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THE ACTION OF ACETYLCHOLINE, DOPAMINE AND 5-HYDROXYTRYPTAMINE ON THE SPONTANEOUS ACTIVITY OF THE CELLS OF RETZIUS OF THE LEECH, *HIRUDO MEDICINALIS*

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Neuropharmacological and neurophysiological studies have been undertaken on both the longitudinal muscle and on the segmental ganglionic neurones of the leech, *Hirudo medicinalis*. The longitudinal muscle of the leech was developed by Minz (1932) from the initial observations of Fuehner (1918) as an assay procedure for acetylcholine. More recently this same preparation has been used as an assay tissue for 5-hydroxytryptamine (Poloni, 1955), and has subsequently been reinvestigated by Schain (1961). Acetylcholine causes the muscle to contract whereas 5-hydroxytryptamine has a relaxing action and reduces the amplitude of contractions produced by acetylcholine. Electrophysiological recordings from the neurones of the leech ventral nerve cord were first performed by Hagiwara & Morita (1962) and by Eckert (1963). Studies concerning the role of the glial cells in this preparation were made by Kuffler & Potter (1964) and Nicholls & Kuffler (1964). Penn & Loewenstein (1966) have studied the role of calcium in maintaining electrical connections between the nerve cells of Retzius. The actions of acetylcholine and 5-hydroxytryptamine on ganglionic transmission in the leech have been studied by Kostowski (1965) using extracellular recording methods. Kostowski suggests that acetylcholine enhances transmission in the leech cord, while 5-hydroxytryptamine may act as an inhibitory agent. The present study was undertaken to investigate the action of possible chemical transmitter agents on the bioelectrical activity of the cells of Retzius (Retzius, 1891). A preliminary report of this investigation has been communicated to the Physiological Society (Kerkut, Sedden & Walker, 1967a).

METHODS

All experiments described in this paper were performed on the ventral segmental ganglia of the medicinal leech, *Hirudo medicinalis*. The animals were obtained from a local dealer and kept in distilled water in an aquarium at a temperature of around 10° C. Before dissection the animals were placed in 10% ethanol for 2–4 min, until they had partially relaxed. They were then removed, pinned on a wax block dorsal side upwards and dissected to show the ventral nerve cord, which lies in a ventral blood sinus. The cord and ventral sinus were separated from the surrounding tissue and divided into three portions, each containing approximately 7 ganglia. These were stored in leech Ringer until required. Fresh leeches for experimentation were prepared each day. The cord

was mounted on a glass slide ventral side uppermost, and viewed under a binocular microscope, magnification 16 or 64. Under the lower power, the ventral sinus wall was carefully slit to expose the ganglion. The neurones can clearly be seen lying beneath a transparent capsule, their soma within giant glial cells (Coggeshall & Fawcett, 1964). For experiments in which the compounds to be tested were added to the bath, no further preparation was made, as it is clear from the work of Nicholls & Kuffler (1964) that compounds can easily reach the neurones when both the capsule and the glia are intact. For the iontophoretic experiments the capsule was carefully slit to expose the soma of the neurones. As far as possible only the two giant cells of Retzius were used in this present study. However, it is possible that on a few occasions adjacent cells were penetrated. The cells of Retzius were identified by their position on the ventral surface of the ganglion, by their size (60–80 μ in diameter), and by the form of their bioelectric potentials. The resting potential lay between 40 and 50 mV, and the action potentials were between 20 and 50 mV in amplitude, generally being between 30 and 40 mV and therefore not possessing an overshoot. Later they could further be identified by their response to compounds applied to the bath. The Ringer used in these experiments had the following composition (Kuffler & Potter, 1964): NaCl, 115 mM ; KCl, 4 mM ; $CaCl_2$, 2 mM ; maleic acid, 10 mM ; glucose, 10 mM ; tris buffer, 10 mM ; NaOH, approximately 10 mM. The Ringer was prepared with the exception of the NaOH, and then adjusted using a pH meter to 7.4 with the NaOH. Before the addition of the NaOH the Ringer had a pH of 2–3.

