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THE MECHANISM OF EXCITATION BY ACETYLCHOLINE IN THE CEREBRAL CORTEX

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SUMMARY

1. The muscarinic depolarizing action of ACh on cortical neurones is associated with an increase in membrane resistance (mean $\Delta V/\Delta R = 3{\cdot}16$ mV/MΩ).

2. ACh also promotes repetitive firing by slowing repolarization after spikes.

3. The depolarizing effect has a mean reversal level of $-86{\cdot}7$ mV (with mean resting potential -56 mV).

4. It is concluded that as a muscarinic excitatory agent, ACh probably acts by reducing the resting K^+ conductance of cortical neurones, and also the delayed K^+ current of the action potential.

5. These results are discussed in relation to the possible role of ACh in cortical function.

INTRODUCTION

The fact that acetylcholine (ACh) applied directly by microiontophoresis can excite certain cortical neurones has been known for a number of years (Krnjević & Phillis, 1963*a*, *b*; Spehlmann, 1963). The characteristic slow time course of this action sharply distinguishes it from the quick excitation produced by glutamate, or indeed from the rapid excitatory action of ACh on Renshaw cells. Another characteristic feature is the remarkably clear muscarinic property of the receptors for the excitatory action in the cortex (Krnjević & Phillis, 1963*c*).

In a subsequent intracellular study (Krnjević & Schwartz, 1967), no clear changes in membrane resistance could be detected when cells were depolarized by ACh; and it was concluded that the primary action of ACh

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may be on some metabolic process which controls the activity of an electrogenic ion pump. In order to test this hypothesis, the effects of a number of metabolic inhibitors were examined; the experiments led to the observation of a selective block of the excitatory action of ACh by 2,4-dinitrophenol (DNP) (Kawamura & Krnjević, 1969); this appeared to confirm the possibility of a metabolic action of ACh.

However, further intracellular studies have shown that DNP causes rather sharp changes in membrane potential and resistance, indicating a selective enhancement of K^+ conductance (Godfraind, Krnjević & Pumain, 1970; Godfraind, Kawamura, Krnjević & Pumain, 1971). This finding suggested the possibility that ACh might have an unsuspected action on the neuronal membrane, and therefore called for a detailed reexamination of the effects of ACh on membrane characteristics. The resulting experiments are the subject of the present paper. Brief reports of this work have already appeared (Godfraind *et al.* 1970; Krnjević, Pumain & Renaud, 1970, 1971*a*).

METHODS

The techniques of intracellular recording, and extracellular iontophoresis have already been described in detail in the two preceding papers (Godfraind *et al.* 1971; Krnjević *et al.* 1971*b*). The intracellular electrodes were filled with 1–3 M solutions of K citrate, K acetate or KCl; while the extracellular micropipettes (mostly two to three-barrelled) contained 1 M-AChCl (British Drug Houses) and various other compounds of interest, such as DNP, Ba^{2+}, glutamate or GABA. The cats were anaesthetized with a mixture of methoxyflurane and N_2O; they were paralysed with succinylcholine and were therefore given artificial respiration.

The extracellular observations were made on cats under Dial (Ciba) or methoxyflurane-N_2O anaesthesia, using five-barrelled micropipettes, as in the experiments of Krnjević & Phillis (1963*a*).

RESULTS

Extracellular observations

The principal characteristics of the excitatory action of ACh are clearly evident in the two traces of Fig. 1. The onset of firing is usually delayed by a period of at least 5–10 sec, even when there is a brisk spontaneous discharge indicating a relatively high level of excitability, and during this initial phase the on-going activity is often sharply reduced for some seconds; this initial depression is more pronounced the more intense the spontaneous firing. The other outstanding feature is a variable, though never negligible after-discharge. The after-discharge may last some 10 sec as in Fig. 1*A*; or have a very much longer duration (1*B*). Not infrequently, the after-discharge appears to persist indefinitely, as a raised level of on-going activity. This is usually seen most clearly with cells which may initially show very little spontaneous firing and little or no response to

ACh; but on repeating the applications, a new and apparently stable state of relatively high activity becomes established. ACh may thus switch on a condition of heightened excitability. This effect is particularly evident with cells whose responsiveness has been depressed by an anaesthetic or other depressant agent (atropine, DNP etc), or with the exceptionally unreactive cells found in isolated or immature cortex (Krnjević, Reiffenstein & Silver, 1970; Krnjević, Randić & Straughan, 1964).

As pointed out in a previous paper (Krnjević & Phillis, 1963*c*) a subthreshold excitatory action of ACh can sometimes be demonstrated by a superimposed application of glutamate. For example, the two units of Fig. 2 could not be excited by a large dose of ACh, even though glutamate

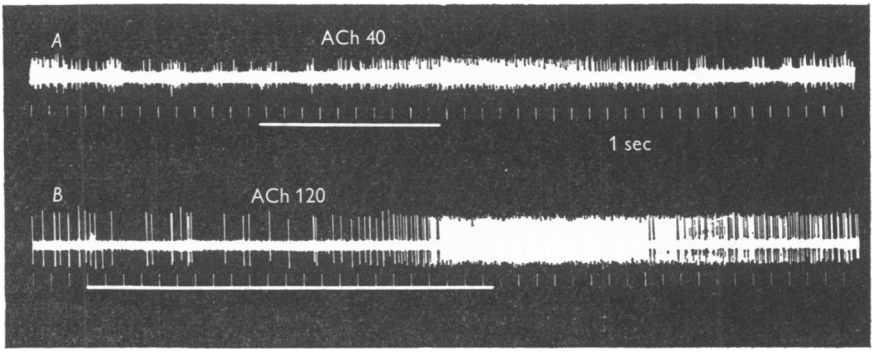

Fig. 1. Typical examples of excitatory effect of ACh on cortical neurones. Extracellular recordings with five-barrelled micropipette, at depth of about 1·0 mm in post-cruciate gyrus of two cats under Dial. Iontophoretic applications are indicated by white lines and currents are given in nA.

excited them readily (*A*). However, for nearly a minute after the end of the release of ACh, the responses to glutamate were strikingly enhanced, both in their intensity and particularly in the duration of the after-discharge. The specific character of this phenomenon is demonstrated by its time course, which corresponds to that of the usual main excitatory action of ACh and also by the fact that it is not shown by the relatively superficial neurones which are typically not excited by ACh (Fig. 3).

