorine also caused typical muscarinic depolarizations when applied by ionophoresis (Figure 1). Estimations of latency were difficult because of the need to use longer ejection pulses than in the case of ACh; however, the time constant of decay of the muscarinic response was the same whichever agonist was used to evoke it (ACh, carbachol, methacholine or oxotremorine) (Figure 1). These observations suggest that the decay of the muscarinic depolarization might not be determined by the rate at which the agonist dissociates from the receptor.

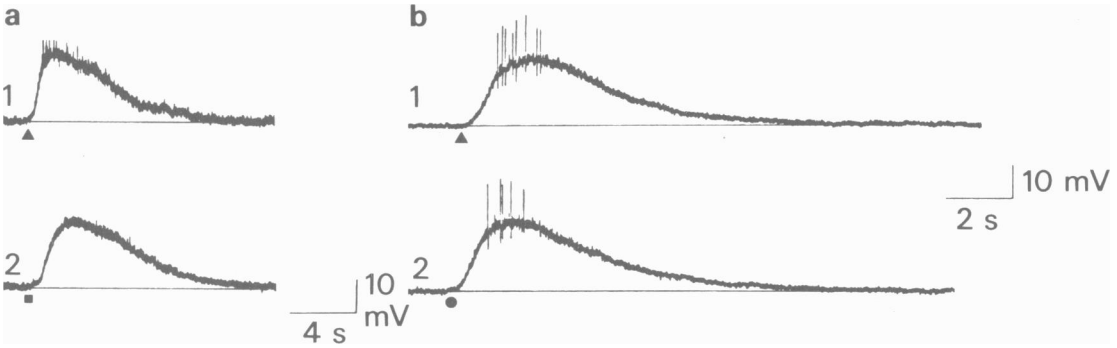

Figure 1 Comparison of the effects of different muscarinic agonists on the same neurone. (a) (1) Acetylcholine (ACh) ionophoresis compared with (2) methacholine ionophoresis. (b) A different neurone. (1) ACh ionophoresis compared with (2) carbachol ionophoresis. The rate of decline of the depolarization was the same for the different agonists.

Time course of hyoscine antagonism

We found previously that perfusion with as little as 1 nM hyoscine could reversibly abolish the muscarinic response to ACh ionophoresis (Morita *et al.*, 1982). In the present experiments we used applications of hyoscine which were brief in comparison to the muscarinic response. We addressed three questions.

Does the time required for diffusion of ACh account for the latency of the response? Unfortunately there is no accurate way to determine the arrival of ACh on the receptor, but the receptor occupancy by hyoscine can be detected by its blockade of the muscarinic response. Hyoscine blocked the muscarinic response when applied by pressure pulse as little as 200 ms prior to ACh. The latency of the ACh