The iontophoretic electrodes were prepared as follows. Ordinary glass microelectrodes were pulled on a horizontal machine and their tips broken to 1–2 μ diameter. These were then placed in distilled water and left for 2 days to allow the tips to fill with water. The acetylcholine chloride or 5-hydroxytryptamine creatinine sulphate at pH 3–4 was then pipetted into the shank of the electrode and the tip placed in a saline solution. 45 V was then applied across the electrode tip, the inside being positive. This aided the diffusion of the compound to the tip of the electrode. After approximately 1 hr, a current reading could be obtained from an avometer placed in the circuit indicating that the compound had reached the electrode tip. Both compounds were ejected as cations. Drugs which were added directly to the bath were made up in leech Ringer. The volume of the bath used was 20 ml. The values given for acetylcholine, 5-hydroxytryptamine and dopamine are the amounts of each compound added to the ganglion. The values given for the antagonists are the final concentrations in the bath, expressed as μg/ml. of leech Ringer.

Intracellular recordings were made using glass microelectrodes, 20–60 MΩ resistance, filled with molar potassium acetate. The potentials were passed through a Medistor cathode follower and displayed on a Tetronix 502A oscilloscope. The results were either filmed or recorded on an Ediswan pen oscillograph.

RESULTS

Acetylcholine

The threshold amount of acetylcholine was 10μg. This produced a slow depolarization and increase in frequency of firing of the action potentials (Fig. 1A). With threshold amounts it took over 40 sec from the time of addition of the acetylcholine before the response became apparent. Addition of higher amounts of acetylcholine—for example, 100 μg (Fig. 1B)—produced an increase in the firing rate of the cell after about 6 sec. Activity induced by 100–500 μg acetylcholine often remained at a raised rate for 10 min or longer, even after repeated washing. At other times the activity of the cell returned to normal after 1 or 2 min. Such activity induced by acetylcholine could be terminated by atropine, 5-hydroxytryptamine or dopamine. Figure 1C and D show the inhibition of acetylcholine induced activity following the application of 100 μg 5-hydroxytryptamine and 100 μg dopamine respectively to the ganglion. The activity was depressed after 5–7 sec and ceased completely after 10–20 sec. After washing the activity returned to the rate before the addition of acetylcholine. Both 5-hydroxytryptamine and dopamine hyperpolarized the membrane.