Randić, Siminoff & Straughan (1964) pointed out that these more superficial neurones are sometimes clearly depressed by ACh (Fig. 4); but we have found this depression to be a rather inconstant phenomenon, as reported recently by McLennan (1970; cf. Phillis & York, 1968).

Intracellular observations

Useful information was obtained from experiments on fifteen cats.

Depolarizing action of ACh

The intracellular records confirmed the extracellular observations. The

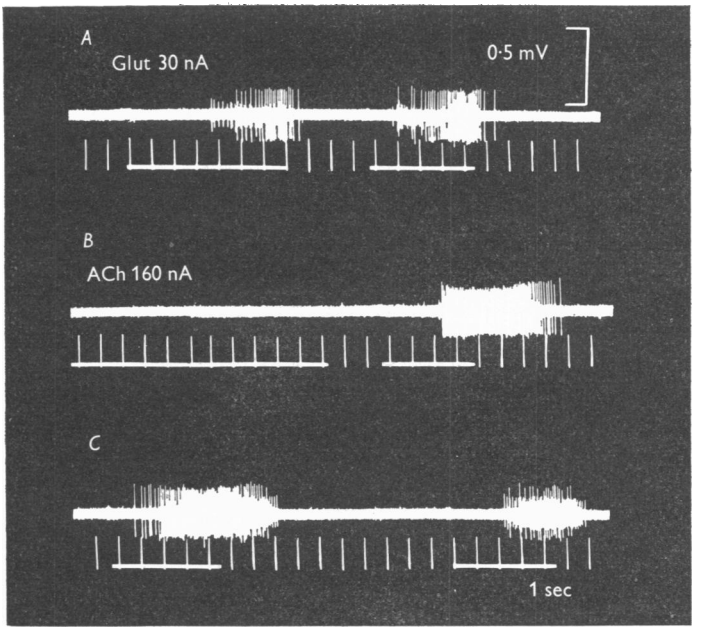

Fig. 2. Marked potentiation and prolongation of glutamate-evoked discharges by subthreshold dose of ACh. *A*: two units in mid-suprasylvian area excited by L-glutamate applied twice. *B*: last phase of a 30-sec application of ACh (first signal). Subsequent tests with glutamate (in *B* and *C*, which are continuous) show enhanced responses, with particularly marked prolongation of after-discharges.

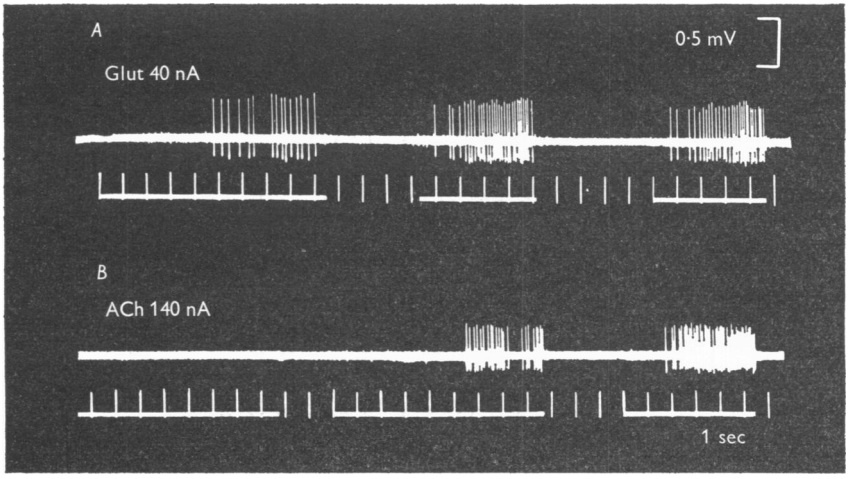

Fig. 3. Suprasylvian unit recorded in same experiment and at similar depth (about 0·5 mm) as those in Fig. 2, but showing no excitatory effect of ACh. *A*: control tests with glutamate. *B*: last portion of 30-sec application of ACh and two subsequent tests of glutamate.

upper trace in Fig. 5 is a good illustration of this: after a delay of some 5 sec, ACh initiated a slow depolarization, which passed the firing threshold 2 sec later; the depolarization and the rate of discharge increased progressively, reaching a peak 1·5 sec after the end of the release of ACh; at the maximum, the cell was depolarized by 17 mV. From the onset of depolarization, the rate of change of potential was about 2·5 mV/sec. Repolarization and the deceleration in firing were very much slower:

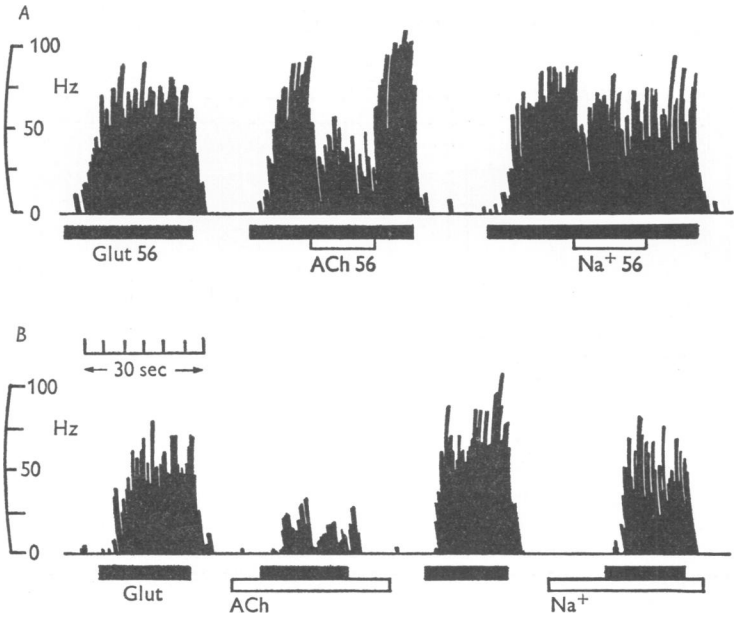

Fig. 4. Pen-writer records of rate of neuronal discharge, showing depressant effect of ACh. In series *A*, ACh release was superimposed on release of glutamate; whereas in series *B*, glutamate test was against a background of ACh release. Current controls are included.

complete return to the base line required some 40 sec, corresponding to a mean rate of only 0·4 mV/sec. The cell showed very little sign of accommodation, the firing being well maintained and the threshold only marginally increased immediately after the phase of intense discharge. We have not been able to detect any clear hyperpolarizing tendency or definite resistance changes immediately after starting applications of ACh, and therefore have no explanation for the frequently observed initial depression of spontaneous discharges.

The same cell could be excited by injecting a depolarizing current through the recording electrode (Fig. 5*B*): the evoked discharge had an extremely sharp onset and termination, with no detectable delay on the

time scale of Fig. 5. A very different effect was seen while releasing ACh after the end of an application of DNP, which caused some hyperpolarization and a fall in excitability (Godfraind *et al.* 1971). The trace in Fig. 5*C* began about 2 sec after the start of the release of ACh (which continued throughout the period shown here); a gradual depolarization is visible, but this amounted to less than 9 mV after 18 sec (equivalent to a mean rate of only 0·5 mV/sec). A depolarizing pulse (slightly smaller than that in *B*) was then applied: there was immediate firing, associated with a progressive increase in depolarization and a corresponding acceleration in firing. At

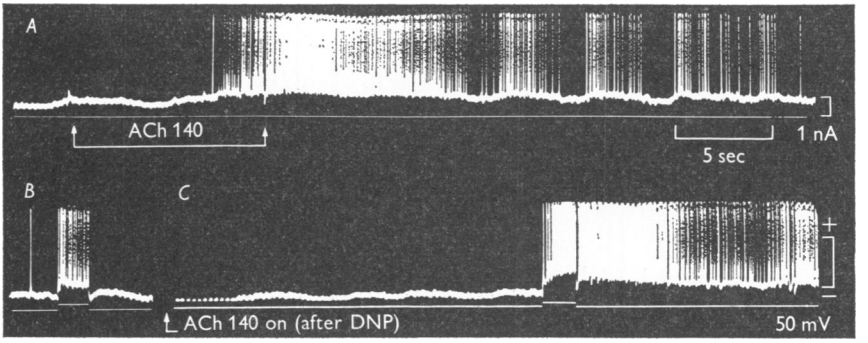

Fig. 5. Intracellular records from pre-cruciate neurone, showing: *A*, excitatory action of ACh released extracellularly; *B*, excitation by intracellular depolarizing current (0·28 nA) monitored on lower trace; *C*, subthreshold excitatory effect of ACh, 1 min after end of an application of DNP; note depolarizing pulse of 0·22 nA; application of ACh began 2 sec before start of *C* and was continued throughout. Resting potential −70 mV.

the end of the current pulse, instead of returning instantaneously to the base line (cf. *B*), the membrane potential remained depolarized by 11·5 mV. If one extrapolates the initial rate of depolarization, one finds that this new level represents a net gain in positivity of 10 mV, directly resulting from the firing. The membrane was partly repolarized during the following 5 sec, the potential reaching a rough plateau some 8 mV above the prefiring level. If the depolarizing effect of ACh had continued at the same rate as during the initial phase (before firing was started by the pulse), by this time it would have produced a change of about +4 mV, or only about half that actually observed.

It thus appears that ACh has a dual action: in addition to having a slow depolarizing effect it delays repolarization after the action potential. When individual spikes are examined more closely, this can be seen sometimes as a pronounced change in shape. For example, in the traces of Fig. 6*B* and *C* (obtained from another neurone) a large positive hump, lasting some 30 msec, appears on the falling phase of the spikes recorded

shortly after the end of an application of ACh. The effect was reversible, the spikes resuming their normal shape within the following minute (cf. Fig. 6*A* and *D*). Evidence of delayed repolarization can also be found in traces *D* and *E* of Fig. 7.

