J. Physiol. (1977), **269**, *pp.* 195–219
With 14 *text-figures*
Printed in Great Britain

RESPONSES OF IDENTIFIED SPINAL NEURONES TO ACETYLCHOLINE APPLIED BY MICRO-ELECTROPHORESIS

BY N. R. MYSLINSKI* AND MIRJANA RANDIĆ†

From the Department of Biochemistry and Pharmacology, Tufts University School of Medicine, Boston, Massachusetts 02111, U.S.A.

(*Received 18 January 1977*)

SUMMARY

1. The responses of identified cells in the cat Clarke's column and dorsal horn to micro-electrophoretically applied cholinomimetics and anticholinergic substances have been investigated.

2. Both antidromically identified (DSCT neurones) and synaptically activated neurones from the region of the Clarke's column of the spinal cord were excited by ACh. However, the proportion of ACh excited cells was greater in units synaptically activated by ipsilateral dorsolateral funiculus stimulation (78%) than in DSCT neurones (50%). In addition, about 55% of neurones activated either antidromically or synaptically by ipsilateral dorsal column stimulation were excited by ACh.

3. In contrast to a relatively weak excitatory potency on the DSCT neurones (maximum firing frequency did not exceed 130% of the control level), ACh proved to be a more potent excitant of units synaptically activated by ipsilateral dorsolateral funiculus stimulation (maximum firing frequency reached 430% of the control level).

4. ACh has a relatively quick and rapidly reversible excitatory effect on Clarke's column neurones and some types of dorsal horn interneurones, which can be obtained also with nicotine. However, the action of nicotine is frequently delayed in onset and recovery. This excitatory action of ACh can be blocked or markedly depressed by dihydro-β-erythroidine. These results and those obtained with acetyl-β-methylcholine and atropine seem to suggest that the receptors mediating excitation of the cholinoceptive spinal cells activated either antidromically or synaptically by ipsilateral dorsolateral funiculus stimulation besides predominantly nicotinic have also weak muscarinic properties.

* Department of Pharmacology, The University of Maryland Dental School, Baltimore, Maryland 21201, U.S.A.

† Department of Veterinary Anatomy, Pharmacology and Physiology, Iowa State University of Science and Technology, Ames, Iowa 50011, U.S.A.

5. Desensitization with repeated applications of ACh and nicotine has been observed in both DSCT neurones and units antidromically activated by ipsilateral dorsal column stimulation.

6. About 11% of units antidromically activated by ipsilateral dorsolateral funiculus stimulation were depressed by ACh. In addition, the depressant effect of ACh was more frequently encountered in the cells unresponsive either to the dorsolateral funiculus or dorsal column stimulation. ACh depression was also seen in units activated either antidromically or synaptically by ipsilateral dorsal column stimulation. In contrast, none of the units synaptically activated by the ipsilateral dorsolateral funiculus stimulation were depressed by ACh. The same was true for spinal neurones receiving convergent peripheral inputs activated either antidromically or synaptically by ipsilateral dorsolateral or dorsal column stimulation.

7. The findings that ACh depression of all tested DSCT neurones is blocked by atropine and readily evoked by acetyl-β-methylcholine indicates that receptors mediating the effect are of muscarinic type.

INTRODUCTION

Biochemical and histochemical studies have demonstrated the presence of acetylcholine (ACh), choline acetyltransferase and acetylcholinesterase in the spinal cord (MacIntosh, 1941; Feldberg & Vogt, 1948; Roessmann & Friede, 1967; Kasa, Mann & Hebb, 1970; Silver & Wolstencroft, 1970, 1971; Odutola, 1972). A number of workers have studied the chemical sensitivity of spinal cord interneurones to ACh, but the results obtained are controversial. Thus Curtis, Phillis & Watkins (1961) could not find any effect of ACh, while Engberg & Ryall (1966) reported that some spinal interneurones were depressed by ACh. Weight & Salmoiraghi (1966) besides confirming the depressant effect of ACh, found interneurones throughout the grey matter facilitated by ACh. On the basis of their findings and the results obtained following systemic application of ACh (Feldberg, Gray & Perry, 1953; Fernandez DeMolina, Gray & Palmer, 1958) Weight & Salmoiraghi suggested that ACh could function as an excitatory transmitter in the spinal cord.

To further investigate this possibility we have studied the chemical sensitivity of the cat Clarke's column neurones and the dorsal horn interneurones to ACh and some cholinomimetic and anticholinergic drugs. We have found that ACh produces both excitation and depression in Clarke's column neurones and some dorsal horn interneurones. The nature of the responses to ACh and the pharmacological properties of receptors mediating excitation and depression in spinal neurones are described in this

paper. In addition an attempt has been made to relate excitation or depression, of identified cat Clarke's column neurones and to a lesser degree some dorsal horn interneurones produced by ACh, to a synaptic input. Preliminary accounts of some of the material presented here have appeared elsewhere (Randić & Myslinski, 1974; Myslinski, Randić & Ledgere, 1974; Randić & Myslinski, 1976).

METHODS

Experiments were carried out on seventy-seven adult cats of either sex. The animals were initially anaesthetized with ether (Mallinckrodt) or halothane (Fluothane, Ayerst). The brain was anaemically destroyed by bilateral occlusion of the common carotid and vertebral arteries. The spinal cord was transected at the first cervical level and the brain rostral to the section was pithed. Thereafter, the animal was maintained on artificial respiration and immobilized by intravenous injections of gallamine triethiodide (Flaxedil, American Cyanamid Co.) or by a constant infusion of succinylcholine chloride (Anectine, Burroughs Wellcome). Body temperature was maintained at 37 ± 1° C by means of an automatic servo-control device. Both carotid blood pressure and end-tidal carbon dioxide concentration were monitored throughout the experiment, the latter being maintained between 4 and 5 %.

The spinal cord was exposed at two regions, one the upper three segments of the thoracic cord and the other the third lumbar segment. The spinal cord was hemisected just above the first thoracic segment and the dorsal column and the left dorsolateral funiculus were dissected for about 1 cm caudal to the section, after which they were prepared separately for electrical stimulation. Electrical recording from the neurones of the dorsal spinocerebellar tract (DSCT) of the Clarke's column was identified by action potentials following stimulation of the ipsilateral dorsolateral funiculus. Movement of the spinal cord was minimized by rigid clamping of the spinal column and the pelvis in addition to performing a bilateral pneumothorax. For further stabilization of the spinal cord, 2 % agar in lactated Ringers solution (Hartmann's solution, Baxter Laboratories) was used.

Activity of the Clarke's column neurones was recorded extracellularly through the central barrel of a five-barrelled glass micropipette, filled with a solution of fast green (FCF, Matheson, Coleman and Bell) saturated in 3 M sodium chloride. The site of recording was thus marked with dye by passing current (10 μA for 10 min) through the recording barrel. The size of the common tip of the five-barrelled glass micro-electrode was between 4 and 6 μm. The construction and filling of the five-barrelled micropipettes have been described by Krnjević & Phillis (1963). The technique and theory of microelectrophoresis in its present application has also been elaborated in detail with attention to its limitations (Curtis, 1964; Salmoiraghi & Stefanis, 1967; Krnjević, 1971). One of the outer barrels of the micropipette contained 1–3 M sodium chloride ('balance' channel) which served as a control for the usual excitatory effect of inward (tip negative) current or the depressing effect of outward (tip positive) current. The remaining outer barrels were filled with aqueous solutions of the various salt complexes to be tested.

The drugs used with their usual concentrations and pH values were as follows: ACh chloride (0·5 or 1·0 M, pH 4·0–4·4, K & K labs; Schwarz/Mann); acetyl-β-methylcholine chloride (0·5 or 1·0 M, pH 4·0, Aldrich Chemical Co.); atropine sulphate (0·01 or 0·2 M, pH 4·4 or 5·5, Schwarz/Mann); carbaminoyl choline chloride (0·5 or 1·0 M, pH 4·4–4·7, Gallard Schlesinger, BDH); dihydro-β-erythroidine hydrobromide

(0·1 M, pH 5·3, Merck, Sharp and Dohme Res. Laboratories); nicotine hydrogen (+)-tartrate (0·5 M, pH 4·0–4·3, B.D.H.; Gallard-Schlesinger).

Sensory inputs to the Clarke's column and dorsal horn neurones were determined either by electrical stimulation of several peripheral nerves dissected out in the left hind limb or by natural (adequate) stimuli applied to the left hind limb with intact innervation. The peripheral nerves prepared for electrical stimulation were four muscle nerves (quadriceps, posterior biceps-semitendinosus, triceps surae and deep peroneal) and three cutaneous nerves (sural, posterior femoral cutaneous and superficial peroneal). Identification of sensory receptor types was primarily based on the criteria described by Burgess, Petit & Warren (1968).