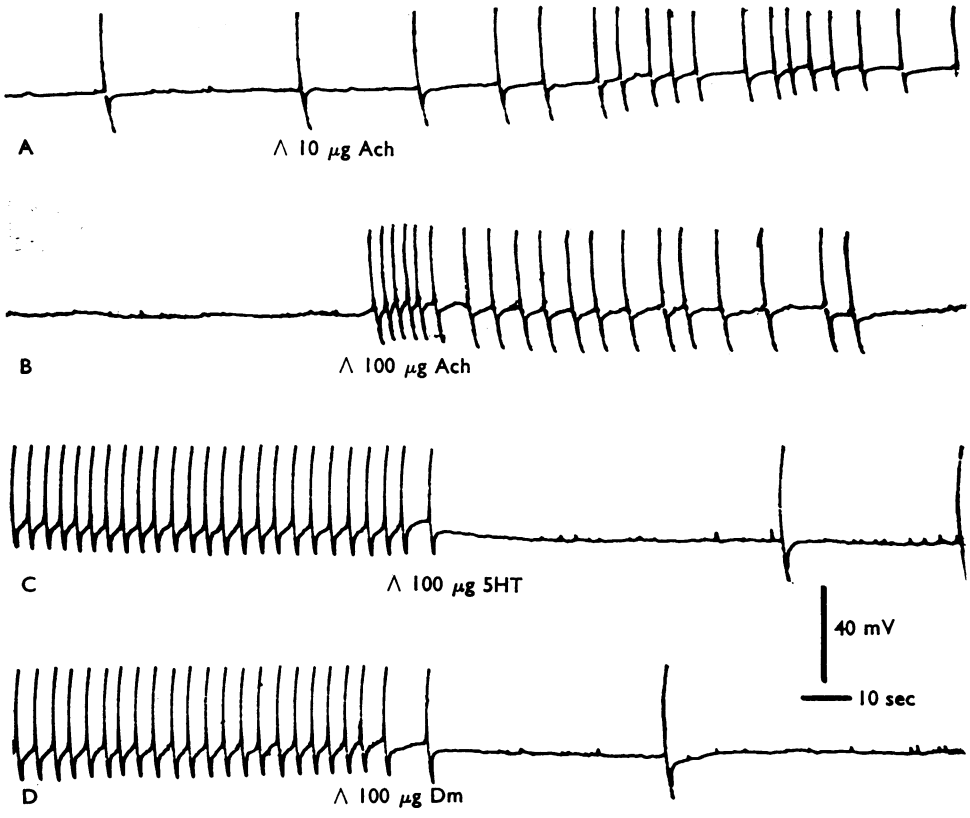

Fig. 1. A, The effect of 10 μg acetylcholine on the spontaneous activity; B, the effect of 100 μg acetylcholine on the spontaneous activity; C, inhibition by 100 μg 5-hydroxytryptamine of activity induced by acetylcholine; D, inhibition by 100 μg dopamine of activity induced by acetylcholine. The values for acetylcholine, 5-hydroxytryptamine and dopamine are the amounts added to the ganglion. All traces are taken from the same cell.

The response to acetylcholine could be reduced or completely blocked by pretreating the preparation with atropine, benzoquinonium, decamethonium, hexamethonium or tubocurarine, the amount of blocking agent required varying greatly from compound to compound. Figure 2 shows the effects on the acetylcholine response of pretreating with hexamethonium (Fig. 2B), tubocurarine (Fig. 2D), and atropine (Fig. 2E). In this case the presence in the bath of 10 μg/ml. hexamethonium reduced the response to the addition of 500 μg acetylcholine. This partial block was easily reversed on washing. In this preparation the presence in the bath of 100 μg/ml. tubocurarine had no effect on the intensity of the acetylcholine response. However, in some preparations, such doses of tubocurarine reduced or abolished the acetylcholine response. Atropine present in the bath at a concentration of 5 μg/ml. completely abolished the response to acetylcholine. This block was readily reversed. In this same preparation the presence in the bath of 0.1 μg/ml. benzoquinonium (not illustrated in Fig. 2) completely abolished the acetylcholine response. Thus for this experiment the order of potency for antagonizing the

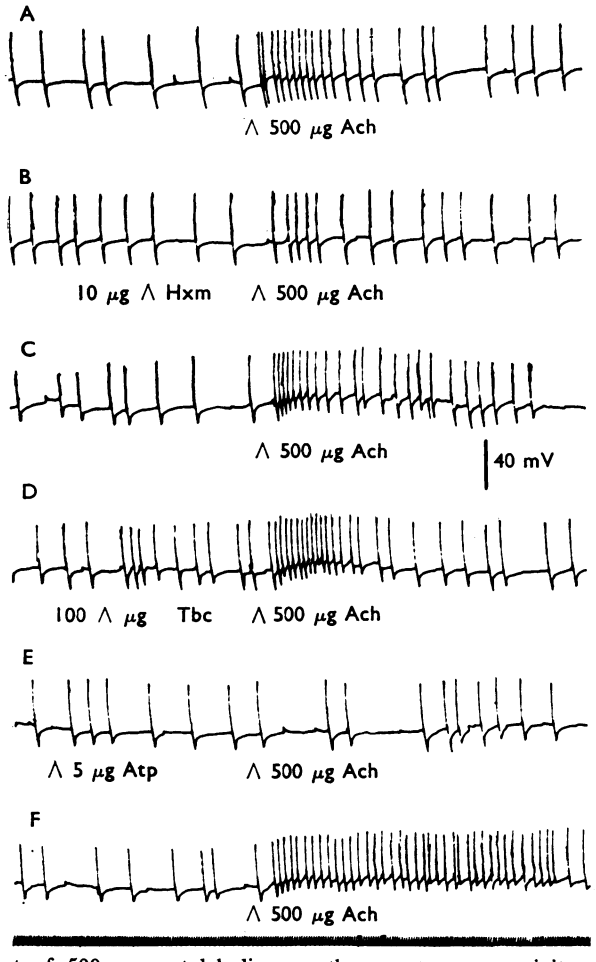

Fig. 2. A, The effect of 500 μg acetylcholine on the spontaneous activity; B, the effect of the presence in the bath of 10 μg/ml. hexamethonium on activity induced by 500 μg acetylcholine; C, the recovery of the acetylcholine response following washing; D, the effect of the presence in the bath of 100 μg/ml. tubocurarine on activity induced by 500 μg acetylcholine; E, the effect of the presence in the bath of 5 μg/ml. atropine on activity induced by 500 μg acetylcholine; F, the recovery of the acetylcholine response following washing. The value of acetylcholine is the amount added to the bath. All traces are from the same cell. Time scale is marked in intervals of 1 sec in this and subsequent figures.

