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CHOLINERGIC TRANSMISSION IN THE FROG SPINAL CORD

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The effect of acetylcholine and other drugs has been tested on the isolated and sagittally hemisected spinal cord of the frog (*Rana temporaria*). The release of acetylcholine from this preparation during stimulation of the spinal roots and whole hemicord has also been studied. After inactivation of the tissue cholinesterases acetylcholine was released spontaneously from the preparation at a steady rate of about 5.5 pmole/15 min/preparation. No increase in this release was obtained by stimulating the dorsal roots, but antidromic stimulation of the ventral roots always gave an increase in the rate of acetylcholine release up to 2.3 times the spontaneous level. Direct stimulation of the spinal cord did not alter the rate of acetylcholine release. Acetylcholine (1 mM) and eserine (10 mM) had weak excitatory actions and prolonged the reflex response of the preparation during dorsal root stimulation, but dihydro-β-erythroidine and atropine had no effect. The response evoked by antidromic ventral root stimulation was potentiated by eserine (10 μM) and depressed by acetylcholine (10 μM) and dihydro-β-erythroidine (1 μM) as in the toad preparation. The evidence supporting cholinergic transmission at some synapses in the spinal cord is discussed in relation to these results.

In a previous paper (Kiraly & Phillis, 1961) it was suggested that the slow depolarizing dorsal root potentials evoked by stimulation of adjacent ventral roots of the toad spinal cord were generated along a neuronal pathway containing at least one cholinergic synapse. It was further suggested that this synapse might be similar, anatomically and pharmacologically, to the synapse between motor axon collaterals and Renshaw cells in the cat.

It is generally considered (Crossland, 1957; Paton, 1958) that, to qualify as a synaptic transmitter agent, the postulated substance should be released during stimulation of the appropriate pre-synaptic elements. Hence it was felt that the demonstration of an increased acetylcholine content in the solution bathing the isolated frog spinal cord during ventral root stimulation would provide further evidence of cholinergic synapses in the ventral root–dorsal root pathway.

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In 1956, Angelucci described the release of an acetylcholine-like substance from the spinal cord of a frog perfused *in situ*. Increased amounts were said to be present in the perfusate during stimulation of the hind foot, and the author used this finding as evidence for an acetylcholine-like compound involved in reflex transmission. This conclusion, however, was but weakly supported by the related findings of Eccles (1947) and Bonnet & Bremer (1952), who showed that high concentrations of acetylcholine are required to cause excitation and that neither atropine nor tubocurarine depress reflex transmission. Further investigations were therefore carried out on the release of acetylcholine during dorsal root stimulation.

Since Kiraly & Phillis (1961) used isolated toad spinal cords, it has also been necessary to confirm their findings on the effects of anticholinesterases, atropine, tubocurarine and dihydro-β-erythroidine on antidromic dorsal root potentials of the frog spinal cord.

METHODS

All experiments have been performed on the isolated spinal cord of the frog (*Rana temporaria*). The spinal cord, after exposure and removal from the vertebral canal, was hemisected longitudinally and one hemicord fixed in a small trough, with a capacity of 0.3 ml., through which solutions were passed. Two solutions were used during the experiments. One,