RESULTS

Excitation by ACh

In total, one hundred and twenty-two spinal neurones located either in the Clarke's column or the dorsal horn were tested with ACh. ACh was expelled from the micropipette as a cation with currents ranging from 20 to 200 nA for periods up to 2 min. In an attempt to define more precisely the type of unit responding to ACh, the responses of cells to electrical stimulation of the ipsilateral dorsolateral funiculus and dorsal column were noted. The results are summarized in Table 1.

Cells antidromically activated by ipsilateral dorsolateral funiculus electrical stimulation. About 75% of these cells were located within the Clarke's column and thus were identified as the dorsal spinocerebellar tract neurones. Identification of units adequately activated by hair movement and/or both hair movement and light touch on the skin of the ipsilateral hind limb and lying dorsolaterally to Clarke's nucleus in the dorsal grey column of the spinal cord was not performed in these experiments by antidromic activation in response to electrical stimulation of the ipsilateral anterior cerebellar cortex. Therefore, we are uncertain about the proportion of DSCT neurones within this group (Randić, Myslinski & Gordon, 1976). Out of the fifty-one neurones antidromically activated by electrical stimulation of the dorsolateral funiculus and receiving excitatory inputs from muscle or joint or cutaneous receptors about 50% of the units were weakly excited by ACh (Table 1). However, five cells receiving convergent inputs (the convergent group includes only those neurones which receive excitatory inputs from more than one of the three sources, i.e. muscle, cutaneous and joint) proved to be insensitive to ACh. Excitation was observed as initiation of firing in previously quiescent cells or as an increase in the rate of spontaneous firing. Excitation had a latency of 1–5 sec and persisted maximally for about 5 sec after ending the ACh ejection. Except for two units, excitation was not seen if ACh was administered with currents of less than 60 nA. In a majority of these neurones, the maximum increase in firing rate produced by ACh

TABLE 1. ACh sensitivity of Clarke's column and dorsal horn neurones

Response of spinal units to electrical stimulation of the ipsilateral dorsolateral funiculus or dorsal column	No. of cells	Input		Acetylcholine effect: Excitation (%)	Depression (%)	Nil (%)
Cells antidromically activated by ipsilateral dorsolateral funiculus stimulation	56	Muscle and/or joint	41	51	13	36
		Cutaneous	10	50	10	40
		Convergent (cutaneous + muscle + joint)	5	—	—	100
Cells synaptically activated by ipsilateral dorsolateral funiculus stimulation	23	Muscle	11	72	—	28
		Cutaneous	4	75	—	25
		Convergent	6	100	—	—
		Unknown	2	100	—	—
Cells synaptically activated by ipsilateral dorsal column stimulation	22	Muscle and/or joint	4	—	—	100
		Cutaneous	12	50	17	33
		Convergent	6	100	—	—
Cells antidromically activated by ipsilateral dorsal column stimulation	5	Cutaneous	4	50	25	25
		Joint	1	100	—	—
Cells *not* activated either by ipsilateral dorsolateral funiculus or dorsal column stimulation	16	Muscle and/or joint	7	57	23	20
		Cutaneous	7	15	70	15
		Convergent	2	—	50	50
Total	122					

did not exceed 130% of the control level. A typical excitatory response to ACh of a unit receiving afferent input from muscle receptors is illustrated in Fig. 1. As seen in Fig. 1, when ACh was applied with a current of 60 nA, there was a latent period of about a second before an increase in firing occurred. On stopping the current, the firing declined to the

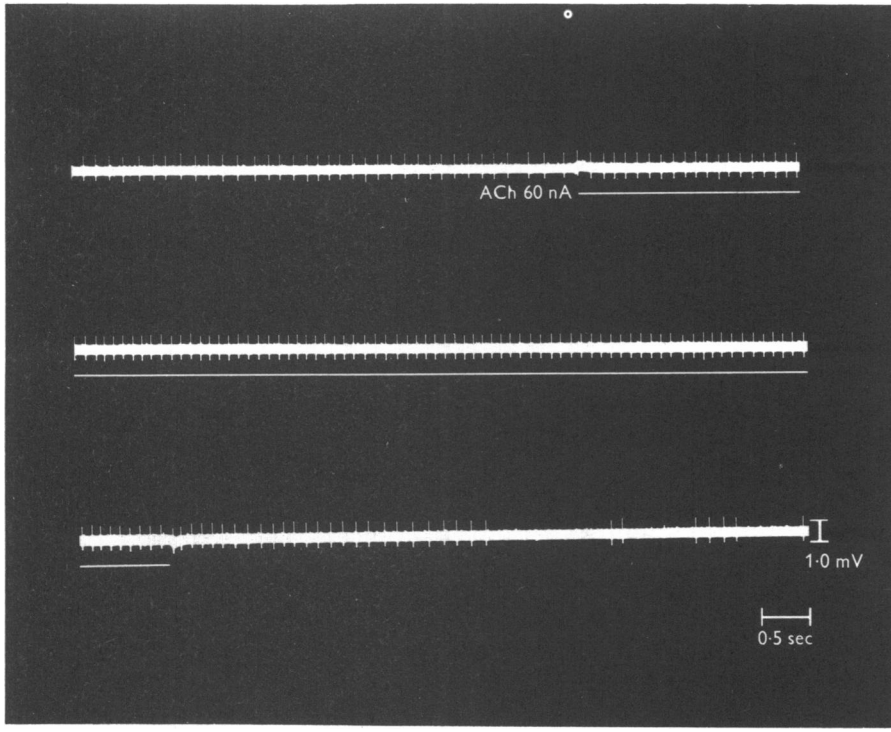

Fig. 1. The excitatory response to ACh (60 nA) of a spontaneously active DSCT cell receiving afferent input from muscle receptors. Note that ACh has a relatively quick and rapidly reversible excitatory effect on this cell. Photographic oscilloscope records of extracellular spike potentials from moving film.

control level within a second. Another remarkable feature was post-excitatory reduction in excitability of the cell, as manifested by the 'pauses' in spontaneous firing, frequently lasting in excess of a second.

In contrast, for example, to pyramidal tract cells (Stone, 1972), a tachyphylaxis was frequently apparent in the DSCT neurones. An example of tachyphylaxis to ACh is shown in Fig. 2, where a decrease in response of a DSCT-muscle unit to successive applications of ACh with currents of either 120 nA (Fig. 2*A*) or 160 nA (Fig. 2*B*) is apparent. With either value

of current, a period of 2–3 min elapsed between successive applications of ACh.

Cells synaptically activated by ipsilateral dorsolateral funiculus electrical stimulation. In contrast to a relatively weak excitatory potency on the DSCT neurones, ACh proved to be a more potent excitant of the Clarke's

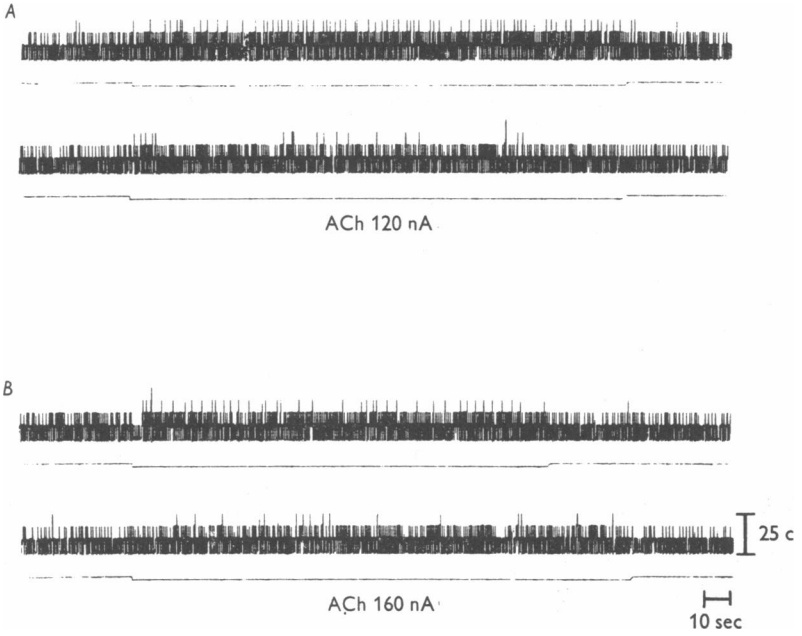

Fig. 2. ACh desensitization in a DSCT neurone receiving afferent input from muscle receptors. Rate-meter record for a spontaneously active unit facilitated by ACh (120 nA in *A*; 160 nA in *B*). Repeated applications of ACh lead to a decline in the excitatory effect (desensitization), as seen in the lower records in *A* and *B*.

column neurones, which were synaptically activated in response to electrical stimulation of the ipsilateral dorsolateral funiculus. Out of twenty-three cells tested, eighteen units (about 78%) were excited with ACh (Table 1). In these neurones the increase in the maximum firing frequency was in the range of 210–430% of the control level and was achieved within 5–20 sec of the onset of ACh application. As in DSCT cells the principal characteristics of the excitatory action of ACh were its relatively quick onset (0·3–3 sec; latency frequently increasing with higher ACh-currents) and cessation within 1–2 sec of the end of ACh application. The tachyphylaxis was also present in these neurones.