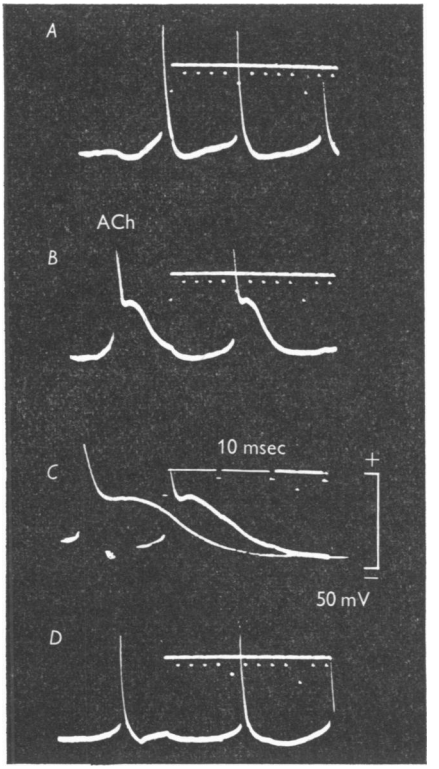

Fig. 6.

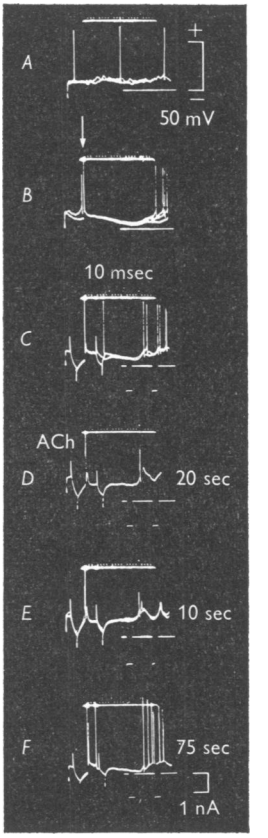

Fig. 7.

Fig. 6. Effect of ACh on spike duration of another neurone. *A*: control. *B–C*: Prolonged spikes recorded 5–10 sec after end of 60 sec application of ACh (200 nA); note faster sweeps in *C*. *D*: control 1 min later. Initial resting potential −48 mV.

Fig. 7. Effect of ACh on membrane potential and resistance of pre-cruciate neurone illustrated in Fig. 5. *A*: spontaneous firing. *B*: IPSP evoked by surface shock at arrow. *C*: two equal pulses of hyperpolarizing currents (monitored on trace below) demonstrate increased conductance during IPSP. *D*: 20 sec after starting release of ACh (140 nA); note depolarizing shift upwards, IPSP is more negative, resistance is increased and there is a delayed repolarization after spike. *E*: these changes are still fully evident 10 sec after switching off current of ACh. *F*: control 1 min later. Micro-electrode contained 1 M-K acetate. Resting potential − 60 mV.

Changes in membrane resistance

In Fig. 7 are also shown the changes in membrane resistance recorded in the cell already illustrated in Fig. 5, but during another application of ACh. From above down, one can see: the initial spontaneous discharge (*A*); its inhibition by a surface shock (at arrow in *B*); the resistance

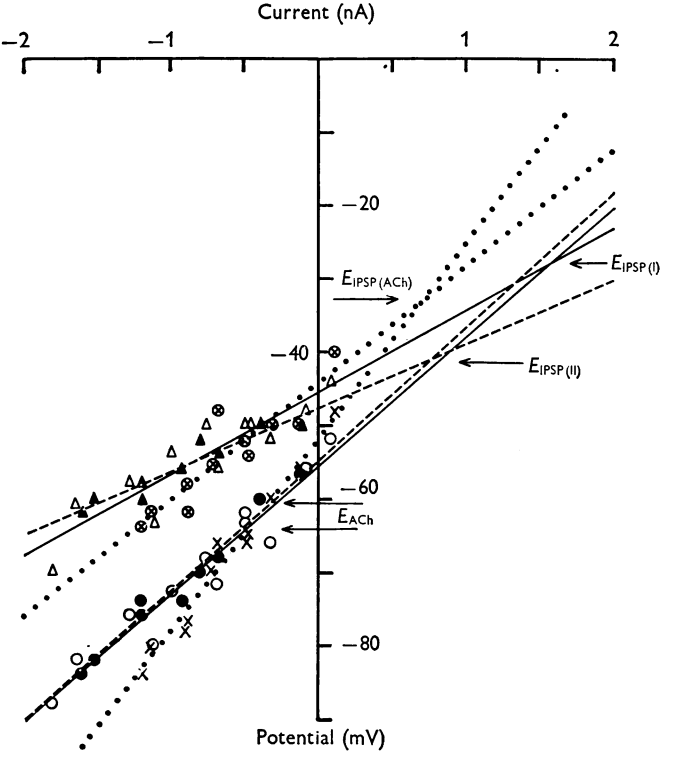

Fig. 8. Voltage–current lines obtained from another neurone by technique illustrated in Fig. 7; vertical axis indicates maximum voltage deflexion evoked by 20 msec pulses of current. Open and filled circles are initial and final 'resting' control points; open and filled triangles are corresponding values near peak of IPSPs; crosses and crossed circles are 'resting' and IPSP points recorded during application of ACh (100 nA). Intracellular micro-electrode contained 1 M-KCl. Regression lines and their equations are as follows. Continuous lines (each based on twelve points): initial controls, resting ($V = 17{\cdot}6\ (\pm 1{\cdot}51)R + 56{\cdot}0\ (\pm 1{\cdot}47)$) and IPSP ($V = 11{\cdot}3\ (\pm 1{\cdot}64)R + 45{\cdot}7\ (\pm 1{\cdot}60)$); dotted lines (each based on eleven points): during ACh, resting ($V = 26{\cdot}6\ (\pm 1{\cdot}46)\ R + 51{\cdot}8\ (\pm 1{\cdot}06)$) and IPSP ($V = 16{\cdot}0\ (\pm 2{\cdot}45)R + 44{\cdot}3\ (\pm 1{\cdot}78)$); dashes (each line based on nine points): final controls, resting ($V = 18{\cdot}1\ (\pm 1{\cdot}17)\ R + 55{\cdot}0\ (\pm 1{\cdot}20)$) and IPSP ($V = 8{\cdot}7\ (\pm 1{\cdot}04)\ R + 47{\cdot}6\ (\pm 1{\cdot}08)$). In all cases, V is negative potential in mV, R resistance in MΩ and values in brackets are S.E. Arrows indicate reversal levels for IPSP or for action of ACh.