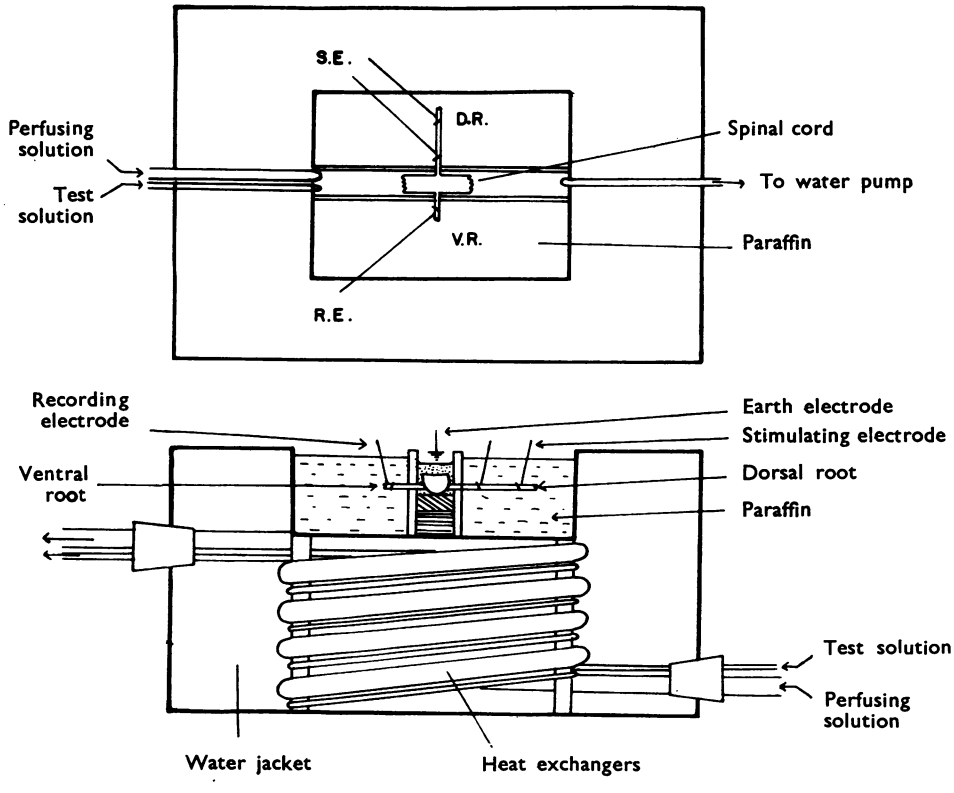

Fig. 1. Plan and sectional views of the waterbath and trough with a hemisected cord in position.

which had the following composition: NaCl 100 mM; KCl 2.5 mM; Na_2HPO_4 0.45 mM; $CaCl_2$ 1.9 mM; $NaHCO_3$ 12 mM; glucose 2.8 mM, was used during some experiments. The second was a standard leech Ringer solution (NaCl 86 mM; KCl 3.1 mM; $CaCl_2$ 1.2 mM; $NaHCO_3$ 1.3 mM; glucose 6.2 mM) and was used in experiments during which the release of acetylcholine from the tissue was being studied. The preparation was able to survive and respond normally for several hours in the latter solution. Solutions were equilibrated with a gaseous mixture of 95% oxygen and 5% carbon dioxide before delivery into the trough. Provision was also made for the direct oxygenation of the solution in the trough during experiments involving the collection of perfusates.

The 8th and 9th ventral and dorsal roots, which, depending on the purpose of the experiment, could be left selectively attached to the sciatic nerve trunks to facilitate stimulation or recording, were laid out on either side of the trough and the lateral walls lowered into position so that the roots passed out through small grooves etched into their lower edges. The compartments on either side of the trough were then filled with liquid paraffin. The trough and paraffin pools formed a portion of the top of a water jacket through which water was circulated to maintain a constant temperature (15 to 16° C). The perfusing fluid and fluid containing drugs to be tested passed through the water jacket before reaching the trough. Pairs of platinum electrodes on the ventral and dorsal roots were used for stimulating and recording; the reflex responses and antidromic dorsal root potentials of the preparations being inspected before acceptance of the preparation for experiments. Ventral root reflexes and dorsal root responses were displayed either on an oscilloscope or on some occasions by a DC paper recorder (Curtis, Phillis & Watkins, 1961a). The responses were similar to those recorded in the toad by Kiraly & Phillis (1961).

Direct stimulation of the preparation was also possible using two electrodes, insulated except at their tips, inserted directly 2 mm apart in the long axis of the spinal cord at the junction of thoracic and lumbar regions.