ACh was equally potent in units receiving sensory inputs from muscle

and cutaneous receptors. However, it is of interest that all six cells having convergent inputs were strongly excited by ACh.

Cells synaptically activated by ipsilateral dorsal column electrical stimulation. Out of twelve cells synaptically activated in response to electrical stimulation of the dorsal column and receiving sensory inputs from various types of cutaneous receptors about 50% were excited by ACh (Table 1).

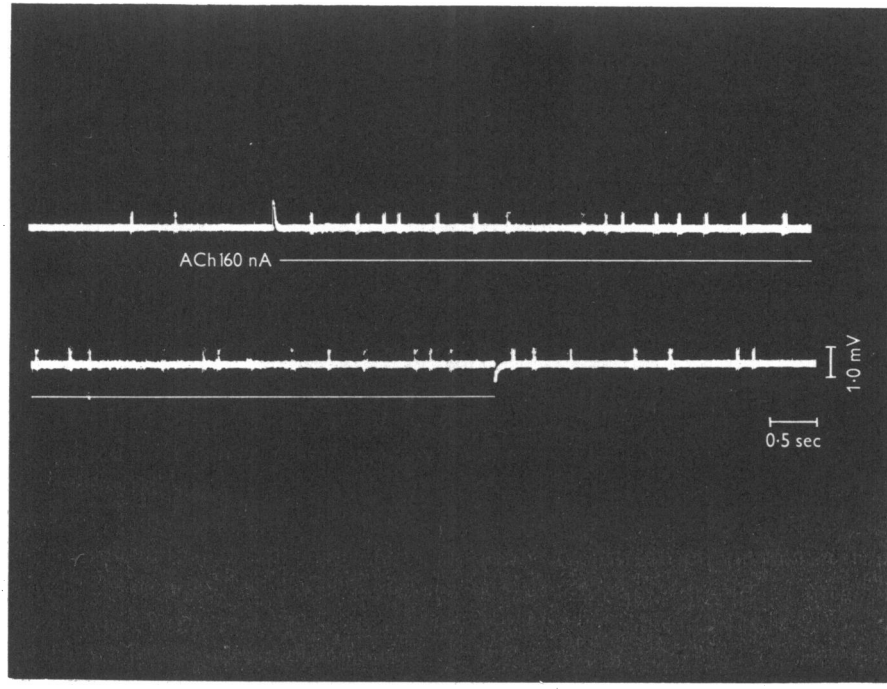

Fig. 3. The excitatory response to ACh (160 nA) of a spontaneously active unit synaptically activated by electrical stimulation of the ipsilateral dorsal column and receiving afferent input from the high threshold mechanoreceptors. Photographic oscilloscope records of spike potentials from moving film.

The characteristics of the excitatory action of ACh were similar to those described above for two other types of spinal neurones. The average increase in the firing rate was 151%. The excitatory response to ACh (160 nA) in a unit activated by a noxious stimulus (strong pinch to the skin of the calf) is illustrated in Fig. 3. In addition, six cells having convergent inputs were all excited by ACh. One example of a unit receiving both, proprioceptive and cutaneous inputs is seen in Fig. 4. In contrast four cells activated from muscle or joint receptors were insensitive to ACh.

Cells antidromically activated by ipsilateral dorsal column electrical stimulation. Out of five cells antidromically activated by electrical stimulation of the left dorsal column, three were excited with a short latency (1–2 sec) by ACh (Table 1). The maximum firing frequency did not exceed 152% of the control level. ACh (80–120 nA applied for 1–2 min) frequently

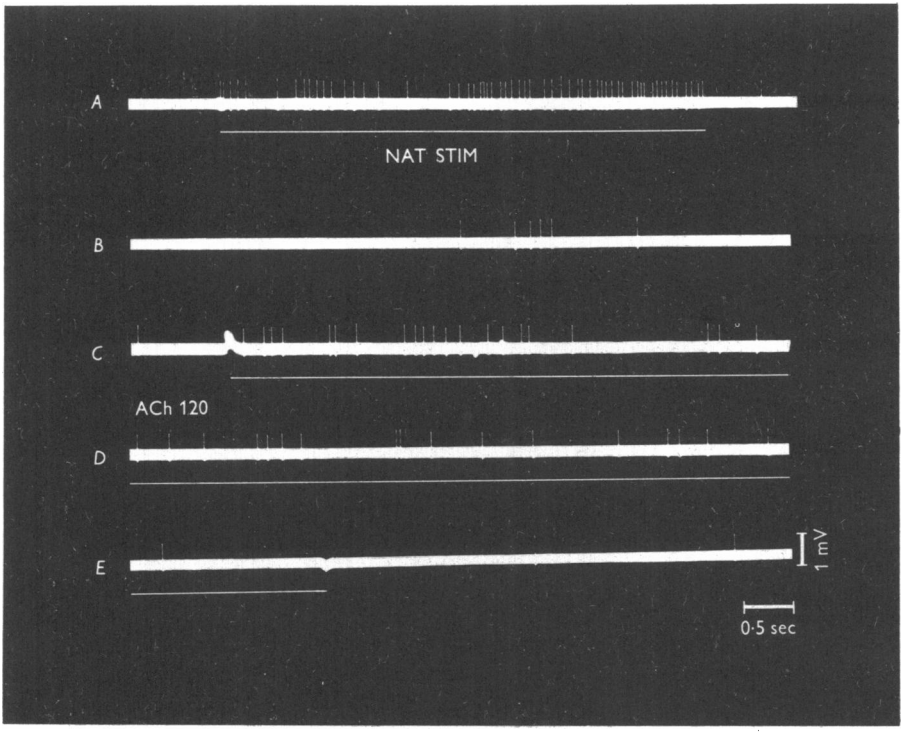

Fig. 4. The excitatory response to ACh (120 nA) of a spontaneously active unit synaptically activated by electrical stimulation of the ipsilateral dorsal column and receiving convergent (muscle, joint and cutaneous) afferent inputs. Unit activated by adequate stimulation (passive movement of knee joint), as shown in *A* (NAT STIM). The horizontal bars below the records indicate the duration of adequate stimulation (*A*) and microelectrophoretic ACh application (*C*, *D*, *E*).

produced a change in the pattern of discharge of the cells activated by cutaneous afferent volleys, which was manifested as an increase both in the number of high frequency bursts and the number of the action potentials within each burst, leaving the number of single action potentials unchanged or slightly increased (Fig. 5). Desensitization with repeated applications of ACh was observed in these neurones, as well.

Cells not activated either by ipsilateral dorsolateral funiculus or dorsal

column stimulation. Out of sixteen units which did not send their axons either in the ipsilateral dorsolateral funiculus or the dorsal column, five cells were strongly excited by ACh. The maximum increase in the firing rate produced by ACh varied between 200 and 300% of the control level. The excitatory effect of 80 nA of ACh on a unit receiving input from the

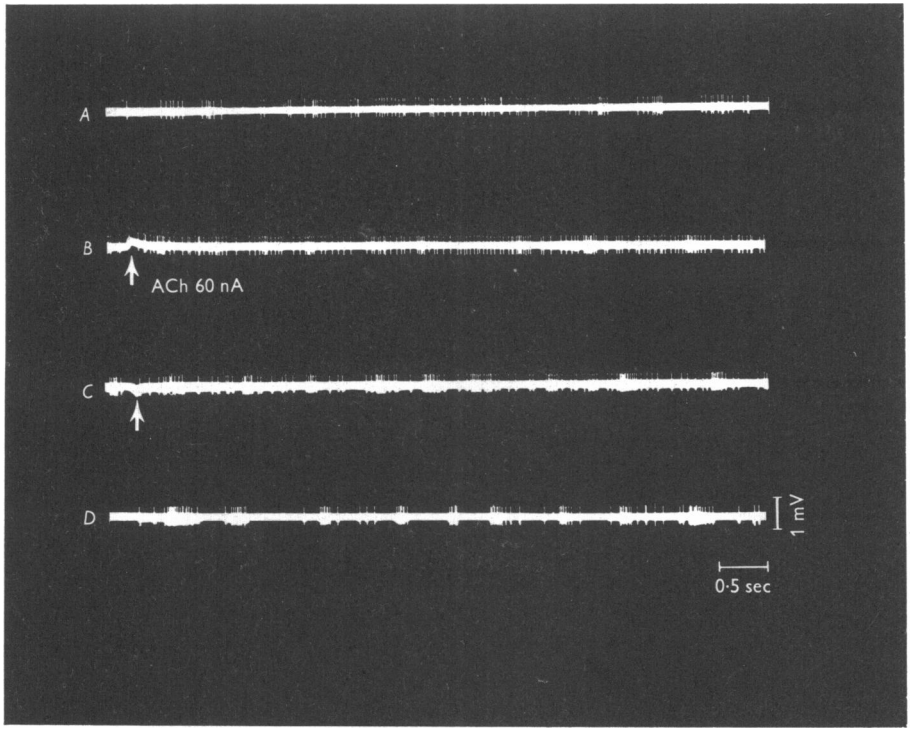

Fig. 5. ACh-produced change in the pattern of a discharge of a cell antidromically activated by electrical stimulation of the ipsilateral dorsal column and receiving cutaneous afferent input. ACh (60 nA) facilitated firing and produced a change in the pattern of discharge, which was manifested both as an increase in the number of high frequency bursts and the number of the action potentials within each burst, leaving the number of single action potentials unchanged or slightly increased.

hip joint is illustrated in Fig. 6. In some units post-excitatory depression of the spontaneous firing lasted for more than 15 sec. Tachyphylaxis with repeated applications of ACh was observed.