response to acetylcholine was: benzoquinonium$\gg$atropine$>$hexamethonium$\gg$tubocurarine. Benzoquinonium and decamethonium were approximately equipotent as antagonists of the acetylcholine response. The presence in the bath of 0.1 μg/ml. decamethonium usually completely abolished the response to the addition of 100 μg acetylcholine (Fig. 3B). This antagonism had largely been reversed after washing for 150 sec (Fig. 3C). The presence in the bath of 0.1 μg/ml. benzoquinonium completely blocked the response to acetylcholine (Fig. 4B). Two equal additions of 100 μg acetylcholine produced only a slight depolarization and a few action potentials (Fig. 4B). Prolonged washing for 9

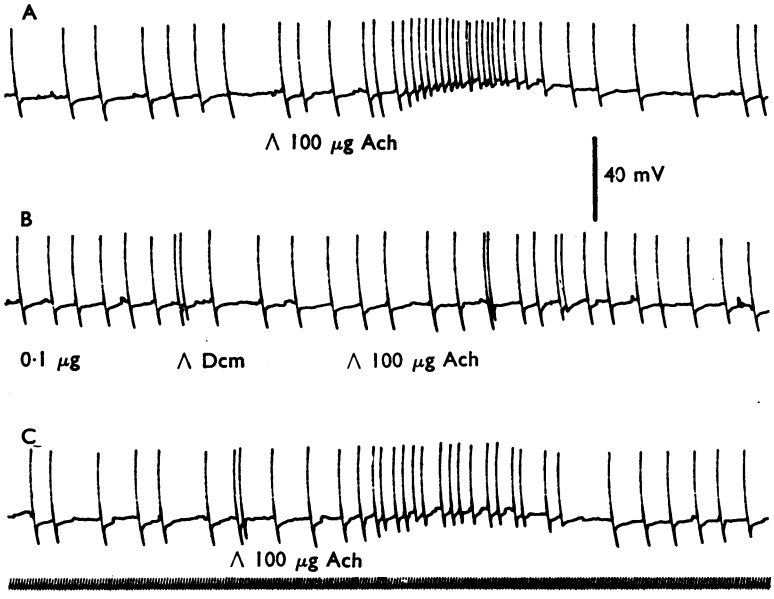

Fig. 3. A, The effect of 100 μg acetylcholine on the spontaneous activity; B, the effect of the presence in the bath of 0.1 μg/ml. decamethonium on activity induced by 100 μg acetylcholine; C, the recovery of the acetylcholine response following washing. The value for acetylcholine is the amount added to the bath. All traces are from the same cell.

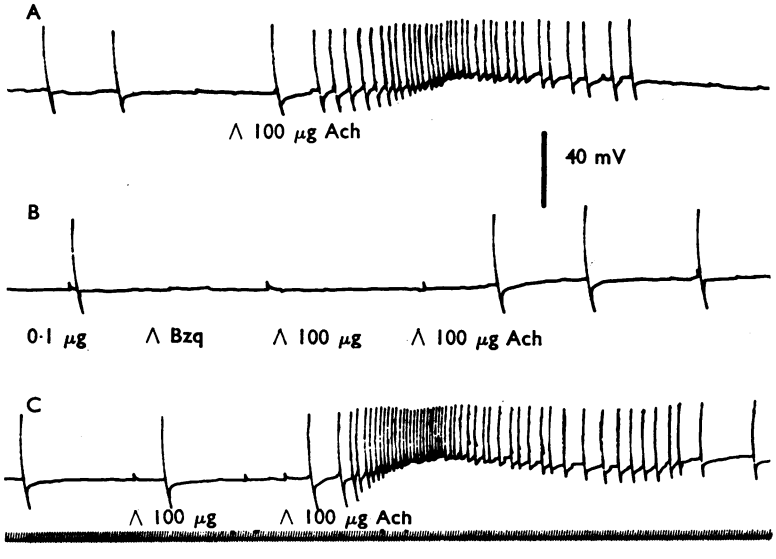

Fig. 4. A, The effect of 100 μg acetylcholine on the spontaneous activity; B, the effect of the presence in the bath of 0.1 μg/ml. benzoquinonium on activity induced by 100 μg acetylcholine; C, the recovery of the acetylcholine response following washing. In traces B and C two doses of acetylcholine were given. The value for acetylcholine is the amount added to the bath. All traces are from the same cell.