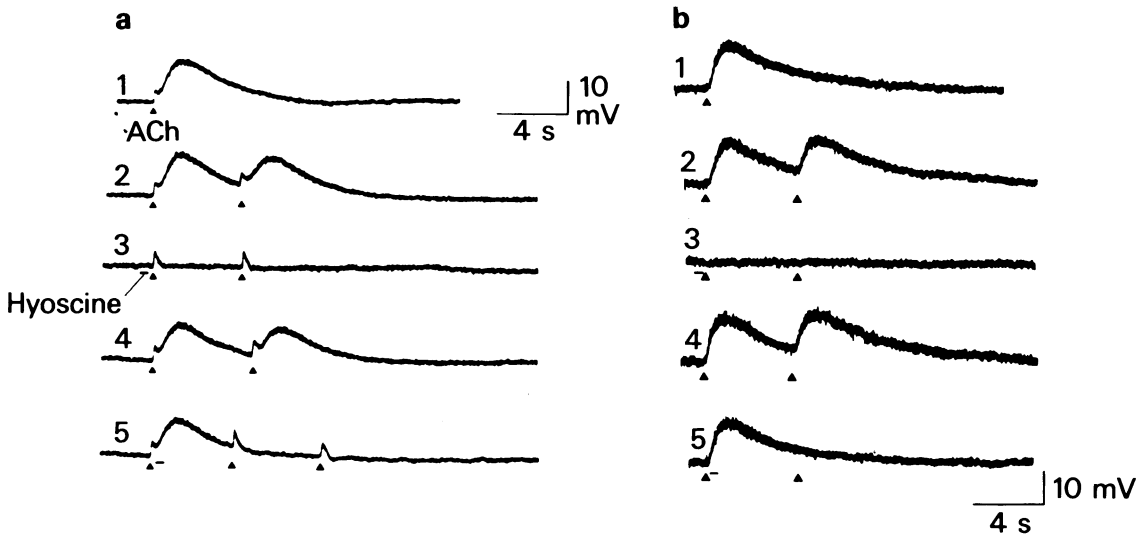

Figure 2 Hysocine is ineffective when applied after the onset of the muscarinic depolarization. (a) (1) Acetylcholine (ACh) ionophoresis (10 nA, 150 ms; ▲) evoked an early (nicotinic) depolarization followed by the muscarinic response. (2) Two applications of ACh at a 5 s interval evoked similar responses. (3) Hyoscine applied by pressure (500 ms solid bar) immediately prior to the first ACh application blocked both muscarinic potentials. Nicotinic responses remain. (4) 10 min later the responses had recovered. (5) Application of hyoscine (same as in 3) immediately after the first ACh pulse did not block the resulting muscarinic depolarization. However, the hyoscine appeared to occupy muscarinic receptors because the effects of a second and third application of ACh were completely blocked. (b) A similar experiment from another neurone in which the nicotinic response was eliminated by hexamethonium (200 μM). In this case ACh application was 20 nA, 20 ms.

action itself often exceeded 200 ms, and the ACh ionophoresis electrode was always much closer to the neurone than the hyoscine containing pipette. It seems unlikely that ACh diffuses much more slowly than hyoscine; therefore, the latency of the ACh response probably does not result from diffusion. After blockade of the muscarinic response by a brief pressure application of hyoscine, the response slowly recovered its initial amplitude. The time for complete recovery was 291 ± 32 s (mean ± s.e.mean, $n = 9$).

Does the latency and slow rising phase of the muscarinic response reflect the time course of receptor occupancy by ACh? We made brief applications of hyoscine immediately after ACh, during the latency and rising phase of the depolarization. Figure 2 illustrates the results of two such experiments. Hyoscine did not affect the muscarinic response when it was applied after ACh, although the same application immediately prior to ACh completely blocked the muscarinic depolarization. This indicates that ACh has already arrived on the receptor even during the latent period.

Does the slow decline of the response reflect dissociation of ACh from the receptor? If ACh continues to occupy the receptors throughout the muscarinic depolarization, then it should prevent their occupancy by hyoscine. The experiment illustrated in Figure 2 suggests that this is unlikely. Hyoscine applied during the rising phase of the ACh depolarization apparently had access to the muscarinic receptors, because a subsequent application of ACh a few seconds later was completely without effect. The effectiveness of ACh ionophoresis in producing a depolarization slowly recovered over the same period of time as that observed when hyoscine was applied prior to ACh. The two equal responses to ACh probably result from occupancy of the same population of receptors, since the ACh is applied essentially instantaneously from a point source close to the cell. However, this assumption is not necessary. If hyoscine occupies only those receptors 'spared' by ACh then the original receptor pool should still be available to respond to the second application of ACh. (In a few experiments we found the same result with pressure application of neostigmine. When applied prior to ACh it increased the amplitude and duration of the muscarinic response, but when applied immediately after the ACh it was without effect.)

In other experiments, ACh was applied by perfusion or by continuous ionophoresis until the muscarinic depolarization reached a steady state. Presumably association and dissociation are now occurring at equal rates and there is a constant level of occupancy. Figure 3 shows that application of hyoscine terminated the ACh depolarization and that the rate of decline of the depolarization was the same as