Location of ACh-excited units. Location of the cell bodies of the ACh-excited Clarke's column neurones and the dorsal horn interneurones was examined by marking the recording site with fast green (see Methods). The site of the recording was marked only when the extracellular response

showed a biphasic configuration (some response) with peak-to-peak amplitude in excess of 1·5–2·0 mV. After marking, the spinal cord was fixed with 10% formalin and sectioned transversely at 20 μm. Relative dye positions marked for twenty-two neurones are reproduced in Fig. 7, using the outline of Clarke's column (interrupted line-oval), the grey-white matter boundary (continuous line) and the central canal (filled

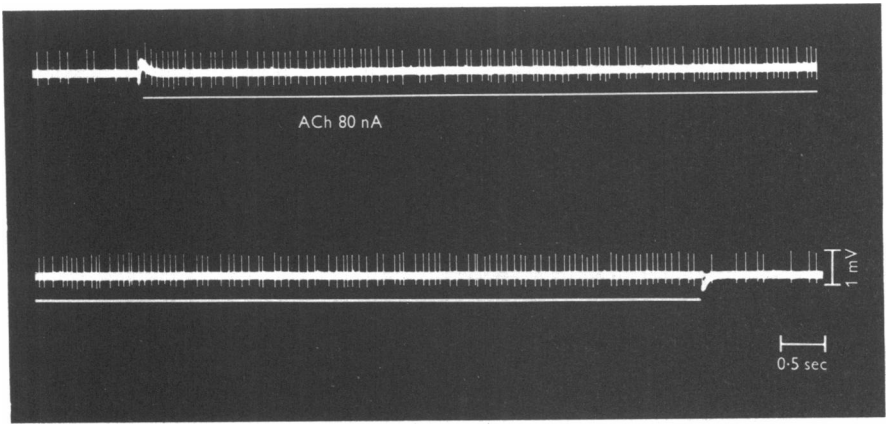

Fig. 6. The excitatory effect of ACh (80 nA) on a spontaneously active unit receiving afferent input from the hip joint. Unit was not activated either by ipsilateral dorsolateral funiculus or dorsal column electrical stimulation. Photographic oscilloscope records of extracellular spike potentials from moving film.

circle) as landmarks. In agreement with our previous findings (Randić *et al.* 1976), the cell bodies of ACh-excited DSCT neurones receiving muscle inputs were always found to be located within Clarke's column, while the DSCT neurones activated by hair movement and/or both hair movement and light touch of the skin on the ipsilateral hind limb were located in the dorsal grey column of the spinal cord. It is of interest that three out of four Clarke's column neurones synaptically activated in response to electrical stimulation of the dorsolateral funiculus and strongly excited by ACh in our experiments were found either within the Clarke's column or in its fringe. The latter finding differs from the previous reports on the location of the synaptically activated Clarke's column neurones (Kostyuk, 1969). In contrast, the Clarke's column neurones, which did not project either in the ipsilateral dorsolateral funiculus or the dorsal column, were found to be located in the fringe of Clarke's column and thus might belong to the border cells.

Depression by acetylcholine

Six out of fifty-six units (i.e. 11%) antidromically activated by the ipsilateral dorsolateral funiculus stimulation were depressed by ACh, if applied with currents between 60 and 160 nA for periods up to 2 min (Table 1). ACh depression could also be demonstrated in the units activated either antidromically or synaptically by the dorsal column stimulation. The depression was manifested as a reduction in the rate of spontaneous firing. Typically the effect had a delayed onset (5–10 sec) and outlasted ACh-ejection for a similar period, as illustrated in Fig. 8. Since

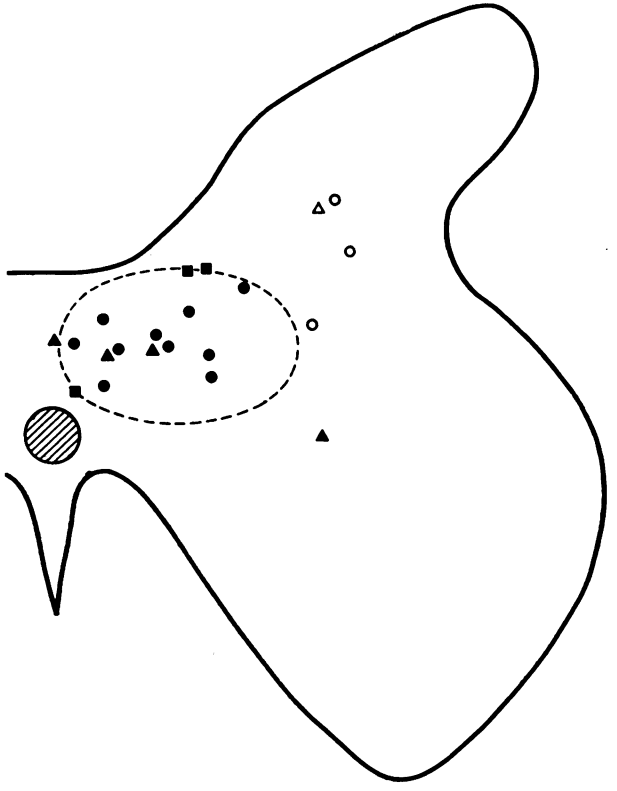

Fig. 7. Distribution of ACh-excited neurones at lumbar spinal levels examined by marking the site of extracellular recording with fast green. Continuous line, the grey-white matter boundary. Dashed oval, Clarke's column. Shaded circle, central canal. Filled circles, DSCT muscle cells; open circles, cells antidromically activated by ipsilateral dorsolateral funiculus electrical stimulation; filled triangles, cells synaptically activated by ipsilateral dorsolateral funiculus stimulation; open triangle, cell synaptically activated by ipsilateral dorsal column stimulation; filled squares, cells not activated either by ipsilateral dorsolateral funiculus or dorsal column stimulation.

the actual passage of outward current may depress the firing of the neurones it is important to distinguish accurately between the effects of ACh itself and the associated current flow. We believe that the depressant action of ACh observed in the spinal neurones described here is not an artifact for the following reasons: (1) The depressant effect of ACh

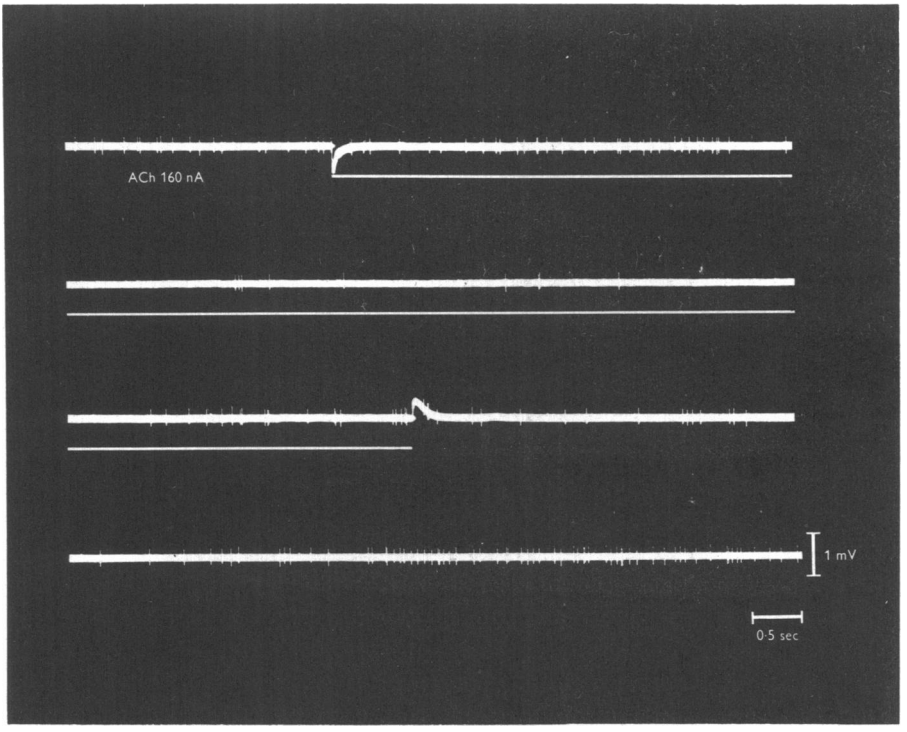

Fig. 8. The depressant effect of ACh (160 nA) on a unit receiving bilateral convergent input. Unit was not activated either by ipsilateral dorsolateral funiculus or dorsal column electrical stimulation. Photographic oscilloscope records of extracellular spike potentials from moving film.