min partially restored the response to acetylcholine (Fig. 4C) so that the application of two doses of 100 μg acetylcholine produced depolarization and increased the firing rate of the cell. A response to the originally effective concentration of acetylcholine did not return until after 20 min. The presence in the bath of 1 μg/ml. benzoquinonium sometimes inhibited the spontaneous activity of the cell and hyperpolarized the membrane. In some experiments the presence in the bath of 0.1 μg/ml. benzoquinonium reduced the response to the addition of 300 μg acetylcholine but did not completely block it. Both benzoquinonium and decamethonium always blocked the response to acetylcholine applied at a concentration 1,000 times greater than the concentration of the blocking agent.

Iontophoretic application of acetylcholine also caused depolarization and excitation of the Retzius cells (Fig. 5A). In this case 100 namps were applied for 10 sec. Repeated applications of acetylcholine approximately every 2 min for 10 sec and at 100 namps

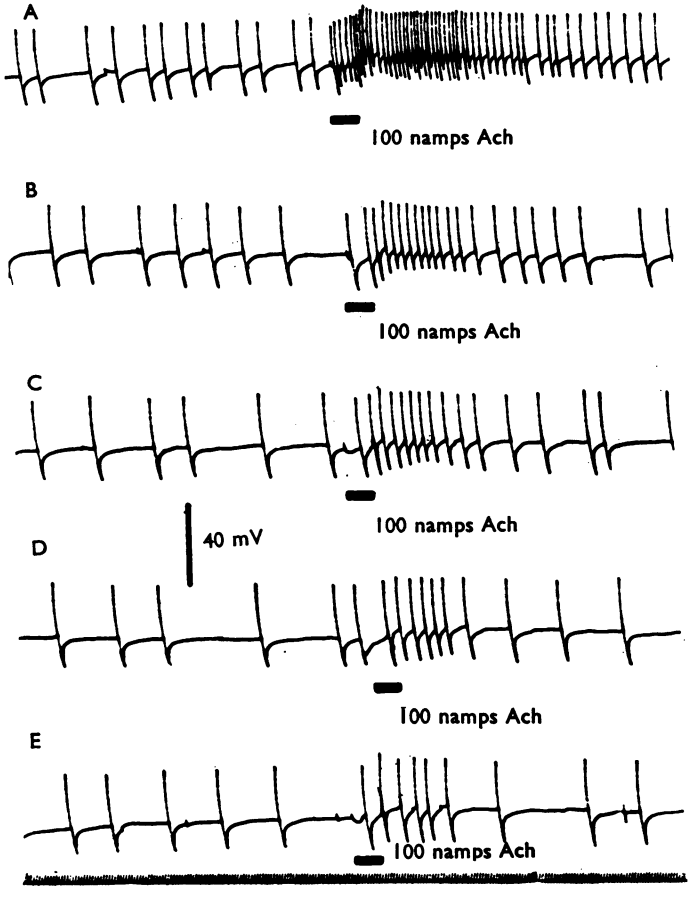

Fig. 5. A, The iontophoretic application of acetylcholine on to a Retzius cell ; B, C, D, E, subsequent injections of acetylcholine given at 2-min intervals. The acetylcholine was injected for 10 sec at a current of 100 namps.

(Fig. 5B–E) resulted in tachyphylaxis of the acetylcholine response. Tachyphylaxis was also sometimes evident after repeated applications of acetylcholine to the bath.

