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ACTIONS OF NORADRENALINE AND ACETYLCHOLINE ON SYMPATHETIC GANGLION CELLS

BY H. KOBAYASHI* AND B. LIBET

From the Department of Physiology, University of California School of Medicine, San Francisco 94122, U.S.A.

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SUMMARY

1. The responses of the post-synaptic membrane of sympathetic ganglion cells to noradrenaline (NA) and to acetylcholine (ACh) were studied in relation to the slow inhibitory post-synaptic potential (S-IPSP) and slow excitatory one (S-EPSP) respectively.

2. NA produced an hyperpolarization of about 4 mV in cells of rabbit superior cervical ganglia.

3. The hyperpolarizing response to NA was not accompanied by any detectable change in membrane resistance, and it was depressed by conditioning depolarization.

4. NA also depressed all the post-synaptic potentials, presumably by an action on presynaptic function.

5. ACh produced a large depolarization in ganglion cells of rabbit and of frog (paravertebral) ganglia, which was accompanied by a large decrease in membrane resistance.

6. When ACh was applied during nicotinic blockade, achieved with high concentration of nicotine (frog ganglia) or D-tubocurarine (rabbit ganglia), it still produced a considerable depolarization. This response could be blocked by atropine, and is presumably a muscarinic type of action.

7. The muscarinic-ACh response was not accompanied by a decrease in membrane resistance. Instead, the frog ganglion cells exhibited increased resistances of up to more than twice the resting value during both the muscarinic-ACh depolarization and the S-EPSP.

8. The muscarinic-ACh depolarization and the S-EPSP were both depressed by conditioning hyperpolarization (in nicotinized frog cells). An initial hyperpolarizing phase now appeared in both of these responses.

9. It is concluded that the hyperpolarizing response to NA and the depolarizing response to muscarinic-ACh action are not generated by

* Present address: Department of Physiology, Tokyo Medical College, Shinjuku-ku, Tokyo, Japan.

increases in ionic mobilities in the post-synaptic membrane; and that these two responses are produced by the same electrogenic mechanisms which underlie the S-IPSP and the S-EPSP respectively.

INTRODUCTION

The synaptic transmitters which act on sympathetic ganglion cells to elicit the slow inhibitory post-synaptic potential (S-IPSP) and the slow excitatory post-synaptic potential (S-EPSP) have been postulated to be, respectively, an adrenergic substance and acetylcholine (ACh) acting muscarinically (Eccles & Libet, 1961; Libet, 1964, 1965, 1967; Tosaka, Chichibu & Libet, 1968). One of the requirements to be fulfilled by a substance before it can be identified as a given synaptic transmitter is that it must produce the same changes in the post-synaptic membrane as those associated with the orthodromically generated PSP itself. The membrane changes involved in both of these slow PSPs have been found to be different in principle from those involved in the better known, fast PSPs (Kobayashi & Libet, 1968); with the slow PSPs there were no increases in ionic conductance of the membrane, and changes in the initial level of membrane potential altered these responses in ways that are different from those predicted from the ionic mobility hypothesis for the fast PSPs (e.g. Eccles, 1964). Uniquely discriminative characteristics are thus provided with which to test the actions of the alleged transmitters. Earlier reports have indicated that adrenergic substances can have the postulated hyperpolarizing action on ganglion cells (Lundberg, 1952; DeGroat & Volle, 1966); and that ACh can have the postulated muscarinic depolarizing action (Takeshige & Volle, 1964; Koketsu, Nishi & Soeda, 1968); these actions were studied further at the single cell level, in both amphibian and mammalian ganglia, in the course of the present study.

In addition, the present investigation has an interest beyond the question of the mediation of the slow PSPs. The actions of NA and of ACh (acting muscarinically) have general physiological and pharmacological significance. It is important to achieve further clarification of the nature of the post-synaptic changes which are involved in these actions where it is possible to do so. Preliminary reports of the present work have been presented (Libet & Kobayashi, 1969*a*, *b*).

METHODS

Superior cervical ganglia of young rabbits and the posterior ganglia in the paravertebral sympathetic chain of the frog were employed in this work. In the frog ganglia only B neurones, i.e. ganglion cells innervated by a preganglionic B fibre and generally giving rise to a post-ganglionic B fibre, were adequately impaled for periods sufficiently long to carry out the present experiments. Such B neurones

characteristically can exhibit an EPSP and a S-EPSP but not a S-IPSP (Tosaka *et al.* 1968). This makes them especially useful for studying the responses to ACh, in relation to the properties of the S-EPSP, without the possible contaminating effects of the inhibitory response to ACh (Eccles & Libet, 1961; Libet, 1967; Takeshige & Volle, 1964).

The techniques employed for intracellular study of sympathetic ganglion cells of frog and rabbit have already been described (Tosaka *et al.* 1968; Libet & Tosaka, 1969; Kobayashi & Libet, 1968). Micro-electrodes were generally filled with 0·6 M-K_2SO_4 and had a resistance of about 100 MΩ, as measured with 60 Hz (30–50 MΩ when filled with 3 M-KCl). Resting membrane potentials were about 45–60 mV, averaging about 50 mV for ganglion cells of both rabbit and frog. These values were similar to those found in the earlier studies in this laboratory.

Pulses of constant current for measuring membrane resistance, as well as steady current for polarizing the cell membrane, were delivered to the recording electrode by means of a bridge arrangement (Ginsborg & Guerrero, 1964). With the electrode tip in the extracellular space before penetrating a cell, the bridge was balanced so that the applied currents produced minimum deflexions of the base line; after an impaled cell had been studied and the electrode withdrawn from the cell, the degree of balance was usually re-checked. Our primary aim was to detect relative changes in membrane resistance that might be produced during a response rather than to measure absolute values. Earlier studies have shown that sympathetic ganglion cells do not exhibit appreciable rectification with changes in membrane potential over the range employed in this work (Kobayashi & Libet, 1968; Nishi & Koketsu, 1960).