was seen when equal iontophoretic currents applied through the control barrel (containing NaCl) had no effect (Fig. 9). It might be argued that this result was due to the tip of the ACh-containing barrel being closer to the unit under study than the tip of the control barrel. However, the depressant effect of ACh was seen with many different electrodes. (2) The delayed effect of ACh with regard to the onset and the end of the iontophoretic current was in sharp contrast with the immediate effect, if any, of NaCl current alone.

It is of interest that the depressant effect of ACh was more frequently encountered in the cells unresponsive either to the dorsolateral funiculus

or dorsal column stimulation. In contrast none of the twenty-three tested units synaptically activated by the ipsilateral dorsolateral funiculus stimulation were depressed by ACh. The same was true for the spinal neurones receiving convergent peripheral inputs, activated either antidromically or synaptically by ipsilateral dorsolateral funiculus or dorsal column electrical stimulation (Table 1).

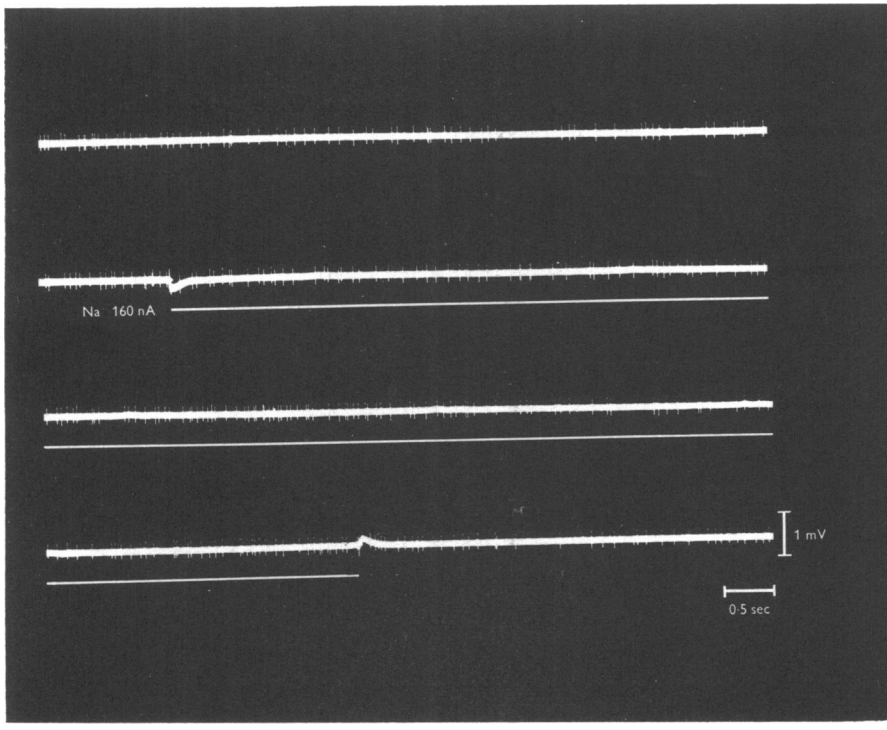

Fig. 9. Same unit as illustrated in Fig. 8. NaCl (160 nA) is without effect on the spontaneous discharge.

Cholinomimetics

The existence of two different types of spinal unit responses (excitation or depression) to microiontophoretically applied ACh suggested that two types of receptors might be present on those cells. Therefore nicotine, acetyl-β-methylcholine (cholinomimetic with muscarinic properties in other systems) and carbaminoylcholine (cholinomimetic with nicotinic properties in other systems) were tested on spinal neurones. As seen in Table 2, thirteen out of twenty cells excited by acetylcholine with ejection currents between 20 and 200 nA were also excited by nicotine applied with similar amounts of positive current. Although similarly as with ACh

TABLE 2. Nicotine sensitivity of ACh-excited Clarke's column and dorsa horn neurones

Response of spinal units to electrical stimulation of the ipsilateral dorsolateral funiculus and dorsal column	No. of cells	Input		Nicotine effect: Excitation (%)	Nicotine effect: Depression (%)	Nicotine effect: Nil (%)
Cells antidromically activated by ipsilateral dorsolateral funiculus stimulation	6	Muscle	4	25	—	75
		Cutaneous	1	100	—	—
		Convergent	1	100	—	—
Cells synaptically activated by ipsilateral dorsolateral funiculus stimulation	3	Muscle	1	100	—	—
		Cutaneous	2	50	—	50
Cells antidromically activated by ipsilateral dorsal column stimulation	2	Cutaneous	2	50	—	50
Cells synaptically activated by ipsilateral dorsal column stimulation	7	Muscle and/or joint	2	50	—	50
		Cutaneous	3	100	—	—
		Convergent	2	50	—	50
Cells *not* activated either by ipsilateral dorsolateral funiculus or dorsal column stimulation	2	Cutaneous	1	100	—	—
		Convergent	1	100	—	—
Total	20					

in some dorsal spinocerebellar tract neurones, a very quick and rapidly reversible excitatory effect is observed with nicotine (Fig. 10), the excitation caused by nicotine frequently is delayed in onset and recovery (latent period varying between 10 and 25 sec, while recovery frequently lasting up to 45 sec; see Figs. 11 and 12), and rather less current is required

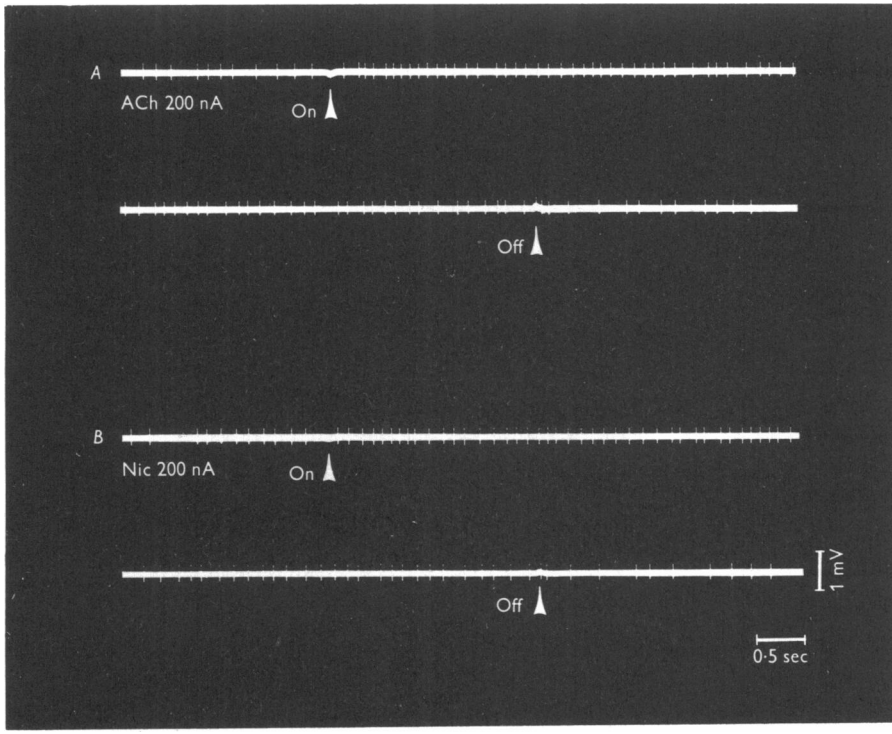

Fig. 10. Excitatory responses to ACh (*A*, 200 nA) and nicotine (*B*, 200 nA) of a spontaneously active DSCT cell receiving afferent input from muscle receptors. Note that both excitations are very rapid in onset and recovery. Photographic oscilloscope records of extracellular spike potentials from moving film.

on the average to cause an acceleration comparable to ACh of the background discharge. Nicotine weakly excited about 50% of the units antidromically activated either by the ipsilateral dorsolateral funiculus or the dorsal column stimulation (maximum increase in the firing frequency did not exceed 123% of the control level) and more strongly excited about 70% of the units synaptically activated, as well as two cells which did not project either in the ipsilateral dorsolateral funiculus or the dorsal column. In these latter two types of cells the maximum increase in the firing frequency varied between 170 and 655% of the control level.