5-Hydroxytryptamine (5-HT)

The threshold amount of 5-HT was 0.1 μg. Such amounts reduced the firing rate of the cell but did not result in hyperpolarization of the resting membrane. Higher amounts of 5-HT produced inhibition of the cell activity for progressively longer periods As with acetylcholine, some preparations exhibited tachyphylaxis to 5-HT. On occasion the addition of 5-HT led to cessation of action potentials but resulted in the production of excitatory postsynaptic potentials of up to 8 mV in amplitude (Fig. 6A). In the experiment of Fig. 6 prolonged washing failed to restore the normal activity (Fig. 6B). The excitatory postsynaptic potentials often exceeded the normal membrane threshold

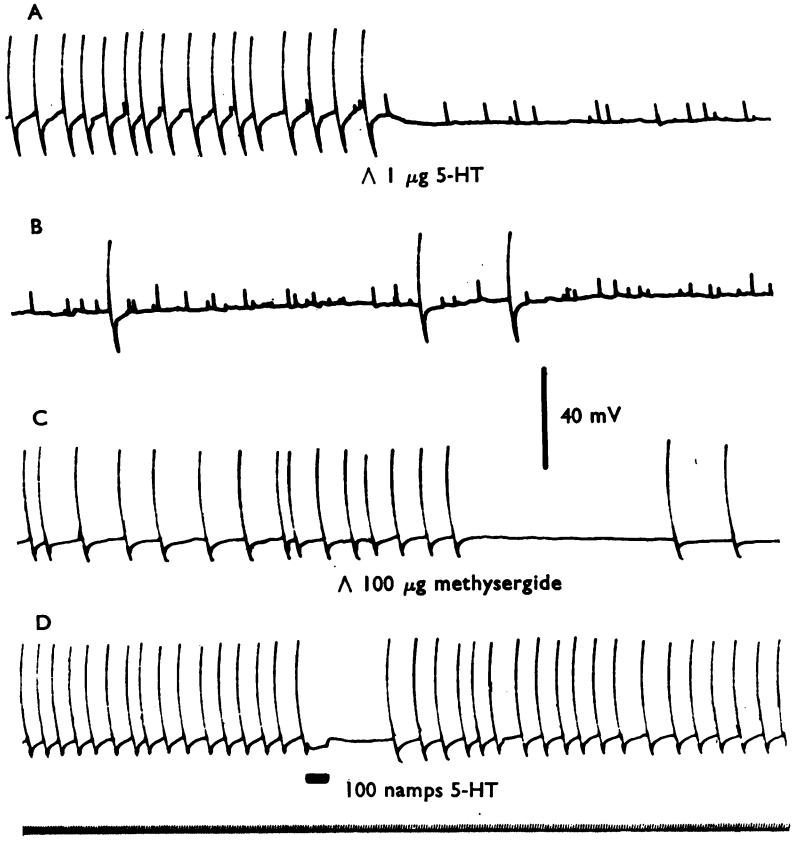

Fig. 6. A, The effect of 1 μg 5-hydroxytryptamine on the spontaneous activity; B, prolonged washing of the cell failed to restore the action potentials—note the presence of excitatory postsynaptic potentials; C, the effect of 100 μg/ml. methysergide on the spontaneous activity; D, the iontophoretic application of 5-hydroxytryptamine on to a Retzius cell; the 5-hydroxytryptamine was applied for 7 sec at a current of 100 namps. The values for 5-hydroxytryptamine and methysergide are the amounts added to the bath. Traces A and B are from the same cell. Traces C and D are from different cells.

for firing, but in the presence of 5-HT they failed to produce action potentials. Methysergide depressed the spontaneous activity of the preparation when applied at a concentration of 100 μg/ml. (Fig. 6C). Large amounts of atropine produced a similar effect. Iontophoretic application of 5-HT (100 namps for 5–7 sec) resulted in inhibition of the spontaneous activity of the cell for 20–30 sec (Fig. 6D). In the experiment of Fig. 6D repeated application of such doses did not result in tachyphylaxis.