Tests of responses of the cell to a substance were made with the micro-electrode in place intracellularly. A small volume of solution (0·3 ml.) containing the substance was introduced slowly (over a few sec) into the open end of the upright gassing tube in the 30 ml. chamber (see Libet & Tosaka, 1969); this could be done without dislodging the micro-electrode from the cell. Similar additions of a dye solution showed that the convection currents, created by the rising bubbles in this tube, rapidly swept the substances into the main chamber and achieved apparent homogeneity of the final concentrations in the chamber within a few seconds. Whenever mentioned, concentrations of the added substances refer to final concentrations in the chamber; these were of course 1% of the concentrations of the substances in the solutions that were added. The testing of substances by such bulk additions to the chamber containing the whole ganglion made it impossble to test a given cell with more than one addition of a substance. All drugs used were obtained in the relatively pure state (i.e. without additives), and were available as the following salts: L-noradrenaline bitartrate (NA), L-adrenaline-HCl, isopropylnoradrenaline-HCl (isoproterenol), acetylcholine-Cl (ACh), acetyl-β-methylcholine-Cl (methacholine or MCh), atropine sulphate, harmine-HCl, D-tubocurarine-Cl (DTC), dihydro-β-erythroidine-HBr.

RESULTS

Adrenergic actions

NA could elicit an hyperpolarizing response in the *mammalian* ganglion cells. However, it only did so with some consistency when the final concentration was sufficiently great (50 μg/ml., i.e. $1{\cdot}5 \pm 10^{-4}$ M) and when a monoamine-oxidase inhibitor (harmine or eutonyl) was previously added. Without an inhibitor no responses were obtained with additions of Na at 1 and 10 μg/ml. in six cells. With an inhibitor present, NA at 1 and 10 μg/ml.

elicited hyperpolarizations of 2–8 mV in eight cells (av. 4·8 mV) and no response in four other cells. Table 1*A* presents the individual responses of twelve cells tested under the finally standardized conditions, with NA added at 50 μg/ml. and in the presence of harmine (5 μg/ml.). In this group, hyperpolarizations of 2·5–6 mV (mean = 4·3 mV) occurred in eleven cells and no response in one cell. The hyperpolarizing response started within about 5 sec after the injection of NA into the chamber and was sustained, for at least some minutes of observation (Fig. 1*b*). The amplitudes of the responses bore no direct relationship to the values of resting membrane potential in the range exhibited by these cells (Table 1*A*), although deliberate alterations in the initial resting potential, by conditioning polarizing currents, can alter the amplitude of the response to NA (see below).

TABLE 1. Hyperpolarizing response of membrane potential to noradrenaline. Rabbits ganglia, K_2SO_4 micro-electrodes; final concentration of NA, 50 μg/ml.; harmine (5 μg/ml.) present*

A No conditioning polarization			*B* During conditioning depolarization of *ca.* 20 mV		
Cell	Initial resting potential (mV)	Maximum hyperpolarization (mV)	Cell	Initial resting potential (mV)	Maximum hyperpolarization (mV)
8–9–1	50	6	10–22–2	58	5
8–9–2	50	5	10–22–5	52	0·7
10–22–1	41	2·5	10–23–3	50	0·5
10–22–7	55	3·5	10–23–4	50	0
10–23–2	56	3	10–25–4	62	1·5
10–23–5	50	4	10–25–6	48	2·8
10–25–1	50	0	10–25–8	46	0
10–25–2	58	6	10–29–1	48	0
10–25–5	52	5	10–30–4	46	0
10–29–2	48	2·8	10–30–6	55	0
10–29–3	50	3·7	10–30–7	50	0·6
10–30–2	49	5·8			

* Partial curarization with DTC (10–15 μg/ml.) was also in force in all cases (but with nicotine, 10^{-4} M, instead of DTC, for cells 10–29–2, 10–29–3, 10–29–1); this was done to permit evaluation of the S-IPSP response to orthodromic impulses in these same cells.

Adrenaline (harmine present) elicited a 4 mV hyperpolarization at 100 μg/ml. in one rabbit cell, and no response at 1 μg/ml. in another. It was not tested more extensively because it has been found, with ganglionic surface recordings, to elicit both hyperpolarizing and depolarizing responses, following close intra-arterial injections (DeGroat & Volle, 1966) and also after intravenous injections (B. Libet, unpublished data). Such a mixture of effects could result in an absence of any

significant net response. *Isoproterenol* has been reported to produce a purely surface negative response after close intra-arterial injection to the superior cervical ganglion of the cat (DeGroat & Volle, 1966); nevertheless, it produced no intracellular response in two rabbit ganglion cells when tested at 100 μg/ml. (harmine present).

In the *amphibian* ganglion cells adrenaline gave generally negative results when added to a chamber similar to the one employed for the rabbit ganglion. In sixteen cells tested with adrenaline at 1–10 μg/ml. (but no harmine present) only one cell exhibited any significant response, a 7 mV hyperpolarization. Even with adrenaline at 50 μg/ml., and harmine present (5 μg/ml.), no response was elicited in five cells so tested; in two of these cells the adrenaline was added during the application of conditioning hyperpolarizing current which increased the membrane potential difference by 20 mV, a procedure that augments the S-IPSP response (Kobayashi & Libet, 1968; Nishi & Koketsu, 1968*b*). NA at 1 and 10 μg/ml. also gave negative results. (It may be noted that adrenaline, rather than NA, is apparently the transmitter for sympathetic effector actions in amphibians; see Falck, Häggendal & Owman, 1963; and MacLean & Burnstock, 1966.)

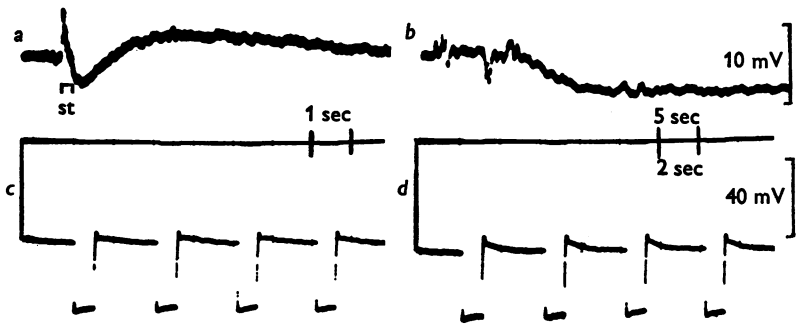

Fig. 1. Response to NA in cells of rabbit's superior cervical ganglion, with *d*TC (15 μg/ml.) and harmine present (5 μg/ml. (*a*) Response to brief train of stimuli, 40/sec (see bar st) to preganglionic nerve; note the summated fast EPSPs during stimulation, with superimposed S-IPSP developing during and after train, followed by the longer lasting S-EPSP. (*b*) Same cell as in (*a*), in resting state, NA added to chamber (to give 50 μg/ml.) as indicated by the mechanical artifacts; prolonged hyperpolarization develops several seconds later. (*c*) Another cell, in resting state, with hyperpolarizing pulses of constant current to measure resistance, set off from a base line by the value of the total transmembrane potential. (*d*) Same as in (*c*), but taken 4 min after addition of NA which produced a steady hyperpolarizing response of about 6 mV in this case. Time calibration in (*d*) holds for (*c*) and (*d*); voltage calibration in (*b*) for (*a*) and (*b*), in (*d*) for (*c*) and (*d*), downward signifying negative polarity of intracellular electrode.