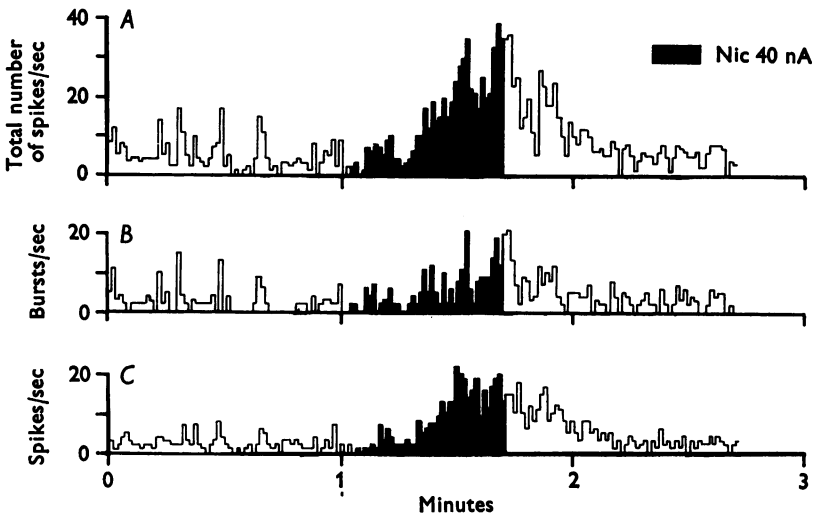

Fig. 11. Excitatory effect of nicotine (40 nA) in a cell synaptically activated by ipsilateral dorsal column stimulation and receiving convergent inputs (cutaneous and joint). Excitation produced by nicotine is delayed in onset and recovery (*A*). It is manifested in this unit as a larger increase in the number of single action potentials (*C*) than in the number of high frequency bursts of electrical spike activity (*B*).

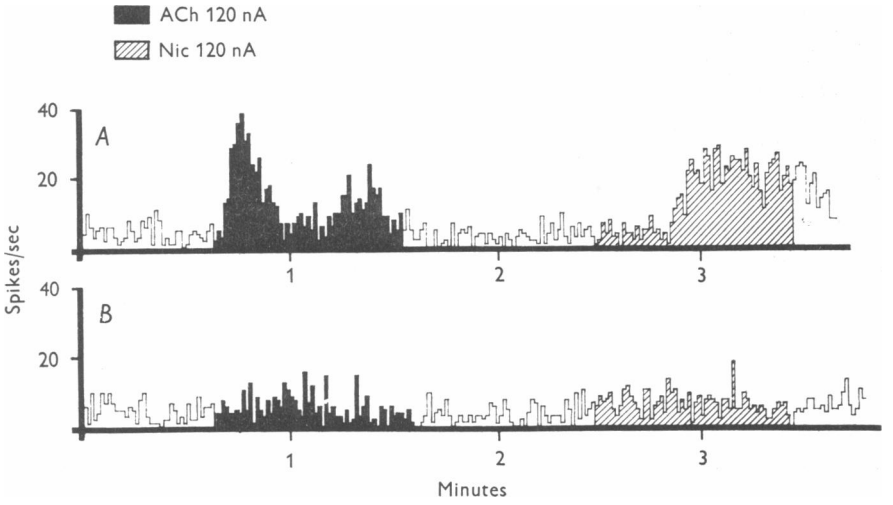

Fig. 12. An example of desensitization to both ACh (120 nA) and nicotine (120 nA) of a cell antidromically activated by ipsilateral dorsal column stimulation receiving cutaneous input. Excitatory responses to both ACh and nicotine are reduced with second application (compare responses to ACh and nicotine in *A* and *B*).

Tachyphylaxis with repeated applications of nicotine was observed (Fig. 12). Nicotine produced a change in the pattern of discharge in several units receiving cutaneous or convergent inputs, which was manifested as a larger increase in the number of single action potentials (Fig. 11*C*) than the increase in the number of high frequency bursts of electrical spike activity (Fig. 11*B*). ACh depressed units were always unresponsive to nicotine (Fig. 13).

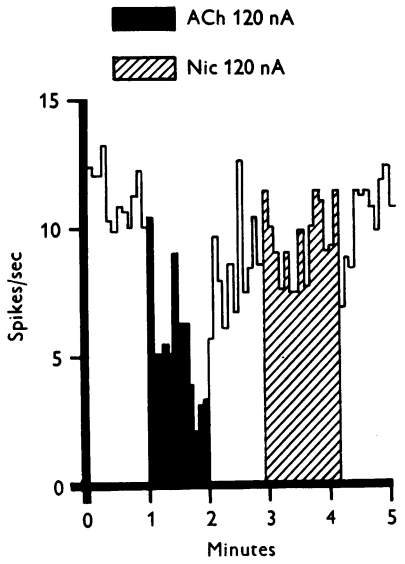

Fig. 13. Depressant effect of ACh (120 nA) in a unit synaptically activated by ipsilateral dorsal column stimulation and receiving convergent afferent input. The unit is unresponsive to nicotine (120 nA).

Acetyl-β-methylcholine was tested in ten cells responding to ACh either with excitation or depression, being activated either antidromically or synaptically during electrical stimulation of the ipsilateral dorsolateral funiculus. Acetyl-β-methylcholine (80–160 nA applied for 1–2 min) was found to depress all cells ($n = 3$) which were also depressed by ACh. The depression produced by acetyl-β-methylcholine was similar in latency and duration to that produced by ACh. In contrast, acetyl-β-methylcholine (120–200 nA applied for 1–2 min) weakly excited only three out of seven cells which were all excited by ACh. The excitation had a latency of up to 60 sec and usually persisted for up to 45 sec after ending the acetyl-β-methylcholine ejection.

Carbaminoylcholine (40–120 nA applied for up to 60 sec) depressed five cells, which responded to ACh with excitation. All cells received

cutaneous afferent inputs and were activated either antidromically or synaptically by the ipsilateral dorsal column stimulation.

Anticholinergic agents – dihydro-β-erythroidine and atropine

In order to get more information about the type of receptors mediating ACh-excitation and ACh-depression in Clarke's column and other dorsal horn neurones we have applied the nicotine receptors blocking agent dihydro-β-erythroidine (DHβE) and the muscarinic receptors blocking agent atropine to these cells. Eight of the cholinoceptive units were tested with DHβE applied either microelectrophoretically as a cation with currents

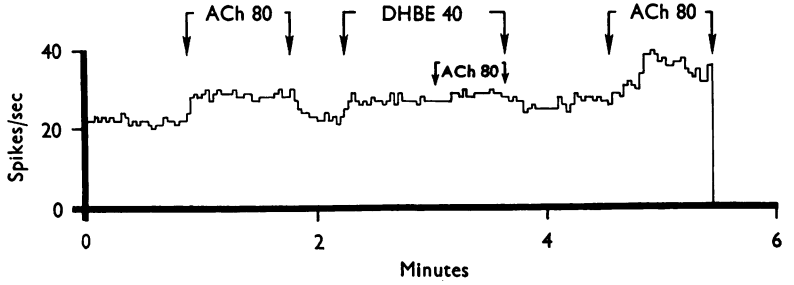

Fig. 14. Antagonism of ACh excitation by dihydro-β-erythroidine (DHBE) in a DSCT cell receiving afferent input from muscle receptors. Note that DHBE (40 nA) administration alone facilitated spontaneous activity.

ranging between 30 and 60 nA for periods of 2–5 min, or systemically (0·1–0·2 mg/kg b.w. I.V.). Five of these cells were antidromically activated by ipsilateral dorsolateral funiculus electrical stimulation, while three cells responded synaptically. In four out of eight tested cells DHβE completely blocked both ACh and nicotine excitations, while in three cells only a partial antagonism to ACh excitatory response was observed. The blocking effect of DHβE (40 nA) on ACh excitation in a DSCT neurone receiving muscle input is illustrated in Fig. 14. ACh depression was not blocked by DHβE. In several cholinoceptive cells a direct excitant effect of DHβE was noted similar to that described for medullary (Salmoiraghi & Steiner, 1963), ventrobasal thalamic (Andersen & Curtis, 1964) and neurones in the pyriform cortex (Legge, Randić & Straughan, 1966).