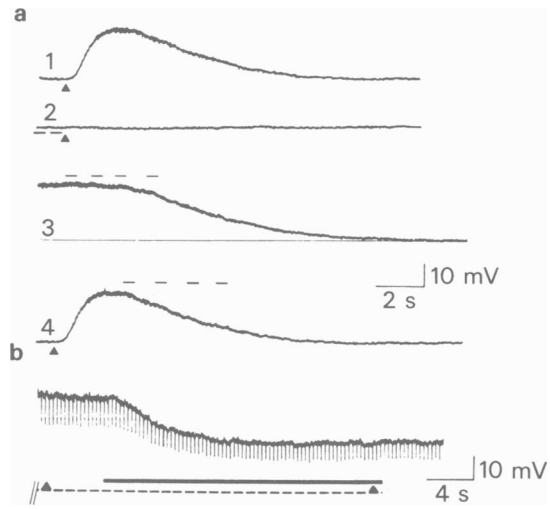

Figure 3 Time course of reversal of muscarinic depolarization is independent of method of application. (a) (1) Control muscarinic acetycholine (ACh) potential (ionophoresis was 10 nA, 90 ms; indicated by ▲). (2) Hyoscine (two pressure pulses of 500 ms duration) completely antagonized the muscarinic response when it was applied just before ACh. The muscarinic ACh potential recovered to control within 5 min (not shown). (3) The perfusing solution contained ACh (3 μM) and the record shows the membrane potential when it had reached its steady-state depolarization (22 mV) after approximately 3 min perfusion. While ACh continued to perfuse the preparation, hyoscine was applied by four applications of pressure (500 ms, solid bars). Reversal of the muscarinic depolarization began within 1.5 s of hyoscine application, and continued with a time constant of 4.5 s (same value as in 1). (4) 30 min after 3. The muscarinic ACh potential had completely recovered (same conditions of ionophoresis as in 1). Hyoscine was applied at the peak of the muscarinic ACh potential. The conditions of hyoscine application were as in (3). The time course was the same as in (1) and (3). Hexamethonium (200 μM) was present throughout. (b) ACh was applied by constant current ionophoresis (20 nA, broken line) until a steady state depolarization (19 mV) was reached. ACh application began 29 s before recording shown. Hyoscine was then applied by constant pressure to the puff pipette (solid bar). The muscarinic depolarization was reversed with a time constant of 5 s. Downward deflections are electotonic potentials, which show the reversal of the muscarinic conductance decrease. Hexamethonium (200 μM) present.

that following a brief ionophoretic application of ACh. The decline of the depolarization from its steady state level might indicate the rate of dissociation of ACh from its receptor, or the rate of decline of other event(s) further down the occupancy response chain. The similarity of the rate to that observed with