measured with 20 msec square pulses at rest and during the IPSP (*C*); and an ACh-evoked depolarization (note upward shift) and increase in resistance – indicated by the greater amplitude of the transmembrane voltage drop – during the release of ACh (*D*) and just after its cessation (*E*); the bottom trace (*F*) is a final control showing a return towards normal levels of potential and resistance. The data of Fig. 7 indicate changes in resistance from 13 MΩ initially, to 16 MΩ during the application of ACh, and 11 MΩ afterwards; this corresponds to a 23 or 45 % increase, depending on whether the initial or the final control is taken as the base line.

In most experiments, sufficient data were obtained by varying the intensity of the applied current pulses to derive voltage–current lines which gave more accurate estimates of changes in resistance (cf. Figs. 8 and 9). A clear increase in resistance was observed during the action of ACh on thirty cells, in twelve different cats. In all but three cases, the rise in resistance was associated with a depolarization (cf. Table 1).

With this technique, positive results are inevitably of greater significance than negative ones. Thus, nearly two-thirds of the cells recorded showed no clear changes in potential or resistance, but we could not readily distinguish the truly 'ACh-insensitive' cells from those that could not be reached by the ACh released, because they were too far from the external electrode or the spread of ACh was impeded by a diffusion barrier. Even cells which appeared to be depolarized by ACh did not always show a definite change in resistance. This can be ascribed to the particular problems associated with resistance measurements. Any mechanical instability (from vascular pulsations etc.) affects the slopes of voltage–current lines much more seriously than the mean resting potential, so that large fluctuations in recorded resistance may easily mask changes in resistance. Furthermore, a failure to record conductance changes is not infrequently clearly due to unfavourable properties of the electrode tip. This is particularly evident when the tip resistance becomes very high (> 50 MΩ); but an electrode may suddenly cease to record IPSP conductance changes, without necessarily showing a serious increase in tip resistance, and yet appears to be able to record satisfactorily the usual potential changes.

When voltage–current lines were obtained at regular intervals (with the help of the computer, by the method illustrated in Fig. 9) the membrane potential and resistance could be plotted continuously as in the graphs of Figs. 10 and 11. The data of Fig. 9 are thus shown in more detail in Fig. 10, where the open symbols indicate the resting potential and resistance, and the closed symbols the values obtained during IPSPs: there was good agreement in the time course of changes in potential and resistance. The resistance values were unusually stable in this experiment. Since they were calculated by fitting regression lines to each series of eight voltage–current points, they were particularly liable to distortion by any mechanical or electrical interference. This can be seen in the graphs of Fig. 11; nevertheless, there was a reasonable approximate agreement between shifts in potential and in resistance.

Reversal level for depolarizing action of ACh

Voltage–current lines obtained from the same cell in the control state and during applications of ACh tended to intersect at a level consistently negative to the resting potential. Such points of intersections are illustrated in Figs. 8 and 9. In Fig. 8, the control points (circles) were obtained before and after the release of ACh. The points recorded during the application of ACh (crosses) indicate a small depolarization (about 4 mV) coupled with a sharp increase in slope (from 18 to 27 MΩ). The two inter-

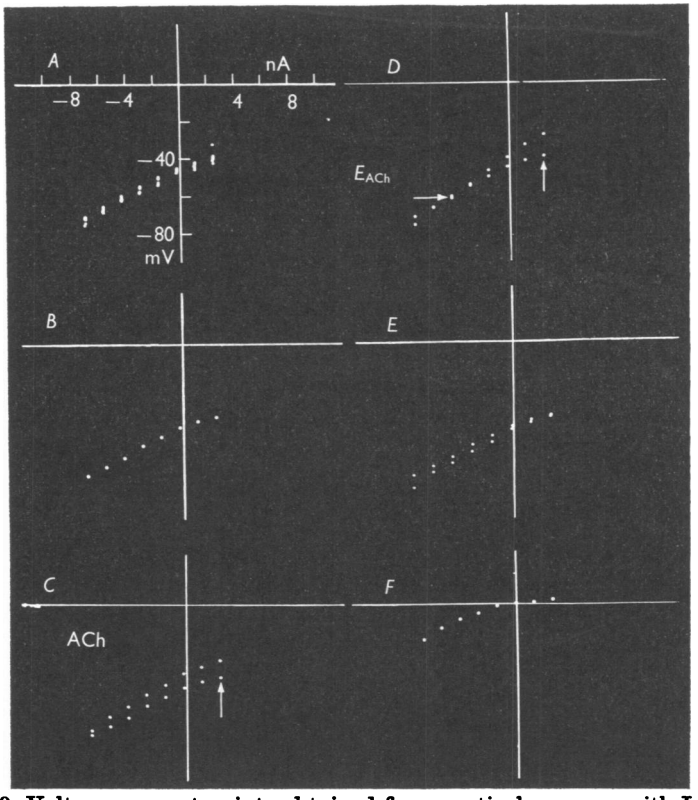

Fig. 9. Voltage–current points obtained from cortical neurone with Linc-8 computer. 30 msec pulses of current of different intensities (indicated by horizontal axis) are applied in series of eight, the corresponding membrane voltage at point of greatest deflexion being given by vertical axis. *A*: eight successive control series of eight such points superimposed photographically to show variability of data. *B*: single control series recorded immediately *before* onset of ACh application (140 nA). *C–E*: series from *B* (marked by vertical arrows) superimposed with three series obtained during and after application of ACh: *C*, 20 sec after onset; *D*, 10 sec after end of ACh release; *E*, 1 min later. *F*: control series recorded outside cell. Horizontal arrow in *D* indicates reversal level for ACh action.

sections between the ACh line (dotted) and the two control (continuous and dashed) lines marks the two possible reversal levels for the action of ACh (E_{ACh}), as shown on the graph.