Both methysergide and atropine when present in the bath at concentrations of 10–100 μg/ml. were found on occasion to block the inhibitory action of 5-HT. Figure 7B shows that the presence in the bath of 10 μg/ml. methysergide reduced the inhibitory action of 5-HT. In this case the methysergide produced a slight increase in the spontaneous activity of the cell. On washing there was a period of inhibition as can be seen

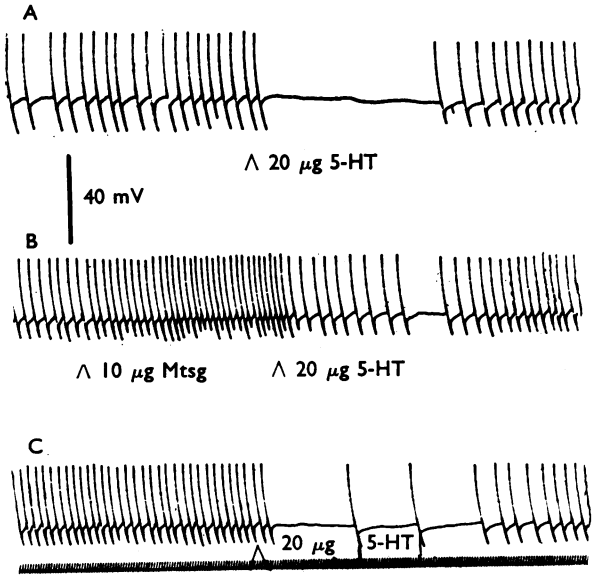

Fig. 7. A, The effect of 20 μg 5-hydroxytryptamine on the spontaneous activity; B, the effect of the presence in the bath of 10 μg/ml. methysergide on the inhibition induced by 20 μg 5-hydroxytryptamine; C, the recovery of the 5-hydroxytryptamine response following washing. The value for 5-hydroxytryptamine is the amount added to the bath. All traces are from the same cell.

in Fig. 7B. After approximately 120 sec the 5-HT response had largely recovered (Fig. 7C). In this experiment washing quickly restored the firing rate of the cell following the addition of 20 μg 5-HT (Fig. 7A). However, this was not always the case, as can be seen in Fig. 8A. In this experiment the presence in the bath of 5 μg/ml. atropine blocked the inhibitory action of 5-HT (Fig. 8B). On many occasions both atropine and methysergide failed to antagonize the action of 5-HT, especially when the cell was sensitive to the addition of 0.1–1 μg 5-HT. Neither of these blocking compounds can be considered to be potent antagonists of 5-HT in this preparation.

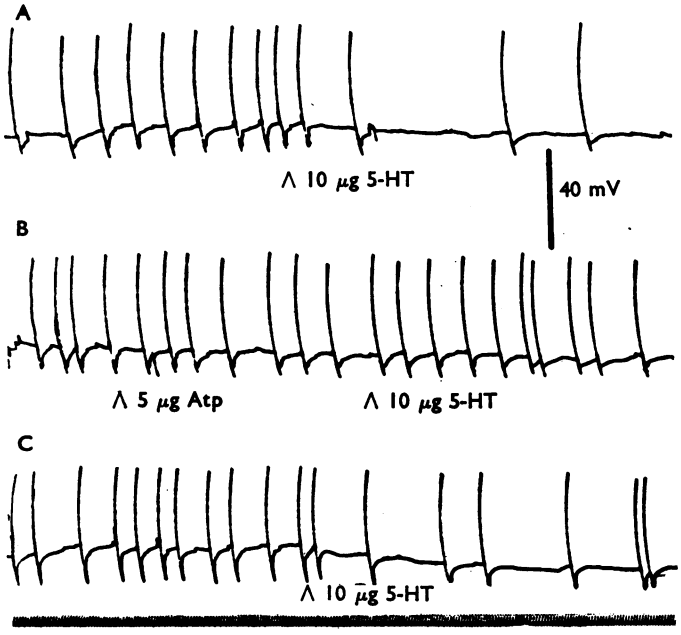

Fig. 8. A, The effect of 10 μg 5-hydroxytryptamine on the spontaneous activity; B, the effect of the presence in the bath of 5 μg/ml. atropine on the inhibition induced by 10 μg 5-hydroxytryptamine; C, the recovery of the 5-hydroxytryptamine response following washing. The value of 5-hydroxytryptamine is the amount added to the bath. All traces are from the same cell.

In addition to acetylcholine, 5-hydroxytryptamine and dopamine, a number of other compounds were tested on the spontaneous activity of the cells of Retzius. These included noradrenaline, adrenaline, isoprenaline, gamma amino-n-butyric acid, glutamic acid, ergothioneine, imidazolyl acetic acid and histamine. None of these compounds had a consistent action on the spontaneous activity of the giant cells.