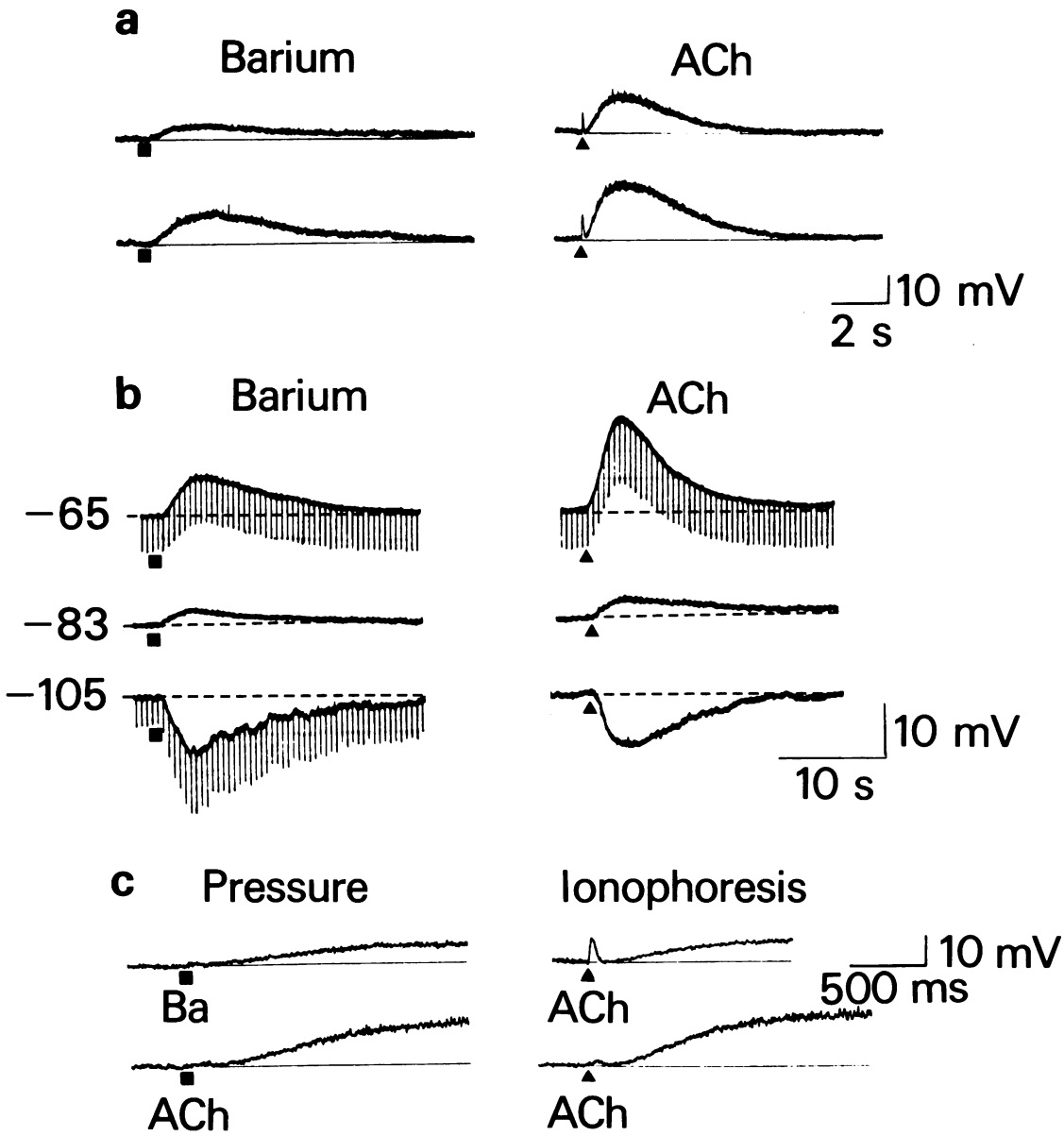

Figure 4 Comparison of barium and acetylcholine (ACh) depolarizations. (a) Barium depolarization was closely similar in time course to the muscarinic potential in the same cell. Depolarizations were evoked by barium (left) and ACh (right). Barium pressure applications (squares) were 50 and 200 ms. ACh ionophoretic applications (▲) were 10 nA for 10 and 30 ms. ACh evoked both nicotinic and muscarinic responses. (b) Barium potential and muscarinic potential reverse polarity at the same membrane potential. ACh ionophoresis current was 10 nA, 500 ms (▲). Barium pressure application was 200 ms (■). The muscarinic ACh potential and barium potential reversed polarities at − 88 mV and − 85 mV, respectively. Hexamethonium (200 μM) present. (c) Latency of barium response. Left side shows comparison between effects of barium (top trace) and ACh (bottom trace) both applied by 30 ms pressure pulses from pipettes positioned at equal distances (50 μm) from the cell. The latency (about 200 ms) was apparent in both cases. Right side shows similar responses evoked by ionophoresis of ACh from a third pipette positioned 3 μm from the cell (top trace: 10 nA, 10 ms; bottom trace: 10 nA, 30 ms, in hexamethonium (200 μM)). Even when applied very close to the cell, the latency of the muscarinic response was not reduced.

transient ionophoretic application leads one to infer that this other event determines the rate. In other words, the decay phase of the muscarinic response contains little information on the dissociation of ACh from its receptor.

Barium depolarizations are not mediated by muscarinic receptors

Barium ions are well known to mimic the muscarinic actions of ACh in central (Krnjevic *et al.*, 1971) and autonomic (Constanti *et al.*, 1981) neurones. We therefore made a comparison of the time course of the barium action with that of ACh. It was first necessary to show that barium effects were independent of the muscarinic receptor.

Barium was applied by brief (30–200 ms) applications of pressure to a pipette with its tip positioned 20–50 μm from the impaled neurone. Closer apposition was usually not possible because of leakage from the 'puff' pipette. Barium depolarizations were closely similar in all respects to those caused by ACh (Figure 4a), but they were not affected by hexamethonium (200 μM), hyoscine (1 μM) or tetrodotoxin (300 nM). Barium depolarizations were reversibly blocked by perfusion with cobalt (2 mM), suggesting that the barium ions might be passing through membrane calcium channels and producing their effects at the inner surface of the membrane. The actual concentration of barium required to depolarize the myenteric neurones was determined by perfusion experiments. The minimally effective concentration was 10 μM, the depolarization caused by 100 μM was approximately 7 mV, and by 1 mM more than 20 mV ($n = 3$).