The voltage–current points in Fig. 9 were recorded on-line from another neurone, with a Linc-8 computer, as described in the Methods. In the first record (*A*) eight successive control series of eight points each have been superimposed to give an indication of the stability of the measurements. In *B–E*, one of these control series is shown for reference, either alone (*B*), or together with a similar series of points recorded 20 sec after the onset of the release of ACh (*C*), 10 sec after the end of the release (*D*) and 1 min later (*E*); the control series *B* is marked with a vertical arrow in *C* and *D*. The depolarizing effect of ACh shown by an upward shift is exaggerated by some coupling artifact during the iontophoretic release of ACh (*C*); the changes in potential and resistance, and the reversal level (at the point of intersection of the control and the ACh lines) are therefore seen most accurately just after the end of the release, when the action of ACh reaches its peak (cf. Figs. 1 and 5). The increase in resistance, the depolarization and the relatively negative reversal levels are thus clearly revealed in *D*. The next record (*E*) shows a substantial return towards the initial state; and the final control series (*F*), recorded outside the cell as a check for the zero potential level and the degree of bridge balance, indicates some non-linear behaviour of the electrode.

The mean of thirty-eight values of E_{ACh} obtained in this way from nineteen different cells was $-74{\cdot}8$ mV (S.D. 31·5), for a mean resting potential of $-48{\cdot}1$ mV (S.D. 18·75, n 41). A more detailed study was made of thirteen cells whose resting potentials were particularly stable, remaining below -40 mV throughout the preceding and final control runs. The corresponding data are given in Table 1. In this relatively normal population, the mean reversal level was -87 mV, or 31 mV negative to the mean resting potential.

ACh and the IPSP

ACh had very little effect on the inhibitory potential and the underlying conductance change. This can be seen from several of the Figures. In general, the IPSP conductance change tended to fix the membrane potential and resistance during inhibition at a relatively constant level. This was especially evident when the IPSP resistance was very low, indicating a particularly potent inhibitory effect and/or close proximity of the inhibitory synapses (cf. Figs. 10 and 11*B*). If the IPSP tended to become positive, as a result of leakage of Cl^- or acetate from the micro-electrode (Kelly, Krnjević, Morris & Yim, 1969), ACh caused it to revert temporarily to its usual negative shape (Figs. 7*D*, *E* and 11*B*). In agreement

TABLE 1. Control values of resting potential and resistance, amount of depolarization and ratio of change in potential to change in resistance ($\Delta V/\Delta R$) during application of ACh (50–200 nA), as well as corresponding reversal potentials (E_{ACh}), all recorded in thirteen cells having resting potentials $\leqslant -40$ mV. Final column gives reversal level of IPSP (E_{IPSP}) for ten cells of same population. Means are given, with S.E. and number of observations in brackets below

Resting potential (mV)	Resting resistance (MΩ)	During ACh action: ΔV (mV)	During ACh action: ΔR (MΩ)	$\Delta V/\Delta R$ (nA)	$(\Delta V/\Delta R).R$ (mV)	E_{ACh} (mV)	E_{IPSP} (mV)
−55·6	12·8	+11·6	5·00	3·16	31·0	−86·7	−62·4
(1·56, 30)	(2·42, 30)	(1·18, 33)	(0·54, 30)	(0·38, 30)	(3·81, 30)	(3·98, 30)	(5·72, 17)