In the experiments on the release of acetylcholine-like substances from the spinal cord, the tissue cholinesterases were inhibited by pretreatment with either eserine sulphate (27 μM) or Sarin (isopropylmethylphosphorofluoridate (71 μM)) at least 30 min before collecting began. When eserine was used it was present throughout the experiment, but Sarin, which causes a long-lasting inhibition of cholinesterase, was only applied for 30 min.

When assaying for the release of acetylcholine the trough was emptied completely and 0.3 ml. of solution added from a pipette. Stimulation was commenced immediately and continued for the 15 min of the collection period. The fluid was removed and stored at 0.5 to 1° C until assayed. Following a collection period, the spinal cord was washed continuously for at least 10 min before recommencing collection to allow all the acetylcholine released by the previous stimulation to diffuse away. Stimulation frequencies used during the present investigation were 1/sec, 4/sec and 20/sec. At all these frequencies the magnitude of the dorsal and ventral root responses of the preparation soon fell during the stimulation period, but at even lower frequencies it was not possible to assay the released activity because of the high background levels.

Solutions were assayed for acetylcholine on the dorsal muscle of the leech set up as described by MacIntosh & Perry (1950) but in a 0.3 ml. muscle bath. Even with these small baths it was not possible to carry out satisfactory controls with the active samples because of their small volume, which prevented more than one application of each perfusate to the leech muscle. However, on two occasions the estimates obtained by this method were confirmed by four-point assays of the same solutions on a microleech preparation as described by Szerb (1961).

Complete identification of the active factor released during these experiments was also hindered by the low levels of activity. However, the following tests, which were carried out in several experiments, indicate that the activity was due to acetylcholine or a related choline ester: (*a*) before the addition of a cholinesterase inhibitor the samples did not contain detectable levels of activity (<2 pmole/ml. acetylcholine); (*b*) the response of the leech muscle

to the samples was potentiated by the addition of an anticholinesterase to the assay bath; and (*c*) treatment of the leech muscle with tubocurarine chloride for 10 min resulted in the abolition of effects of previously active pooled solutions as well as those of the equivalent acetylcholine control solutions. A more thorough identification of acetylcholine released from a toad brain and spinal cord preparation during direct stimulation has been reported by Li (1938). However, as the spinal cord was not stimulated independently of the brain, the relevance of his results to the present investigation cannot be ascertained.

Although it has not been possible to identify the released activity as acetylcholine rather than a very similar choline ester, it is for convenience subsequently referred to as acetylcholine. All results have been expressed in terms of acetylcholine chloride (Roche Products).

RESULTS

Spontaneous release

After inactivation of the tissue cholinesterases acetylcholine could be detected in the collection fluid from all the spinal cords tested. During the early phases of each experiment, when collecting started within about 30 min of setting up the preparation, relatively high levels of activity appeared in the perfusate (up to 30 pmole acetylcholine/15 min). However, during subsequent collection periods the level of activity declined quite sharply to a lower but stable level of about 5.5 pmole/15 min/preparation. Since the initial spontaneous release was relatively high and subject to rapid alteration, electrical stimulation of the preparation was not commenced until the spontaneous release had reached a stable level.

Dorsal root stimulation

The relative lack of action of drugs which modify transmission at cholinergic synapses on the reflex responses of the frog spinal cord has already been described by several authors. Acetylcholine has an excitant action, but only when applied in relatively high concentrations (Eccles, 1947; Angelucci, 1956). Anticholinesterases have been reported to have either excitant (Eccles, 1947; Bonnet & Bremer, 1952; Kolmodin & Skoglund, 1953) or no obvious actions (Angelucci, 1956), and antagonistic drugs such as atropine and tubocurarine were either inactive (Angelucci, 1956) or had excitant actions (Eccles, 1946; Bonnet & Bremer, 1952). In the present series of experiments it has been confirmed that acetylcholine (1 mM) and eserine (10 μM) have weak excitant actions causing an increase in the spontaneous activity and prolongation of the reflex responses of the preparation. Dihydro-β-erythroidine (100 μM) and atropine (100 μM) had no obvious action on reflex transmission. The failure to demonstrate a significant increase in the release of acetylcholine during dorsal root stimulation was therefore not surprising (Table 1). The variations in the amount of acetylcholine were small and within the margins of error of the bioassay, which at these levels of activity was $\pm 20\%$.