Membrane resistance (r_m). No appreciable change in ohmic resistance of the membrane was detected during the hyperpolarizing action of NA on mammalian ganglion cells. An example may be seen in Fig. 1*c*, *d*, showing an absence of change in amplitude of the transmembrane p.d.s produced by the long-lasting pulses of constant current. A similar absence of change in r_m had also been found with the S-IPSP (Kobayashi & Libet, 1968).

Conditioning depolarization. To compare the effects of conditioning polarization on the S-IPSP with those on the response to NA, both responses were tested during passage of a constant depolarizing current which lowered the initial resting potential by about 20 mV. This condition has been found (Kobayashi & Libet, 1968) to depress the S-IPSP in rabbit ganglion cells almost completely, as may be seen in Fig. 2*a–c*. The responses to NA that were obtained during such conditioning depolarization were also very small (e.g. Fig. 2*d*, to be compared with Fig. 1*b*). The group of individual responses for all cells tested with NA during conditioning depolarization (Table 1*B*) may be compared with the control group of

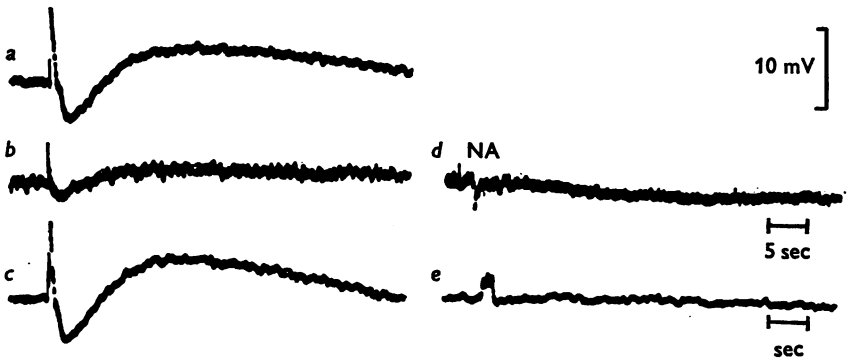

Fig. 2. Conditioning depolarization on a rabbit's ganglion cell with DTC and harmine present, as in Fig. 1. Responses to supramaximal preganglionic stimulation, 0·3 sec train at 40/sec, in (*a*) unconditioned cell with resting potential of 62 mV; in (*b*) during conditioning depolarization of about 20 mV; in (*c*) shortly after conditioning depolarization was turned off; in (*e*), after addition of NA (50 μg/ml.), but with no conditioning polarization on. (*d*) Response to NA added during conditioning depolarization of 20 mV, to be compared to orthodromic response obtained during similar conditioning in (*b*), and to NA response (in Fig. 1*b*) obtained in an unconditioned cell. Time calibration in (*e*), for (*a*), (*b*), (*c*), as well; voltage calibration same for all.

responses for cells tested in the unconditioned state (Table 1*A*). The average response of the conditioned group is much lower in amplitude (1 mV), with eight cells out of the eleven tested exhibiting virtually no response. In the only two conditioned cells (nos. 10–22–2 and 10–25–6 in Table 1*B*) that exhibited relatively 'normal' amplitudes (5 and 2·8 mV) of response to NA, the S-IPSP response was also much less effectively depressed by the conditioning depolarization than it was in the other cells. If these two cells are therefore removed from the list in Table 1*B*, the mean value becomes 0·4 mV. Calculations of the S.E. of the difference of the means of the NA responses (for control and depolarized groups) gave

a value of 0·7 with all cells included, or of 0·6 with cells 10–22–2 and 10–25–6 in Table 1*B* excluded. The *t* test for significance of the means gives a *P* considerably < 0·001, even with all cells included. There is therefore little doubt that the hyperpolarizing response to NA was markedly depressed by conditioning depolarization, particularly in those cells in which the S-IPSP was also so depressed.

Effects of NA on post-synaptic responses. When the post-synaptic responses to a brief train of preganglionic impulses were re-tested after the addition of NA (50 μg/ml.) to the chamber, all PSPs were found to be markedly depressed. However, the S-IPSP and S-EPSP were generally more depressed than the summated (fast) EPSPs (e.g. Fig. 2*e* vs. 2*c*). The effects of NA on the PSPs were apparently reversible; this could be inferred from the fact that after testing a cell by addition of NA to the chamber and subsequently washing out the chamber with fresh Ringer solution, the next cell impaled consistently exhibited all PSPs in the normal range of amplitudes.

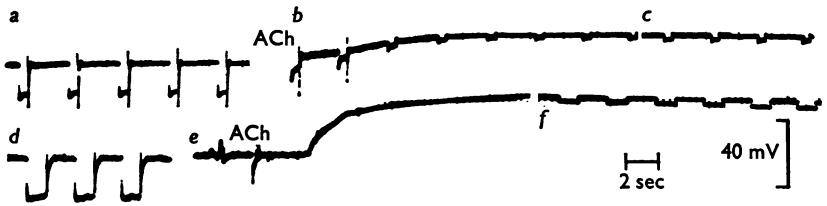

Fig. 3. ACh action in absence of nicotinic blocking agent. (*a*) Frog ganglion cell, with resistance-testing pulses on resting potential of 55 mV, eserine (2 μg/ml.) present; (*b*) same, but recording starts some seconds after adding ACh (10^{-3} M); and (*c*), about 20 sec after (*b*). (*d*) Rabbit ganglion cell, resting potential of 50 mV, eserine (2 μg/ml.) present; (*e*), same, without the testing pulses, ACh (10^{-3} M) added as indicated; (*f*) continues a few seconds after (*e*), with superimposed resistance testing pulses of same constant current as in (*d*).