The effect of atropine was examined on twelve units activated either antidromically or synaptically during electrical stimulation of the ipsilateral dorsolateral funiculus. Atropine was applied microelectrophoretically as a cation with currents ranging between 20 and 40 nA for 1 min (ten units) or intravenously (0·2 mg/kg b.w., two units). We found that atropine reversibly blocked both ACh and acetyl-β-methylcholine

depressions in all four tested DSCT cells. In addition, atropine partially antagonized ACh excitation in four out of eight cells synaptically activated by ipsilateral dorsolateral funiculus stimulation. In the remaining four units, atropine was without any effect. Atropine often depressed neuronal activity which might be due to its local anaesthetic action (Curtis & Ryall, 1966).

DISCUSSION

Acetylcholine excitation

The literature has long predicted the presence of ACh excitable neurones within the Clarke's column and adjacent regions of the dorsal horn of the spinal cord. Thus biochemical and histochemical studies have demonstrated the presence of ACh, choline acetyltransferase and acetylcholinesterase in the spinal cord (MacIntosh, 1941; Feldberg & Vogt, 1948; Roessmann & Friede, 1967; Kasa *et al.* 1970; Silver & Wolstencroft 1970, 1971; Odutola, 1972). Although the chemical sensitivity of Clarke's column neurones was not studied until now at the cellular level, effects of ACh on dorsal horn interneurones were investigated but the results obtained are controversial. Some of the earliest studies found ACh to be ineffective in cat spinal interneurones (Curtis *et al.* 1961) or causing mainly depression in a certain proportion of tested units (Engberg & Ryall, 1966). In addition, facilitatory effect of ACh was noted in spinal interneurones following both systemic (Feldberg *et al.* 1953; Fernandez de Molina *et al.* 1958) and microelectrophoretic application (Weight & Salmoiraghi, 1966).

In this study we have found that both antidromically identified (DSCT neurones) and synaptically activated neurones from the region of the Clarke's column of the spinal cord are excited by microelectrophoretic application of ACh. However, the proportion of ACh excited cells was greater in units synaptically activated by electrical stimulation of the ipsilateral dorsolateral funiculus (about 78%) than in antidromically identified DSCT neurones (about 50%). In addition about 55% of units either antidromically or synaptically activated by electrical stimulation of the ipsilateral dorsal column were found to be excited by ACh.

In contrast to a relatively weak excitatory potency on the DSCT neurones (the maximum increase in firing rate produced by ACh did not exceed 130% of the control level), ACh proved to be a more potent excitant of the Clarke's column neurones, which were synaptically activated in response to electrical stimulation of the ipsilateral dorsolateral funiculus (in these neurones the increase in the maximum firing frequency was in the range of 210–430% of the control level). In relation to our findings of differential sensitivity of Clarke's column neurones the results

of Silver & Wolstencroft (1971) are of interest to be mentioned. Namely, these investigators have found that the acetylcholinesterase activity in the large cells of the cat Clarke's column (presumably the cells of origin of the dorsal spinocerebellar tract) varied from slight to moderate and the staining appeared to be on the cell surface. Scattered among these large cells were a few small neurones with strong staining in the cell bodies and processes. On the basis of these findings they have suggested that certain small neurones in Clarke's column might be considered as being cholinergic, while the large neurones (possibly DSCT cells) which stain weakly or moderately are unlikely candidates for cholinergic neurones.

Our microelectrophoretic studies with ACh are of interest since they have shown that ACh has a relatively quick and rapidly reversible excitatory effect on Clarke's column neurones and some particular types of dorsal horn neurones, which can be obtained also with nicotine. However, the action of nicotine is frequently delayed in onset and it lasts much longer, presumably because nicotine is not broken down by cholinesterases. This excitatory action of ACh in units activated either antidromically or synaptically during ipsilateral dorsolateral funiculus stimulation can be blocked or markedly depressed by nicotine antagonist dihydro-β-erythroidine, applied either systemically or microelectrophoretically. These findings point to the existence of nicotinic receptors mediating ACh excitation of spinal units activated either antidromically or synaptically by ipsilateral dorsolateral funiculus or dorsal column stimulation. Since the introduction of the microelectrophoretic technique, ACh has been tested on a large number of cells of many different kinds throughout the mammalian central nervous system. Many cells have proved sensitive to ACh (Phillis, 1971, Krnjević, 1974) but so far the nicotinic action of ACh has been seen in its clearest form only in the Renshaw cells (Eccles, Fatt & Koketsu, 1954; Curtis & Eccles, 1958), although some unusually rapid responses have been observed in the lateral geniculate of the cat (Phillis, Tebēcis & York, 1967) and in the brain stem of the rat (Bradley & Dray, 1972). In several other areas, the actions of ACh can be reproduced with nicotine and blocked effectively by DHβE, e.g. in the medulla (Salmoiraghi & Steiner, 1963), the lateral geniculate (Curtis & Davis, 1963) thalamus (Andersen & Curtis, 1964), the cerebellum (McCance & Phillis, 1968) and the supraoptic nucleus of the hypothalamus (Barker, Crayton & Nicoll, 1971; Dreifuss & Kelly, 1972). Although, the most striking excitatory actions of ACh in the cerebral cortex are muscarinic (slow in onset and recovery and responses are readily blocked by atropine) some nicotinic excitations have been observed in the cingulate gyrus (Krnjević, 1965) or in the superficial layers (Stone, 1972).

We have found that some of the cholinoceptive cells activated either antidromically or synaptically by ipsilateral dorsolateral funiculus stimulation are weakly excited by acetyl-β-methylcholine. In addition, in four out of eight cells synaptically activated by ipsilateral dorsolateral funiculus stimulation atropine caused a partial reduction of the response to acetylcholine (30–50%). Thus these results obtained with acetyl-β-methylcholine (cholinomimetic with muscarinic properties in other systems) and atropine (selective blocking agent of muscarinic ACh receptors) seem to suggest that the receptors mediating excitation of the cholinoceptive spinal cells, activated either antidromically or particularly synaptically by ipsilateral dorsolateral funiculus stimulation besides nicotinic have also weak muscarinic properties.

The decline or the loss of the excitatory effect of ACh, either during an application, or with repeated applications as described in the present results, is probably due to desensitization. Such desensitization or tachyphylaxis has been observed with repeated applications of ACh at the motor end-plate (Katz & Thesleff, 1957) and in the Renshaw cell system (Curtis & Ryall, 1966). In contrast, little tachyphylaxis was apparent in the pyramidal tract cells (Stone, 1972). Marked tachyphylaxis has been seen with other excitatory substances applied by microelectrophoresis at sites where they are believed to be transmitters, e.g. monoamines (Phillis & Tebēcis, 1967; Roberts & Straughan, 1967).

Zieglgänsberger & Reiter (1974) have studied the mode of operation of ACh in the spinal neurones of cats combining intracellular recording with extracellular iontophoresis. Similar to the findings of Krnjević, Pumain & Renaud (1971) in the cerebral cortex of cats, they found that the majority of spinal neurones (motoneurones, Renshaw cells and neurones giving rise to ascending tracts) showed depolarization by ACh associated with no detectable change in membrane conductance or even a slight conductance decrease. Krnjević *et al.* (1971) suggested that the mechanism underlying the depolarization of cortical cells involved a decrease of resting K^+ conductance. They deduced from alterations of the declining phase of the spike that the delayed K^+ current of the action potential is also altered. On the basis of these data Zieglgänsberger & Reiter (1974) have suggested that ACh may play a role as an excitatory modulator of spinal cord neuronal activity rather than as a transmitter of fast excitatory phenomena.

Similar intracellular studies of the membrane potential and conductance changes induced either by ACh or by the synaptic action should be undertaken in order to find out more about a possible excitatory transmitter or modulator role of ACh in Clarke's column neurones.

ACh depression

Besides excitation, application of ACh resulted in depression of 11% of units antidromically activated by the ipsilateral dorsolateral funiculus stimulation. In addition, the depressant effect of ACh was frequently encountered in the cells unresponsive either to the dorsolateral funiculus or dorsal column stimulation. In confirmation of previous findings (Engberg & Ryall, 1966; Weight & Salmoiraghi, 1966), ACh depression could also be demonstrated in the units activated either antidromically or synaptically by the dorsal column stimulation. In contrast none of the tested units synaptically activated by the ipsilateral dorsolateral funiculus stimulation were depressed by ACh. The same was true for all tested spinal neurones receiving convergent peripheral inputs, activated either antidromically or synaptically by ipsilateral dorsolateral funiculus or dorsal column stimulation.

Since the actual passage of outward current may depress the firing of the underlying neurones, it is very important to distinguish accurately between the effects of ACh itself and the associated current flow. We believe that the depressant action of ACh on Clarke's column and some dorsal horn interneurones is not an artifact for the following reasons. (1) The depressant effect of ACh was seen when equal current applied through the control barrel containing NaCl had either no effect, or an excitatory effect. (2) Also the delayed effect of ACh with regard to the onset and the end of microelectrophoretic current was in sharp contrast with the immediate effect, if any, of NaCl current alone. (3) ACh depressant effect was reversibly blocked by atropine in the DSCT neurones.