Ventral root stimulation

Antidromic stimulation of the 8th and 9th ventral roots in the amphibian spinal cord evokes a slow depolarizing potential change in the adjacent dorsal roots. The characteristics of this potential in frog dorsal roots have already been described (Barron & Matthews, 1938; Eccles & Malcolm, 1946; Lloyd & McIntyre, 1949;

TABLE 1

THE SPONTANEOUS RELEASE OF ACETYLCHOLINE FROM THE FROG SPINAL CORD AND THE CHANGES OBSERVED DURING ELECTRICAL STIMULATION

* These values represent the group average of the spontaneously released acetylcholine immediately before and after the period of stimulation. † The values in this column represent the group average of the change in release during stimulation

Maximal stimulation of	Frequency of stimulation/ sec	Number of experiments	Average spontaneous release* pmoles/15 min/ hemisected cord	% change† during stimulation
Dorsal roots	1	6	6·5	−13
	4	4	9·0	+10
Ventral roots	1	4	5·5	+52
	4	19	5·5	+93
	20	5	4·5	+132
Spinal cord	1	3	6·0	−20
	4	4	8·0	−7

Koketsu, 1956) and are similar to those that can be recorded in the dorsal roots of the toad spinal cord (Kiraly & Phillis, 1961). In the course of the present investigation it has been shown that the effect of drugs on the response is apparently also identical in both species of amphibia. Eserine (10 μM) potentiated the response, a finding which had previously been described by Koketsu (1956), and dihydro-β-erythroidine (1 μM) depressed the potential. Katz & Miledi (1962) have also described a depressant action of tubocurarine chloride on the antidromic dorsal root potential of the frog, confirming a similar finding by Kiraly & Phillis (1961) on the toad. Acetylcholine (10 μM) also depressed the potential as in the toad.

Antidromic stimulation of the ventral roots of the 28 preparations used in this series of experiments invariably resulted in an increased release of acetylcholine from the spinal cord. The results at several frequencies of stimulation are shown in Table 1. In each case the percentage increase in release of activity has been calculated on the basis of the average of spontaneously released acetylcholine immediately before and after stimulation. The values shown in Table 1 represent the average of the percentage increases.

Stimulation at a frequency of 1/sec of four preparations resulted in an average increase in acetylcholine of 52%. In another experiment during which the ventral roots were stimulated at this frequency, a 660% increase in release resulted. However, since only one assay of the sample was possible and since increases of this order of magnitude were unique, this result has not been included in Table 1. In the majority of experiments the ventral roots were stimulated at a frequency of 4/sec; this series resulted in an increased release of 93%. A further five experiments during which the ventral roots were stimulated at 20/sec yielded a 132% increase in the release of acetylcholine.

In Table 2 the results obtained during two individual experiments are shown. These experiments have been selected because they also illustrate the failure of dorsal root stimulation to cause a demonstrable release of acetylcholine from preparations which responded to ventral root stimulation with an increased release. Both experiments also illustrate another feature of the effect during ventral root

TABLE 2

THE CHANGES FOUND IN ACETYLCHOLINE RELEASE (pMOLE/15 MIN COLLECTION/ HEMISECTED SPINAL CORD) FROM FROG SPINAL CORD PREPARATIONS

US=Unstimulated. VRS=Stimulation of ventral root. DRS=Stimulation of dorsal root

Expt. 1		Expt. 2	
US	8	US	2
DRS (4/sec)	4·5	VRS (4/sec)	4·5
VRS (4/sec)	8	US	2
US	3	VRS (4/sec)	5·5
VRS (20/sec)	5·5	US	2
US	2·5	DRS (1/sec)	1
		US	1
		VRS (4/sec)	2
		US	2

stimulation, namely, the decline in the amount of acetylcholine release during repeated periods of stimulation of the ventral root. For this reason the majority of preparations were only stimulated once and then discarded.