Action of ACh in absence of nicotinic blocking agents

The depolarizing action of ACh on single cells of frog ganglia has been described by Ginsborg & Guerrero (1964), who also demonstrated that membrane resistance was markedly reduced during such action. Our findings with frog ganglion cells are similar to theirs (e.g. Fig. 3*a* to *c*). The values of the maximal depolarizing responses for the different cells tested are collected in Table 2*A*. Mammalian ganglion cells were also found to respond in a similar fashion (Fig. 3*d* to *f*).

Action of ACh in presence of nicotinic blocking agents

It has been found that the S-EPSP (known earlier as the 'late-negative' or LN wave in recordings at the ganglionic surface) is relatively insensitive

to curarizing agents in rabbit ganglia (Eccles, 1952; Libet, 1964, 1967) and, at least to weaker concentrations of these agents, in frog ganglia (Libet, Chichibu & Tosaka, 1968). Also, nicotine (about 10^{-4} M) completely blocks the (fast) EPSP even after the initial depolarization it produces has sub-

TABLE 2

A Depolarizing responses to ACh (frog ganglion cells)

Cell	Electrode solution	Final concn. of ACh (mole/l.)	Eserine (μg/ml.)	Atropine (μg/ml.)	Initial resting potential (mV)	Maximum depolarization (mV)
3–26–3	KCl	10^{-5}	—	—	56	10
3–26–5	KCl	10^{-4}	—	—	54	17
3–26–6	KCl	10^{-4}	—	1	44	3
3–26–7	KCl	10^{-4}	—	1	65	20
3–28–2	KCl	10^{-4}	—	—	48	12
3–28–3	KCl	10^{-4}	—	—	36	8
3–28–4	KCl	10^{-4}	—	—	54	14
7–30–1	K_2SO_4	10^{-4}	2	—	53	7
11–6–1	K_2SO_4	10^{-3}	2	—	55	16
3–6–1*	KCl	10^{-3}	1	—	50	28
3–6–2*	KCl	10^{-4}	1	—	44	10
	B Responses to ACh in presence of nicotine†					
9–17–1	K_2SO_4	10^{-3}	2	—	50	14
9–17–4	K_2SO_4	10^{-3}	2	—	56	8
9–17–5	K_2SO_4	10^{-3}	2	20	60	0
9–17–6	K_2SO_4	10^{-3}	2	20	50	0
11–6–2	K_2SO_4	10^{-3}	2	—	48	11
11–6–6	K_2SO_4	10^{-3}	2	—	50	9
11–7–3	K_2SO_4	10^{-3}	—	—	46	3
11–8–1	K_2SO_4	10^{-3}	—	—	45	2

* Dihydro-β-erythroidine present at 1 μg/ml. This weak concentration of curarizing agent only partially depresses the fast EPSPs. A large fraction of the nicotinic response to ACh, especially when added in these high concentrations, undoubtedly remained unblocked here.

† Nicotine added at least 30 min before ACh tests; concentration of nicotine 6×10^{-5} M in cells 9–17–1, 4, 5, 6, and 10^{-4} in the last four cells.

sided (Ginsborg & Guerrero, 1964), but the S-EPSP can still be elicited at this time (Nishi & Koketsu, 1968*a*; see Fig. 4*d*). On the other hand, the S-EPSP is highly sensitive to blockade by atropine at about 0·1 μg/ml., even though much higher concentrations of this drug (10–20 μg/ml.) had little or no effect on the (fast) EPSP (Eccles & Libet, 1961; Libet *et al.* 1968; Tosaka *et al.* 1968).

It was demonstrated in the present study that ACh could still elicit a substantial depolarization in ganglion cells which have been subjected to

a strong concentration of a nicotinic blocking agent, but that such a depolarization has different characteristics from that elicited in the absence of such a blocking agent. The maximal values for depolarizing responses of *frog ganglion cells* to ACh in the presence of nicotine are given in Table 2*B*, and example recordings are shown in Fig. 4*a* to *c*. The mean peak value for the four cells with eserine (but not atropine) present was 10·5 mV. Atropine appeared to block the response of the nicotinized cells to ACh (see also Koketsu *et al.* 1968). *Rabbit ganglion cells* responded to

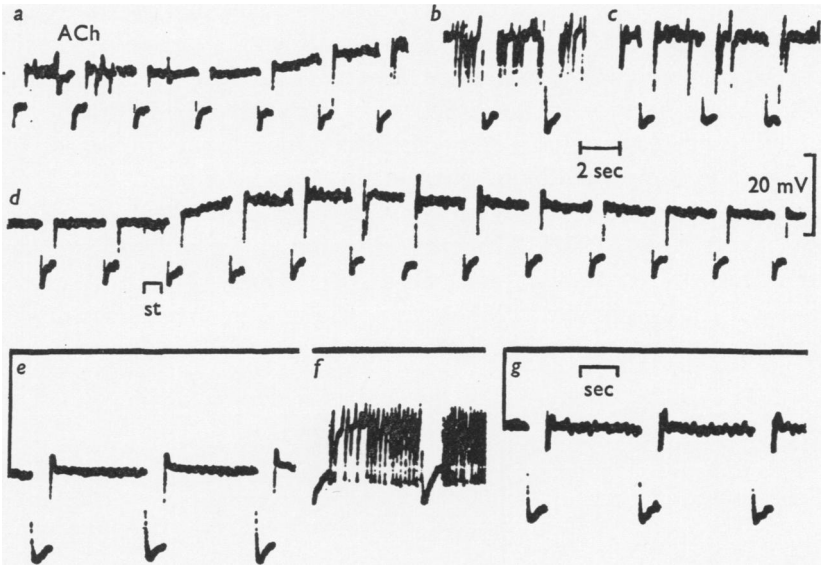

Fig. 4. ACh action, in presence of nicotinic blocking agent. (*a* to *d*) Frog ganglion cell, with resistance-testing constant current pulses superimposed on resting potential of 50 mV, nicotine (10^{-4} M) and eserine (2 μg/ml.) present: (*a*) before and after addition of ACh (10^{-3} M) as indicated; (*b*) few seconds after (*a*); (*c*) record continued 60 sec after (*b*); (*d*) S-EPSP response to preganglionic stimulation for 1 sec at 80/sec delivered during st (obtained before the ACh addition in (*a*). (*e* to *g*) Rabbit ganglion cell, resting potential 50 mV, with resting pulses (top line represents arbitrary base line), DTC (50 μg/ml.) and eserine (2 μg/ml.) present: (*e*) before ACh; (*f*) about 10 sec after adding ACh (10^{-3} M); (*g*) about 80 sec after the addition of ACh. The spontaneous firing in (*f*) tends to obscure the new level of membrane potential, but the latter is probably close to that seen in (*g*). Time calibration 2 sec for (*a*) to (*d*), 1 sec for (*e*) to (*g*); voltage calibration same for all. Tips of spikes in (*b*), (*c*) and (*f*) are not visible.