The finding that ACh depression of all tested DSCT neurones is blocked by atropine and readily evoked by acetyl-β-methylcholine indicates that receptors mediating the depressant effect are predominantly of muscarinic type. Substantial evidence has now accumulated from microelectrophoretic experiments that ACh can depress the firing of certain central neurones (Krnjević, 1974).

This work was supported by grants from the National Science Foundation (GB 37864), the National Institutes of Health (R01 NS 12972-01), and the Iowa State University Research Foundation.

REFERENCES

ANDERSEN, P. & CURTIS, D. R. (1964). The pharmacology of the synaptic and acetylcholine-induced excitation of ventrobasal thalamic neurones. *Acta physiol. scand.* **61**, 100–120.

BARKER, J. L., CRAYTON, J. W. & NICOLL, R. A. (1971). Noradrenaline and acetylcholine responses of supraoptic neurosecretory cells. *J. Physiol.* **218**, 19–32.

BRADLEY, P. B. & DRAY, A. (1972). Short-latency excitation of brain stem neurones in the rat by acetylcholine. *Br. J. Pharmac.* **45**, 372–374.

BURGESS, P. R., PETIT, D. & WARREN, R. M. (1968). Receptor types in cat hairy skin supplied by myelinated fibres. *J. Neurophysiol.* **31**, 833–848.

CURTIS, D. R. (1964). Microelectrophoresis. In *Physical Techniques in Biological Research*, vol. 5, ed. NASTUK, W. L., pp. 144–190, New York: Academic.

CURTIS, D. R. & DAVIS, R. (1963). The excitation of lateral geniculate neurones by quaternary ammonium derivatives. *J. Physiol.* **165**, 62–82.

CURTIS, D. R. & ECCLES, R. M. (1958). The excitation of Renshaw cells by pharmacological agents applied electrophoretically. *J. Physiol.* **141**, 435–445.

CURTIS, D. R., PHILLIS, J. W. & WATKINS, J. C. (1961). Cholinergic and non-cholinergic transmission in the mammalian spinal cord. *J. Physiol.* **158**, 296–323.

CURTIS, D. R. & RYALL, R. W. (1966). The excitation of Renshaw cells by cholinomimetics. *Expl Brain Res.* **2**, 49–65.

DREIFUSS, J. J. & KELLY, J. S. (1972). The activity of identified supraoptic neurones and their response to acetylcholine applied by iontophoresis. *J. Physiol.* **220**, 105–118.

ECCLES, J. C., FATT, P. & KOKETSU, K. (1954). Cholinergic and inhibitory synapses in a pathway from motor-axon collaterals to motoneurones. *J. Physiol.* **126**, 524–562.

ENGBERG, I. & RYALL, R. W. (1966). The inhibitory action of noradrenaline and other monoamines on spinal neurones. *J. Physiol.* **185**, 298–322.

FELDBERG, W., GRAY, J. A. B. & PERRY, W. L. M. (1953). Effects of close arterial injections of acetylcholine on the activity of the cervical spinal cord of the cat. *J. Physiol.* **119**, 428–438.

FELDBERG, W. & VOGT, M. (1948). Acetylcholine synthesis in different regions of the central nervous system. *J. Physiol.* **107**, 372–381.

FERNANDEZ DEMOLINA, A., GRAY, J. A. B. & PALMER, J. F. (1958). Effects of acetylcholine on the activity of the lumbosacral cord of the cat. *J. Physiol.* **141**, 169–176.

KASA, P., MANN, S. P. & HEBB, C. O. (1970). Localization of choline acetyltransferase. *Nature, Lond.* **226**, 812–816.

KATZ, B. & THESLEFF, S. (1957). A study of the 'desensitization' produced by acetylcholine at the motor end-plate. *J. Physiol.* **138**, 63–80.

KOSTYUK, P. G. (1969). On the function of dorsal spino-cerebellar tract in cat. In *Neurobiology of Cerebellar Evolution and Development*, ed. LLINÁS, R. Chicago: American Medical Association.

KRNJEVIĆ, K. (1965). Action of drugs on single neurones in the cerebral cortex. *Br. Med. Bull.* **21**, 10–14.

KRNJEVIĆ, K. (1971). Microiontophoresis. In *Methods in Neurochemistry*, vol. 1, ed. FRIED, R., pp. 129–172. New York: Marcel Dekker, Inc.

KRNJEVIĆ, K. (1974). Chemical nature of synaptic transmission in vertebrates. *Physiol. Rev.* **54**, 418–540.

KRNJEVIĆ, K. & PHILLIS, J. W. (1963). Iontophoretic studies of neurones in the mammalian cerebral cortex. *J. Physiol.* **165**, 274–304.

KRNJEVIĆ, K., PUMAIN, R. & RENAUD, L. (1971). The mechanism of excitation by acetylcholine in the cerebral cortex. *J. Physiol.* **215**, 247–268.

LEGGE, K. F., RANDIĆ, M. & STRAUGHAN, D. W. (1966). The pharmacology of neurones in the pyriform cortex. *Br. J. Pharmac.* **26**, 87–107.

MACINTOSH, F. C. (1941). The distribution of acetylcholine in the peripheral and the central nervous system. *J. Physiol.* **99**, 436–442.

MCCANCE, I. & PHILLIS, J. W. (1968). Cholinergic mechanisms in the cerebellar cortex. *Int. J. Neuropharmac.* **7**, 447–462.

MYSLINSKI, N. R., RANDIĆ, M. & LEDGERE, M. E. (1974). Chemical sensitivity of the dorsal spinocerebellar tract neurones in relation to various sensory inputs. *4th Ann. Meet. Soc. Neurosci.* Abstract 481. Bethesda: Society for Neuroscience.

ODUTOLA, A. B. (1972). The organization of cholinesterase-containing systems of the monkey spinal cord. *Brain Res.* **39**, 353–368.

PHILLIS, J. W. (1971). The pharmacology of thalamic and geniculate neurones. *Int. Rev. Neurobiol.* **14**, 1–48.

PHILLIS, J. W. & TEBĒCIS, A. K. (1967). The responses of thalamic neurones to iontophoretically applied monoamines. *J. Physiol.* **192**, 715–745.

PHILLIS, J. W., TEBĒCIS, A. K. & YORK, D. H. (1967). A study of cholinoceptive cells in the lateral geniculate nucleus. *J. Physiol.* **192**, 695–713.

RANDIĆ, M. & MYSLINSKI, N. R. (1974). Microiontophoretic studies of neurones in the Clarke's column. *XXVI Int. Cong. Physiol. Sci.* Abstract 894, Faridabad: Thompson Press (India), Ltd.

RANDIĆ, M. & MYSLINSKI, N. R. (1976). Microelectrophoretic studies on neurones in the Clarke's column. In *Drugs and Central Synaptic Transmission*, ed. BRADLEY, P. B. & DHAWAN, B. N., pp. 235–244. London: Macmillan.

RANDIĆ, M., MYSLINSKI, N. R. & GORDON, J. H. (1976). Spinal localization of neurones receiving inputs from cutaneous afferents in the cat hindlimb. *Brain Res.* **105**, 573–577.

ROBERTS, M. H. T. & STRAUGHAN, D. W. (1967). Excitation and depression of cortical neurones by 5-hydroxytryptamine. *J. Physiol.* **193**, 269–294.

ROESSMANN, U. & FRIEDE, R. L. (1967). The segmental distribution of acetyl cholinesterase in the cat spinal cord. *J. Anat.* **101**, 27–32.

SALMOIRAGHI, G. C. & STEINER, F. A. (1963). Acetylcholine sensitivity of cat's medullary neurones. *J. Neurophysiol.* **26**, 581–597.

SALMOIRAGHI, G. C. & STEFANIS, C. N. (1967). A critique of iontophoretic studies of central nervous system neurones. *Int. Rev. Neurobiol.* **10**, 1–30.

SILVER, A. & WOLSTENCROFT, J. H. (1970). Cholinesterases and choline acetylase in the spinal cord of the cat. *J. Physiol.* **210**, 92–93*P*.

SILVER, A. & WOLSTENCROFT, J. H. (1971). The distribution of cholinesterases in relation to the structure of the spinal cord in the cat. *Brain Res.* **34**, 205–227.

STONE, T. W. (1972). Cholinergic mechanisms in the rat somatosensory cerebral cortex. *J. Physiol.* **225**, 485–499.

WEIGHT, F. F. & SALMOIRAGHI, G. C. (1966). Responses of spinal cord interneurones to acetylcholine, norepinephrine and serotonin administered by microelectrophoresis. *J. Pharmac. exp. Ther.* **153**, 420–427.

ZIEGLGÄNSBERGER, W. & REITER, CH. (1974). A cholinergic mechanism in the spinal cord of cats. *Neuropharmacology* **13**, 519–527.