Direct stimulation of the spinal cord

A series of 7 experiments was carried out during which the preparation was stimulated by electrodes placed directly in the spinal cord. Short latency reflex responses in the ventral roots following stimulation of the lateral column have been described (Machne, Fadiga & Brookhart, 1959 ; Brookhart & Fadiga, 1960) and it was felt that stimulation of this pathway would yield further evidence of the presence or absence of cholinergic synaptic junctions on to the motoneurones. Moreover, stimulation in this manner might be expected to excite pathways which, though not involved in reflex transmission, nevertheless contained cholinergic synapses.

However, as can be seen in Table 1, direct stimulation of the spinal cord at frequencies of 1/sec and 4/sec resulted only in small changes in acetylcholine output, within the error of the assay technique.

DISCUSSION

The use of *in vitro* preparations such as the isolated amphibian spinal cord makes it possible to avoid many of the difficulties associated with *in vivo* central nervous tissues. Thus the problem of blood-brain barrier impermeability is circumvented. Furthermore, peripheral actions or actions on the vascular system within the spinal cord of pharmacologically active compounds, with consequent indirect modification of neuronal responses, are avoided.

The pharmacological results on dorsal root–ventral root reflex responses described in this paper have largely confirmed those reported by previous authors (Bonnet & Bremer, 1952 ; Eccles, 1946 ; Kolmodin & Skoglund, 1953 ; Angelucci, 1956) and are similar to those of the toad (Kiraly & Phillis, 1961). Thus acetylcholine itself has relatively little action unless it is applied in high concentrations, and anti-cholinesterases, although slightly increasing reflex responses, have little potentiating action on acetylcholine. Atropine, dihydro-β-erythroidine, and tubocurarine do not depress reflex responses as would be expected if acetylcholine is involved in synaptic

transmission along this pathway. This latter finding confirms the results of Eccles (1946), Bonnet & Bremer (1952) and Angelucci (1956), but not those of Wright & Taylor (1949), who reported that dihydro-β-erythroidine blocks synaptic transmission in the isolated frog spinal cord. The latter authors also failed to see an excitant action of curare on the preparation, as described by Eccles (1946) for the frog and Kiraly & Phillis (1961) for the toad.

From these results it is evident that cholinergic synapses play only a minor moderating role in the conduction of activity from dorsal to ventral roots. This conclusion has been supported by the demonstration that there was no significant increase in the release of acetylcholine from the preparation during dorsal root stimulation. Likewise the failure to demonstrate an increased release of activity during direct stimulation suggests that acetylcholine is not involved to any great extent in the synaptic activity of the preparation.

On the basis of their results on the pharmacology of the antidromic dorsal root potential, Kiraly & Phillis (1961) suggested that there might be a cholinergic synapse interposed in the pathway from ventral to dorsal roots in the toad spinal cord. It was postulated that this synaptic link corresponded to the motor-axon collateral–Renshaw cell synapse in the cat (Eccles, Fatt & Koketsu, 1954; Eccles, Eccles & Fatt, 1956; Curtis, Phillis & Watkins, 1961b). The presence of motor axon collaterals in the frog has been confirmed by Silver (1942).