ACh (10^{-3} M), in presence of DTC (50 μg/ml.; i.e. 6×10^{-5} M) and eserine, with 10–15 mV depolarizations (e.g. Fig. 4*e* to *g*). (In one of the four rabbit cells so tested, an initial hyperpolarization developed after the usual

latency; this hyperpolarization had a peak amplitude of 12 mV, lasted about 10 sec, and was followed by a sustained depolarization of about 10 mV. Such an initial hyperpolarizing phase in a mammalian ganglion cell is consistent with the observations of Takeshige & Volle, 1964, in responses to muscarinic-ACh action by intra-arterial injection, and with the role of ACh in mediating the S-IPSP as postulated by Eccles & Libet, 1961).

During the depolarization produced by the S-EPSP (Fig. 4*d*) or by the muscarinic-ACh action (Fig. 4*a* to *c*) there were usually repetitive spontaneous discharges of the nicotinized frog cells. This demonstrates at the level of the ganglion cell that the muscarinic-ACh action, even when generated by preganglionic impulses, is capable of eliciting an actual post-ganglionic discharge. Evidence for this capability had already been obtained for whole ganglia, with recordings of population responses of the whole ganglia to preganglionic stimulation (Trendelenburg, 1966; Brown, 1967; Nishi & Koketsu, 1968*a*) as well as to intra-arterial injection of ACh (Takeshige & Volle, 1962). The greater tendency for repetitive firing to occur in nicotinized than in unblocked cells agrees with an observation made by Nishi & Koketsu (1968*a*) for post-ganglionic discharge of whole ganglia.

D-tubocurarine (DTC) in high concentrations appeared to depress all response to ACh in the amphibian ganglion cells, in contrast to the mammalian cells. ACh at 10^{-3} M (eserine present) produced no change in two frog cells when DTC at 50 μg/ml. was used instead of nicotine (though there was temporary initial depolarization in one case). With DTC at 20 μg/ml. (and eserine) present, some depolarization did appear in two out of five cells tested with 1 to 2×10^{-4} M ACh. At 20 or 50 μg/ml., DTC did not block all of the S-EPSP elicited by preganglionic impulses; but the S-EPSP was consistently larger in those frog ganglion cells treated with nicotine (10^{-4} M) than in those subjected to strong concentrations of DTC. Thus, DTC at high concentrations appears to block to a considerable degree the muscarinic receptors of frog ganglion cells; it does this to a much lesser degree, if at all, in the rabbit ganglion cells (see above and below; also Libet, 1967).

Methacholine (MCh) has been found to elicit a surface positive followed by negative response in mammalian ganglia, after a close intra-arterial injection (Takeshige *et al.* 1963); those responses were blocked by atropine. In four rabbit ganglion cells tested in the present study, addition of MCh at 10^{-3} M was followed by a depolarization, with a mean peak value of 7·5 mV (range 5–12 mV). With atropine present (20 μg/ml.) a fifth cell showed no response to MCh. In frog ganglion cells, addition of MCh (10^{-4}–10^{-5} M) was followed by slow depolarizations (mean peak value about 10 mV) which were more variable in amplitudes and time courses among different cells than were the responses to ACh.

Membrane resistance (r_m). In the nicotinized ganglion cells of frog the depolarizing response to ACh was not accompanied by any decrease in r_m, in contrast to the non-nicotinized cells (Fig. 3*a* to *c*). Instead there was

actually an increase in r_m up to about twice the resting value (Fig. 4*a* to *c*). This was similar to the changes in r_m occurring during the S-EPSP itself (Fig. 4*d*); a small increase rather than a decrease in r_m had already been reported to accompany the S-EPSP in curarized frog ganglion cells (Kobayashi & Libet, 1968), and larger increases were now observed in many cells (see Fig. 5). The time course of the increase in r_m seemed to parallel

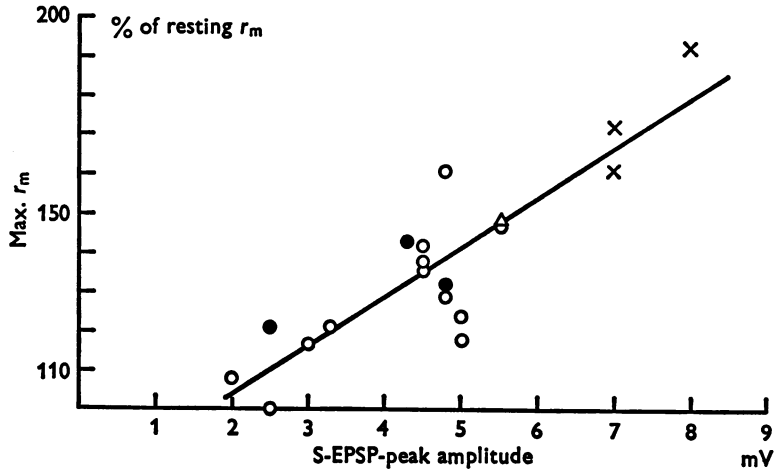

Fig. 5. Relationship between the increased membrane resistance (r_m), achieved during the S-EPSP in frog ganglion cells, and the amplitude of the S-EPSP. Ordinate values are for maximum r_m during the response, given as a percentage of the resting r_m before the response. Abscissa gives values of peak amplitude of the S-EPSP (in mV). Each symbol represents a separate ganglion cell: ○, those with no blocking agents added; ●, cells curarized (with dihydro-β-erythroidine, 2–5 μg/ml.); △, nicotine present (no eserine), 10^{-4} M; ×, nicotine plus eserine (2 μg/ml.) present. The line is drawn through points calculated by the least squares method. (The curarizing agent does not depress the nicotinic fast EPSP response as thoroughly as the nicotine, but the summated fast EPSPs do not last long after the end of the train of preganglionic impulses; this nicotinic-ACh action is therefore finished well before the peak of the S-EPSP response is reached.)