DISCUSSION

Of the compounds investigated, the most consistent excitatory compound is acetylcholine, while the most consistent inhibitory agents are 5-HT and dopamine. The other compounds tested gave no clear consistent result when tested on the cells of Retzius. There is a striking difference in the sensitivity of these cells to acetylcholine and 5-HT. In general the cells will respond to 100–1,000 times more dilute solutions of 5-HT than of acetylcholine. It would appear that both compounds can reach the cells with approximately the same delay of about 5 sec. It is clear from the use of antagonists that the acetylcholine action resembles that at the vertebrate neuromuscular junction rather than that in vertebrate autonomic ganglia or nerve smooth muscle junctions, since the neuromuscular blocking drugs, decamethonium and benzoquinonium, were more effective antagonists than were atropine or hexamethonium. In view of this, it is surprising that tubocurarine was relatively ineffective as an antagonist. In the longitudinal muscle of the leech, the acetylcholine response is antagonized by tubocurarine, but not by atropine. In

fact it is claimed that atropine enhances the acetylcholine response (Minz, 1955). In the leech ganglion atropine does antagonize the acetylcholine response but the concentration required is about 50 times greater than that of benzoquinonium.

There are a number of criteria which have to be satisfied before a compound can be considered to be a chemical transmitter. 5-HT has been shown to be present in the leech ganglion by Welsh & Moorhead (1960), and using fluorescence microscopy 5-HT has been located in 6 cells in each ganglion, 2 of which are the cells of Retzius (Kerkut, Sedden & Walker, 1967b). 5-HT has a clear inhibitory action on the spontaneous activity of the cells of Retzius when 0.1 μg was added to the ganglion. Higher amounts of 5-HT hyperpolarize the membrane. However, there are no obvious spontaneous inhibitory postsynaptic potentials (ipsps) present in these cells. One of the criteria for identifying an inhibitory transmitter is that it should produce an ipsp similar to that produced by nerve stimulation. The connectives leading into the ganglion have not yet been stimulated to test whether they produce ipsps.

Acetylcholine has been shown to be present in the leech nervous system (Carayon-Gentil & Gautrelet, 1938), and it has been collected in the perfusate after stimulation of the nerves leading to the dorsal muscles of the leech (Bacq & Coppee, 1937). Since acetylcholine mimics nerve stimulation, it has been suggested that this is a cholinergic system, although no electrophysiological investigations have been performed on it. However, recently intracellular recordings have been made from the dorsal muscle fibres (Washizu, 1967). Pharmacologically the leech nerve muscle junction is considered to be nicotinic (Minz, 1955). Nicotine mimics the action of acetylcholine at low concentrations while at higher concentrations nicotine blocks the cholinergic response. The acetylcholine effect is antagonized by tubocurarine, and tubocurarine antagonizes neuromuscular transmission following stimulation of the nerve which innervates the muscle. However, in other annelids—for example, *Sipunculus* and *Aphrodite*—neuromuscular transmission is antagonized by atropine (Bacq, 1947), suggesting that muscarinic cholinergic systems are present in some annelids. From the present study it would appear that in the leech the central cholinoceptive system is nicotinic. Thus both central and peripheral cholinoceptive systems are nicotinic.

SUMMARY

1. Recordings from the cells of Retzius of the leech, *Hirudo medicinalis*, show that they have resting potentials of between 40 and 50 mV, with action potentials ranging in amplitude from 20 to 50 mV.

2. The spontaneous activity of the cells of Retzius is increased following the application of acetylcholine. The threshold amount of acetylcholine is 10 μg.

3. The most effective antagonists of the acetylcholine response are benzoquinonium and decamethonium. These compounds antagonize the acetylcholine response when present at a concentration 1,000 times less than the concentration of acetylcholine.

4. Atropine, hexamethonium and tubocurarine also antagonize the action of acetylcholine. Atropine antagonizes the acetylcholine response when present at a concentration 20 to 100 times less than the concentration of acetylcholine.

5. The spontaneous activity of the cells of Retzius is decreased following the application of 5-hydroxytryptamine. The threshold amount of 5-hydroxytryptamine is 0.1 μg.

6. Both methysergide and atropine antagonize the effect of 5-hydroxytryptamine in some preparations.

7. The present investigation indicates that it is possible that a nerve cell could be excited synaptically by a given chemical and also have the same chemical as its own transmitter.

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