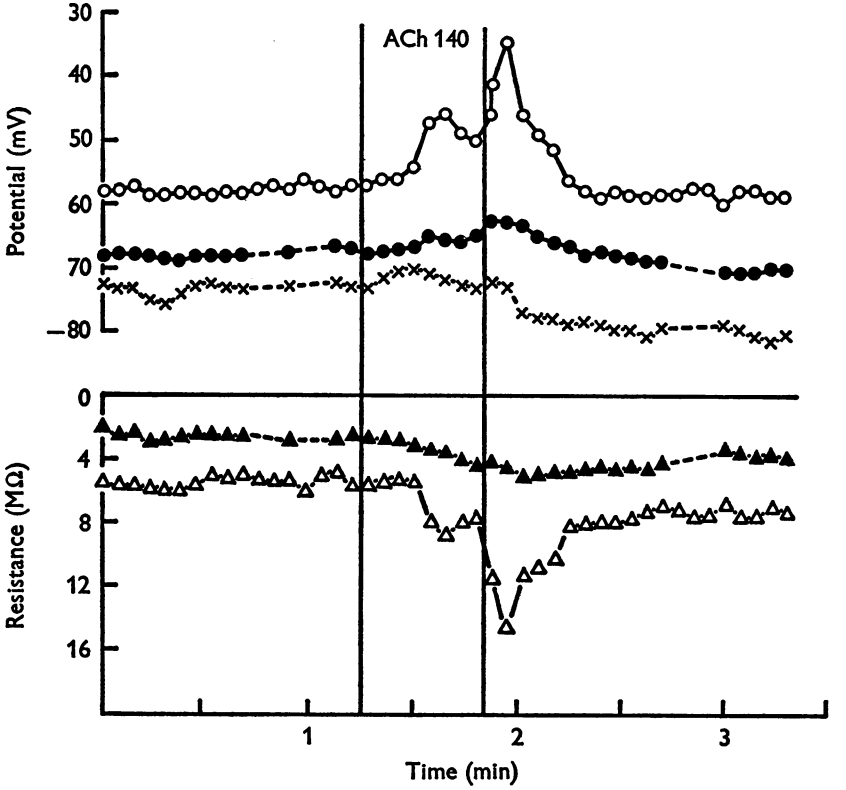

Fig. 10. Another run showing time course of changes produced by ACh in neurone illustrated in Fig. 9: membrane potential at rest (open circles) and during IPSP (filled circles), IPSP reversal level (crosses) and membrane resistance at rest (open triangles) and during IPSP (filled triangles). Each point calculated from regression lines drawn through series of eight voltage–current points (cf. Fig. 9). Note increasing resistance downward.

with these observations, the reversal potential for the IPSP (E_{IPSP}) was not significantly altered during applications of ACh (Figs. 8, 10 and 11*B*); but there was a possibly significant tendency for E_{IPSP} to shift towards somewhat greater negativity *afterwards* (cf. same Figures).

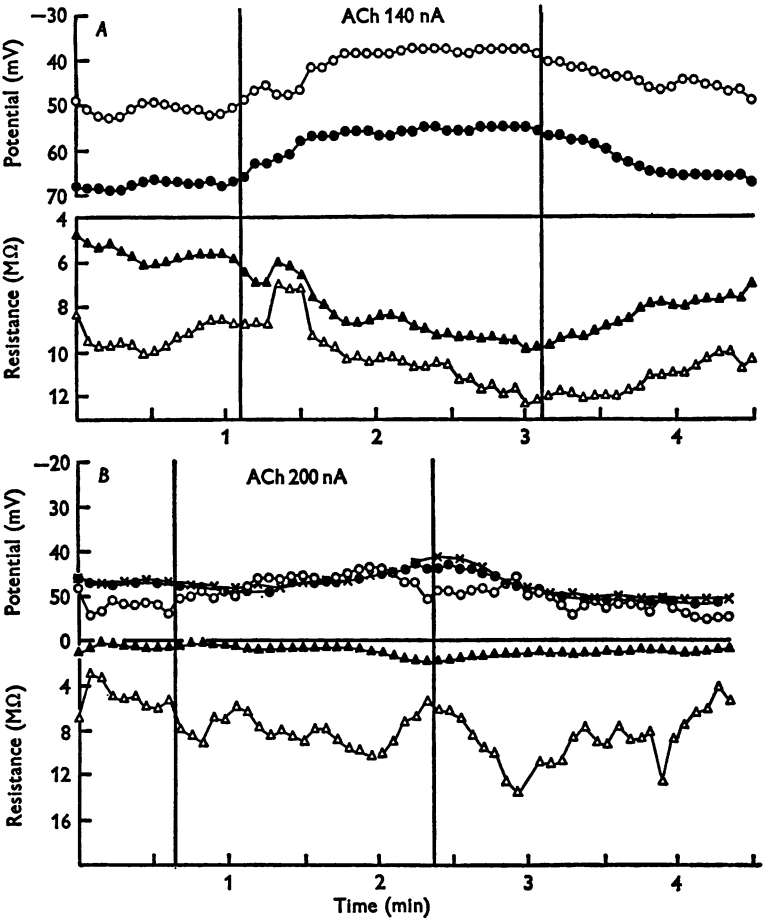

Fig. 11. Effects of ACh on two other neurones. Symbols as for Fig. 10. Recording micro-electrodes contained 1 M-K citrate (*A*) or 3 M-KCl (*B*).

The reversal potential for the IPSP recorded with K citrate electrodes did not usually differ significantly from E_{ACh}. But whenever KCl or K acetate electrodes were used, E_{IPSP} was consistently much more positive (cf. Fig. 8). Hence, in the group of observations summarized in Table 1, for which both types of electrodes were utilized, the mean value of E_{IPSP} was highly significantly more positive (by 24 mV) than the average E_{ACh} (see

also Table 3 in Godfraind *et al.* 1971), where E_{ACh} and E_{IPSP} differed by 42 mV).

Effects of ACh on unresponsive cells (neuroglia)

In thirteen experiments, stable recording from nineteen unresponsive cells made possible tests of ACh, applied in doses of 140–200 nA, for at least 1 min. In half the cases, there was no significant change. Ten applications of ACh resulted in a reversible increase in membrane resistance, varying between 10 and 80% (mean +43·7%), in four cases associated with a reversible change in membrane potential: either a depolarization (by 5–15 mV, three times) or a small hyperpolarization. One further test gave a reversible *fall* in resistance, but no change in potential.

DISCUSSION

The intracellular observations presented here confirm the special character of the excitatory action of ACh on cortical neurones, already apparent from extracellular studies. The outstanding features are a slow depolarizing action, which may or may not lead to an outright discharge, but which strongly potentiates the effects produced by other excitatory inputs; and a tendency to repetitive firing, apparently due to some slowing of repolarization at the end of spikes.