closely the onset and increase of the depolarization, whether in response to ACh or during the S-EPSP response to a brief train of preganglionic impulses. However, there were instances observed in which the maximum value of r_m was attained before the peak of the S-EPSP. The values of the maximum r_m, expressed as percentages of the values before the response, correlated roughly with the maximum values of depolarization achieved. This was the case both for the responses to ACh and for the S-EPSPs; the r_m changes are plotted against amplitude of S-EPSP in Fig. 5. Extrapolation of the curve in Fig. 5 would indicate that the S-EPSPs with low amplitudes of about 2 mV would be accompanied by little or no increase

in r_m. A decrease in r_m during a S-EPSP was never observed. Shifting the initial resting potentials by 10–20 mV, with conditioning polarizing current, can produce changes in the S-EPSP (Kobayashi & Libet, 1968; Nishi, Soeda & Koketsu, 1969; see also below); but the small spontaneous differences in resting potentials (which were in the range of 42–50 mV) among the different cells whose responses are plotted in Fig. 5 did not correlate with the differences in amplitudes of slow EPSPs and accompanying changes in r_m.

With DTC (20 μg/ml.) instead of nicotine applied to *frog ganglia*, the two cells that exhibited depolarizations (of 6 and 7 mV) following additions of ACh (10^{-4} M) showed in one case no change in r_m, and in the other a small increase in r_m of about 15 %. In three of the four *rabbit* ganglion cells that were tested with ACh (10^{-3} M) in the presence of DTC (50 μg/ml.) and eserine, r_m was measured. In the case shown in Fig. 4*e* to *g*, a small decrease in r_m was apparent during the first 20 sec or so (Fig. 4*f*) after adding ACh. However, r_m returned to the resting value during the following 20 sec and remained so until observation ended at 1 min 45 sec, even though the ACh-induced depolarization of 11 mV remained unchanged (Fig. 4*g*). A second cell, tested for r_m only at about 1 min after addition of ACh, showed a slight increase in r_m (of about 10 %) associated with the 10 mV depolarizing response which had developed by this time. The third cell exhibited no change (or possibly a slight decrease) in r_m during its depolarizing response of 15 mV.

These results with DTC in the frog and rabbit ganglion cells can be explained as being due to a variable mixture of the muscarinic action of ACh with a small remaining nicotinic action; the latter would be not completely blocked by the DTC but perhaps became more rapidly desensitized than the muscarinic action. *D*TC at 50 μg/ml. does not eliminate completely the fast EPSP in rabbit ganglia (Eccles, 1952) or, at 20 μg/ml., the fast EPSP of B neurones in frog ganglia (B. Libet, unpublished data); thus it would not be surprising to find some nicotinic action of ACh still effective even at the concentrations of DTC used above. On the other hand, nicotine, in the concentrations used for the frog cells, completely abolished the fast EPSPs.

Conditioning hyperpolarization. It has been found in frog ganglion cells that the S-EPSP was suppressed if elicited during an applied steady hyperpolarization of 10–30 mV (Kobayashi & Libet, 1968). In the present study the effects of conditioning hyperpolarization on both the S-EPSP and the response to muscarinic-ACh action were compared in the same cells. This was done in six cells of frog ganglia, all in the presence of nicotine (10^{-4} M) and eserine (2 μg/ml.), using K_2SO_4-filled micro-electrodes.

The effects of conditioning hyperpolarization (20 mV in most cases) *on the S-EPSP* were observed before testing for the ACh responses. In each case, the depolarization that normally constitutes the S-EPSP was depressed (Fig. 6*b*), as we have already reported previously (Kobayashi & Libet, 1968). (Such an anomalous depressant effect of conditioning hyperpolarization on the S-EPSP has now been confirmed by Nishi *et al.* 1969, although an opposite result had been reported earlier by Koketsu & Nishi, 1967.) In addition to the depression of the depolarizing S-EPSP, each of

these nicotinized cells exhibited an initial hyperpolarizing phase in the response (Fig. 6*a* vs. *b*), i.e. the S-EPSP appeared to show a reversal of polarity in at least the initial period. (Such reversals had not been observed in our earlier study on weakly curarized cells, Kobayashi & Libet, 1968.) The peak amplitude of the initial hyperpolarizing phases averaged about 6 mV (e.g. Fig. 6*b*), with a range of 3–8 mV except for one at 14 mV. (The latter cell also had the largest hyperpolarizing phase in the ACh response.) These initial hyperpolarizing responses seemed to have distinctly shorter latent periods than the purely depolarizing S-EPSPs of unconditioned cells; the former appeared to start early in the 1 second period of preganglionic stimulation in contrast to the latter (compare Fig. 6*a* with 6*b*).

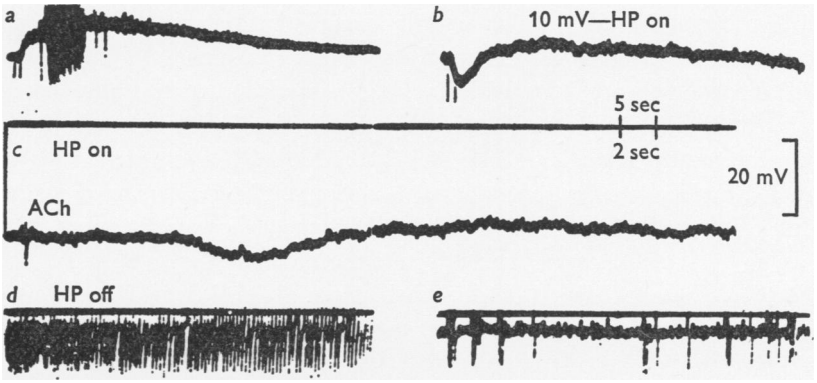

Fig. 6. Conditioning hyperpolarization on S-EPSP and ACh response, in nicotinized (and eserinized) B neurone of frog ganglion, resting potential 50 mV. (*a*) Response to preganglionic stimulation, 1 sec train of 80/sec (stimuli applied between the two markers); (*b*) same, but during conditioning hyperpolarization of 10 mV; (*c*) response to ACh (10^{-3} M, addition begun at artifact) during similar 10 mV hyperpolarization; (*d*) continuation of record in (*c*) but after turning off the conditioning hyperpolarization; (*e*), continuation of same recording a few seconds after that in (*d*). The upper straight line in (*c*) to (*e*) is an arbitrary base line for reference to shifts in intracellular potential; the depolarized level seen in (*d*) to (*e*) is real, as any shifts due to the conditioning hyperpolarizing currents are balanced out by adjustments in the bridge before penetrating the cell. Tops of spike discharge during the depolarizing response in (*d*) to (*e*) are not visible. Time calibration, 5 sec for (*a*) to (*b*); 2 sec for (*c*) to (*e*).