It was found that anticholinesterases potentiate the antidromic dorsal root potential and dihydro-β-erythroidine depresses it. Acetylcholine itself also depresses the potential, probably due either to a desensitization of the synapse or else to an occlusion of the synaptically evoked firing by the high level of background firing initiated by the artificially applied acetylcholine. The existence of this cholinergic synaptic link is further supported by the results on the release of acetylcholine during ventral root stimulation. It has been shown that ventral root stimulation at three different frequencies resulted in an increased level of activity in the perfusate and that the release/stimulus declined rapidly with increased frequencies of stimulation. A similar finding has been reported for the neuromuscular junction (Straughan, 1960; Krnjević & Mitchell, 1961) and cerebral cortex (Mitchell, 1962). The amount of acetylcholine released during successive periods of ventral root stimulation was found to decline, indicating that there was a progressive failure in the replacement or mobilization of the transmitter. Attempts to avert this failure of release by the addition of choline chloride to the perfusing fluid (D. A. Brown, personal communication) did not yield conclusive results.

The possibility that some of the acetylcholine released during the present experiments came from the ventral roots has not been excluded. Several investigators (including Calabro, 1933; Bergami, 1936; and Lissak, 1939) have reported that *in vitro* stimulation of various nerves liberates acetylcholine, although Gaddum, Khayyal & Rydin (1937) concluded that this only happened when the nerve underwent degenerative changes as a result of the stimulation. Thus, although Lissak (1939) has recorded that 5.5 pmoles of acetylcholine were released from the frog's sciatic nerve by stimulation for 10 min, there are several reasons for believing that this was not the major source of the acetylcholine in these experiments. First, the

spinal cords were placed in the narrow trough so that the ventral roots passed directly into the paraffin pool. Thus only those portions of the motor axons which were actually incorporated in the tissue of preparation would have been present in the trough. Second, Lissak was unable to demonstrate any resting release of acetylcholine from intact regions of unstimulated nerves. Third, the nerves were able to respond to repeated periods of stimulation with a constant release of acetylcholine.

The relatively high levels of spontaneous release during the early phases of the experiments confirm the findings of Angelucci. However, his conclusions are different from ours regarding the importance of the cholinomimetic factor released during dorsal root–ventral root reflex transmission. The technique used in his experiments would have resulted in the stimulation of both afferent and efferent fibres in the limb, and the increased release of the factor observed may well have been due to this antidromic activity in the efferent fibres.

The failure to observe a significant increase in the release of acetylcholine during dorsal root stimulation which would also have activated motoneurones is not altogether surprising, since it is unlikely that all the motoneurones whose axons discharged during antidromic volleys would also have responded to the less direct dorsal root afferent volley.

A similar argument can be applied to the result obtained with direct stimulation of the spinal cord. The greatest density of motoneurones in the amphibian spinal cord lies in the regions of brachial and lumbar enlargements from which the nerve plexuses to the limbs arise (Silver, 1942), with fewer in the intervening area. As the stimulating electrodes were located in this immediate area, the direct pulses would not have been expected to excite as many pre-synaptic cholinergic fibres if these are in fact the motor axon collaterals.

The weak excitant actions of acetylcholine and anticholinesterases on dorsal root–ventral root reflex transmission may well be the result of actions at the postulated cholinergic synapses. Since the long latency of the antidromic dorsal root potential suggests that there are several synapses along the generating pathway apart from the first, transmission at these may well be non-cholinergic. However, excitation at the initial synapse by antidromic ventral root stimulation or the application of acetylcholine and anticholinesterases with the consequent excitation of the other related cells would be expected to increase the general level of excitability of the preparation, and hence increase reflex responses.

Such a facilitation has been demonstrated by Katz & Miledi (1962), who were able to increase the ventral root reflex responses to sub-maximal dorsal root stimulation by preceding it with an antidromic volley in another ventral root. The discharge of efferent volleys down adjacent ventral roots after antidromic ventral root stimulation (Washizu, 1960) is further evidence of the excitatory influence of activity in this pathway on other neurones. In this respect, however, the amphibian preparations apparently differ from mammalian tissues, for Renshaw cells in the cat are known to inhibit motoneuronal discharges (Renshaw, 1941; Eccles *et al.*, 1954), and the occasional late facilitation is probably in fact a "dis-inhibition" (Wilson, Diecke & Talbot, 1960; Wilson & Burgess, 1962).

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