The barium depolarization was associated with a conductance decrease and reversed its polarity at -87.5 ± 2.7 mV (mean ± s.e.mean, $n = 4$) (Figure 4). In these four neurones, the muscarinic ACh potential reversed at -88.3 ± 1.6 mV. Thus, barium closes potassium channels which are essentially voltage-independent under our experimental conditions, and which cannot be distinguished from those affected by ACh. However, the lack of effect of hyoscine showed that barium did not exert its effects through the muscarinic receptor. Thus, the contribution of the ACh-receptor interaction to the kinetics of the muscarinic response might be approached by comparison with the kinetics of the barium response.

Latency It was difficult to determine accurately the latency of the barium depolarization, because leakage from the pipette often depolarized the neurone when the tip was brought within 20 μm. Therefore, two pipettes were used for pressure ejection, one containing ACh and the other barium. These were held at a distance of about 20 μm, and a third pipette was positioned for ACh ionophoresis with its tip 3–5 μm from the neurone. Figure 4c shows the result of such an experiment. The response to barium appeared to be associated with a latency similar to that observed for ACh, applied either by pressure or by ionophoresis. More typically, the barium pipette was kept 50–100 μm from the impaled neurone and the latency to the onset of depolarization was between 200 and 1000 ms.

Time course The barium depolarization was closely similar in time course to the ACh potential in the same neurone (Figure 4). The overall response could be described by $y = A[\exp(-t/\tau_2, + \exp(-t/\tau_1)]$ where y is the depolarization in mV, A is a scaling constant and τ_2 and τ_1 are the time constants of the falling and rising phase. Values for τ_1 and τ_2 were 784 ± 34 ms and 2530 ± 23 ms (mean ± s.e. of mean, $n = 27$), which were similar to those reported for the muscarinic ACh potential (Morita *et al.*, 1982; North & Tokimasa, 1982).

Effects of coooling

If barium and ACh operate by a similar intracellular mechanism, they might be expected to have the same sensitivity to temperature changes. Reduction of the temperature from 36 to 26°C prolonged the latency, reduced the peak amplitude, and increased the time constants τ_1 and τ_2 of both the muscarinic and the barium depolarization. The magnitudes of these effects were not different, the Q_{10} (36°C *vs* 26°C) values for τ_1 being 3.03 ± 0.45 (muscarinic, $n = 6$) vs 2.50 ± 0.5 (barium, $n = 3$) and for τ_2 2.97 ± 0.34 (muscarinic, $n = 6$) vs 3.10 ± 0.3 (barium, $n = 3$). Quantitative measures of latency of the barium response were difficult for the reasons mentioned above. However, even when the delay was already several hundred ms, it was markedly prolonged by temperature reduction. These effects of cooling on the muscarinic responses are similar to those described by others (Hartzell *et al.*, 1977; Hill-Smith & Purves, 1978; Morita *et al.*, 1982).

Discussion

The results suggest that the long latency and slow decay of the effects of muscarinic agonists are likely to be due to events beyond the initial interaction of agonist with the muscarinic receptor (i.e. to intracellular events or to the behaviour of membrane channels). The strongest evidence in support of this view is that barium produced a slow depolarization and potassium conductance decrease with the same response kinetics as ACh, and this effect of barium was independent of the muscarinic receptor. The potas-

sium channels closed by barium and ACh appear to have the same characteristics, and both are sensitive to the intracellular calcium concentration (North & Tokimasa, 1983b).

The present findings strongly suggest that diffusion and agonist-receptor interaction are not major contributors to the time course of the muscarinic depolarization. This may be different from the situation in which muscarinic agonists increase potassium conductance. In the heart, the kinetics have been attributed to diffusion coupled with a low density of receptors (Trautwein *et al.*, 1981); and in mudpuppy cardiac ganglion cells the decay of the response is thought to reflect the dissociation of the agonist (Hartzell, 1981). Our findings do not allow us to conclude whether channel lifetimes themselves, or the availability of an intracellular intermediate are the main determinants of the time course. There is an interesting correlation between the rate at which the depolarization occurs during the muscarinic response and the time course of decline in potassium conductance which has been transiently increased by a brief entry of calcium ions into the cell (North & Tokimasa, 1983b). It is possible that a movement of calcium ions between two or more intracellular compartments, one of which determines the membrane potassium conductance, could underlie the kinetics of the response. Further experiments are needed to test such a proposal quantitatively.

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