To test the effects *on the muscarinic-ACh* response, conditioning, hyperpolarization of about 20 mV was started before and sustained during, and for about 30 sec after, the addition of ACh (10^{-3} M). Addition of ACh was followed by an initial hyperpolarizing phase in five of the six cells (Fig. 6*c*), instead of the initial depolarizing one obtained in unconditioned, nicotinized cells (e.g. Fig. 4*a* to *c*). The hyperpolarizing response had an average

maximum amplitude of about 6 mV (3 to 13 mV) and appeared to last about 5–10 sec in the different cells, though a late small tail may have gone on for a longer time in some instances. In all six cells there was either no actual depolarization or only a small one, relative to the level before adding ACh (e.g. Fig. 6*c*), while the conditioning hyperpolarization remained on. The whole response to ACh was thus altered in a way exactly comparable to the changes in the S-EPSP that were seen to be produced by conditioning hyperpolarization in these same ganglion cells. When the conditioning current was turned off (with ACh of course still present in the medium) the membrane potential rapidly moved to a depolarized level, 20 mV or more from control level, and considerable spontaneous discharging occurred for a time (e.g. Fig. 6*d*, *e*). This showed that the conditioning hyperpolarization merely modified the depolarizing response to the ACh and did not permanently abort it.

The clear finding of depression of the depolarizing response to muscarinic-ACh action by conditioning hyperpolarization appears to be contradictory to the report by Koketsu *et al.* (1968). These authors reported that the amplitude of the slow, atropine-sensitive depolarization that was produced by an iontophoretic pulse of ACh was 'directly proportional to the value of the resting potential', in a manner similar to that found for the fast, curare-sensitive ACh depolarization. It is difficult to explain the discrepancy between our and their results, especially since Nishi *et al.* (1969) have now confirmed our finding (Kobayashi & Libet, 1968) that the S-EPSP, which has characteristics very similar to those of the muscarinic-ACh depolarization, is depressed by conditioning hyperpolarization of the membrane.

DISCUSSION

In both the hyperpolarizing response to NA and the depolarizing response to muscarinic-ACh action there is an absence of any detectable increase in ionic conductance of the membrane; this appears to represent the actual responses at the post-synaptic membranes upon which these substances act (see below). Secondly, both of the responses are altered by shifts in the initial membrane potential in directions opposite to those expected from an ionic diffusion hypothesis for their electrogenesis (see further below), i.e. the NA response is depressed by conditioning depolarization and the depolarizing muscarinic-ACh response is depressed by conditioning hyperpolarization. The electrogenic mechanisms for both of these responses thus appear to differ in principle from that developed by the nicotinic-ACh action on these cells, and from those developed by transmitter actions that produce the fast post-synaptic responses in general (e.g. Eccles, 1964); thus, the potentials developed by NA or muscarinic-

ACh actions are not due to movements of ions down their electrochemical gradients. Similar differences from the fast EPSP had been found for both the S-IPSP and S-EPSP by Kobayashi & Libet (1968), and were recently confirmed for the S-EPSP by Nishi *et al.* (1969). Krnjević & Schwartz (1967) had reported earlier that the atropine-sensitive depolarizing response of cortical pyramidal cells to ACh can occur without a detectable decrease in r_m. In their case, however, it is more difficult to eliminate the possibility that such an observation is explainable by locations of the ACh receptor sites at points remote from the recording electrode, although the available evidence appeared to be against such an explanation (Krnjević, 1969).

Changes in membrane resistance. In addition to the one offered above, two possible alternative explanations for the absence of any detectable decreases in r_m, during responses of sympathetic ganglion cells to NA or muscarinic-ACh, should be considered. (*a*) The appropriate receptor sites for action by NA and ACh (muscarinic) might have been remote from the intracellular location of the micro-electrode. (See the analysis of this kind of problem, for EPSPs in motoneurones, by Smith, Wuerkar & Frank, 1967.) This possibility has already been considered with respect to the similar difference in detectable decreases in r_m between the slow PSPs and the fast EPSP in sympathetic ganglion cells (Kobayashi & Libet, 1968). Frog ganglion cells are unipolar and lack the dendritic branching needed to provide remote receptor sites; in addition, the muscarinic-ACh action does elicit a considerable conductance change in these cells, but it is opposite in direction to that of the nicotinic-ACh action. The same amplitudes of steady polarizing currents applied through the recording electrode for altering the fast EPSP were also sufficient for altering the NA response and the S-IPSP (rabbit) or the muscarinic-ACh response and the S-EPSP (frog). (Such similarities should be compared to the differences in effectiveness of polarizing currents in altering different EPSPs with presumably different receptor loci in spinal motoneurones (Nelson & Frank, 1967).) This indicates strongly that the receptor sites for the fast EPSP and the slow PSPs are not far apart electrically. (*b*) The absence of a detectable change in membrane resistance might represent a rough balance of increased conductance(s) for some specie(s) of ion(s) and decreased conductance(s) for other ion(s); these opposing changes in ionic conductances could give rise to a net change in e.m.f. and to the observed response in the membrane potential. However, the effects of conditioning polarizing currents on the responses to the transmitter substances were different from those to be expected with such changes in ionic conductances.

The *increase* in r_m, that accompanied the depolarizing response to muscarinic-ACh and the S-EPSP in frog ganglion cells, is not simply an

instance of anomalous rectification which becomes manifest because of the depolarization developed in these responses. No such increase in resistance was observed during passive depolarization by external currents (Kobayashi & Libet, 1968).

Changes with shifts in initial membrane potential. The apparently anomalous changes in amplitudes of response that are induced by conditioning polarizations cannot be explained by induced changes in membrane resistance. In the rabbit ganglion cells the amplitudes of the action potentials, elicited ortho- or antidromically, were not changed by conditioning depolarizations or hyperpolarizations (of 10–30 mV) beyond that expected from the shift in resting potential relative to the peak of the unconditioned action potential. In addition, the amplitude of the resistance-testing pulses of constant current applied across the unconditioned membrane were roughly similar in amplitude whether the pulses were in the hyperpolarizing or depolarizing direction. In the frog ganglion cells there is not any obvious anomalous rectification to account for the effects of hyperpolarization. No appreciable decrease in resting membrane resistance could be demonstrated in frog cells over the range of hyperpolarization employed (Kobayashi & Libet, 1968; Nishi & Koketsu, 1960). In addition, the summated fast EPSP in partially curarized cells did not decrease during conditioning hyperpolarization, but increased as expected from the ionic hypothesis (see Kobayashi & Libet, 1968). It appears, therefore, that conditioning depolarization and hyperpolarization produced changes in the responses to NA and to muscarinic-ACh actions, respectively, which reflected real differences between these responses on the one hand, and the responses to the nicotinic-ACh action that elicits the fast EPSP on the other.

The reversal of polarity of the initial phase of ACh response and of S-EPSP, produced by conditioning hyperpolarization in nicotinized frog ganglion cells, has not been observed in lightly curarized cells studied with KCl micro-electrodes (Kobayashi & Libet, 1968). Therefore, it is perhaps of secondary, albeit intriguing, interest as an attribute of the muscarinic-ACh action. It should be noted that the reversal of polarity of response towards one of hyperpolarization, by a hyperpolarizing shift in membrane potential is, like the depression of the depolarizing response, contrary to that expected on the ionic hypothesis.

Transmitters for the fast and slow PSPs. ACh had already met the requirements demanded of the transmitter for the fast EPSP in sympathetic ganglia (e.g. Volle, 1969), including the production of a drop in membrane resistance accompanying the depolarization of (amphibian) ganglion cells (Ginsborg & Guerrero, 1964); we have confirmed the latter observation for amphibian cells and have also demonstrated its occurrence in

mammalian ganglion cells. The S-EPSP, on the other hand, requires a transmitter that will elicit a depolarization that occurs even during nicotinic blockade but is blocked by atropine (Eccles & Libet, 1961), and one that is not accompanied by any increase in membrane conductance (Kobayashi & Libet, 1968). The present study demonstrates that the two kinds of depolarization elicited by ACh, i.e. without and with nicotinic blockade present, are in fact developed with precisely such differences. In addition, conditioning hyperpolarization modified the muscarinic type of ACh action in the same peculiar manner that it affected the S-EPSP itself. It should be noted that the drop in r_m during ACh action when no nicotinic blocking agent is present does not mean that the muscarinic action is absent without such blockade. This change is what would be expected if the nicotinic and muscarinic actions were mediated at different sites on the membrane which are in parallel electrically. Similarly, the fact that the amount of muscarinic-ACh-depolarization is such a large fraction of the ACh-depolarization without nicotinic blockade does not mean that nicotinic-ACh depolarization alone would be small, if the e.m.f.s generated by nicotinic and muscarinic actions are in parallel.

The present work also shows that NA elicits a response, in mammalian ganglion cells, that has the same extraordinary characteristics found for the S-IPSP itself, i.e. NA elicits an hyperpolarizing response which does not involve any detectable change in ionic conductance of the membrane, and this response is depressed rather than enhanced by conditioning depolarization. This similarity of the actions of NA to those of the actual S-IPSP transmitter on the ganglion cells, when added to other kinds of evidence available (Eccles & Libet, 1961; Libet, 1965; Siegrist, Dolivo, Dunant, Foroglou-Kerameus, de Ribaupierre & Rouillier, 1968; Matthews & Raisman, 1969; but see Volle, 1969) provides strong additional support for the hypothesis that NA, or some related adrenergic substance, is the transmitter. The alternative more unlikely interpretations are (*a*) that NA pharmacologically mimics the unique actions of the actual transmitter, with the latter chemically unrelated to NA, or (*b*) that NA brings about the release of the actual transmitter. The final concentration of NA that was needed to achieve a consistent response by the rabbit ganglion cells was relatively high (10–50 μg/ml., or *ca.* 10^{-4} M, although 1 μg/ml. was also effective). However, this is explainable on the basis of diffusional and enzymic barriers in the path of the substance from the external surface of the ganglion to the ganglion cells; too slow a build-up of concentration at the cell might not produce a recognizable response. Even with tests of ACh, whose role as the transmitter for the EPSPs is not in doubt, large final concentrations of ACh and the presence of eserine were required in order to obtain optimal responses even in the smaller frog ganglia (see also

Ginsborg & Guerrero, 1964). The comparative absence of an hyperpolarizing response to adrenaline or to NA in most of the B neurones tested in the frog ganglia does not provide evidence against the postulated role of an adrenergic substance as the transmitter for the slow IPSP in these structures. The S-IPSP response has been found only in the C neurones in frog ganglia (Tosaka *et al.* 1968). C neurones are very difficult to impale adequately and none were tested with adrenaline in this study. On the other hand, the present findings do indicate that most B neurones lack the receptors for responding to adrenaline.

The depression of all the PSPs of rabbit ganglion cells by NA (50 μg/ml.) indicated that NA at this concentration was also affecting presynaptic function, in addition to its production of a post-synaptic hyperpolarization. The hyperpolarization of the ganglion cell by NA should augment, rather than depress, the fast EPSP and, in rabbit ganglia, both of the slow PSPs (Kobayashi & Libet, 1968). Clear cut evidence for depression of presynaptic function in rabbit ganglia by adrenaline (10^{-5} M) has recently been reported by Christ & Nishi (1969; see also Weir & McLennan, 1963). Adrenaline is far more active than NA in depressing the post-ganglionic discharge that is elicited by preganglionic impulses (Weir & McLennan, 1963). It remains to be seen whether any presynaptic action that was produced by NA was merely a pharmacological effect, dependent on the relatively high concentration that had to be added to achieve an observable post-synaptic response under the conditions employed in the present study.

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