Systematic tests have shown that the membrane resistance tends to rise when cortical cells are depolarized by ACh. Moreover, the voltage–current lines, which respectively characterize the membrane in its normal state and under the action of ACh, intersect at points consistently more negative than the resting potential. These findings indicate a change in membrane conductance for an ion having a highly negative equilibrium potential. In view of the close agreement between E_{ACh} and E_K the simplest explanation for these results is that ACh lowers the resting membrane conductance for K^+ (g_K), and also the delayed K^+ currents associated with the action potential (Hodgkin & Huxley, 1952).

Effect of changes in g_K on the membrane potential

One can describe the resting potential (V) as follows (cf. Hodgkin & Horowicz, 1959):

$$V = E_K T_K + E_{Na} T_{Na} + E_{Cl} T_{Cl}, \quad (1)$$

where E is the equilibrium potential for each ion (defined by the Nernst relation), and T the corresponding transport number ($T = g/G$, where $g_{Na} + g_K + g_{Cl} = G = 1/R$, g being the individual ionic conductances and R the membrane resistance).

There is reason to believe that in the resting state T_{Cl} is relatively small

($< 0{\cdot}1$), since injections of Cl^- affect the resting potential very much less than the IPSP. Precise measurements of the resting T_{Cl} are made difficult by the usually unstable conditions of recording; but the data obtained from two cells in which E_{Cl} was progressively altered by Cl^- leakage from the micro-electrode, gave slopes of $\delta V/\delta E_{IPSP}$ which did not differ significantly from zero. We therefore have some justification for assuming that the resting T_{Cl} may be negligible, so that eqn. (1) can be simplified to

$$V = E_K T_K + E_{Na} T_{Na}. \tag{1a}$$

In the steady state, there is no net membrane current,

$$\begin{aligned} I &= I_K + I_{Na} \\ &= g_K(E_K - V) + g_{Na}(E_{Na} - V) \\ &= 0. \end{aligned}$$

But if g_K is reduced by Δg_K, the K^+ current (I_K) is correspondingly diminished, and a net inward current is established

$$I = \Delta g_K(E_K - V). \tag{2}$$

The magnitude of this depolarizing current clearly depends both on the reduction in g_K and on g_{Na} (which determines the effective driving force $E_K - V$), and it tends to zero as V approaches E_K, its direction reversing if $E_K - V$ becomes positive. A depolarizing action generated by this mechanism should thus have a reversal potential identical with E_K.

Rate of depolarization

The slow depolarizing action of ACh is not a simple consequence of the proposed mechanism. From eqn. (2),

$$C(\mathrm{d}V/\mathrm{d}t) = \Delta g_K(E_K - V), \tag{3}$$

$$= \Delta G(E_K - V) \tag{4}$$

(C is the membrane capacitance). Multiplying both sides of (4) by R_1 (the initial resistance),

$$R_1 C(dV/\mathrm{d}t) = (\Delta R/R_2)(E_K - V) \tag{5}$$

and

$$\mathrm{d}V/\mathrm{d}t = -\Delta R(E_K - V)/R_2\tau, \tag{6}$$

where R_2 is the final resistance and $\tau = RC$ is the membrane time constant. Since $\Delta R/R_2$ is typically 0·28 (Table 1), $E_K - V$ is -35 mV and $\tau \approx 10$ msec (Lux & Pollen, 1966), $\mathrm{d}V/dt$ should be about 1 V/sec. Clearly, the slow observed depolarizations (cf. 2·5 mV/sec in Fig. 5) must be a function of the progressive increase in resistance (cf. Figs. 10 and 11). This phenomenon can be ascribed to a slow interaction between ACh and membrane receptors, and also perhaps to further K^+ inactivation by depolarization. Although cortical neurones do not show anomalous rectification under normal conditions (see below), if partly blocked by the action of

ACh (or some other blockers such as Ba^{2+}), g_K may be further reduced by depolarization. This would contribute to the depression of the delayed K^+ current and may provide a further explanation for the remarkably persistent excitatory effect ('switching-on action') which is sometimes observed. The marked voltage-dependence of the duration of 'humped' spikes recorded in the presence of Ba^{2+} or TEA (Krnjević *et al.* 1971*b*) is in keeping with this suggestion.

E_K and E_{ACh}

The concentration of K^+ inside neurones in the cerebral cortex of cats has been estimated at 166 mM (Bourke & Tower, 1966). However, since 10–15 % of the tissue K^+ is 'slowly exchangeable' (cf. these authors), the effective cytoplasmic $[K^+]$ can be taken as 150 mM. Assuming that extracellular $[K^+]$ is in equilibrium with the $[K^+]$ of cerebrospinal fluid (Davson, 1963), we can use the $[K^+]_{c.s.f.}$ values of 3·8 or 2·66 mM given respectively by Bourke & Tower (1966) and Ames, Higashi & Nesbett (1965). The corresponding reversal potentials for K^+ (E_K), calculated from the Nernst relation, are -98 and -108 mV. Since the lower estimate of $[K^+]_{c.s.f.}$ was based on a larger number of observations (Ames *et al.* 1965) and is presumably more representative, the more negative value of E_K is probably nearer the truth. For the purpose of calculations, we have therefore taken a round figure of -105 mV for E_K.

Some resting potentials of -70 mV (or even more negative) have been recorded in most studies of cortical neurones (Phillips, 1959; Li, 1961; Creutzfeldt, Lux & Nacimiento, 1964; Jasper & Stefanis, 1965; Koike, Okada, Oshima & Takahashi, 1968). Assuming then that the normal resting potential of the undamaged cells is -70 mV, the mean of $-55{\cdot}6$ ($\pm 1{\cdot}6$) mV in Table 1 indicates a 26 % average shunting effect of membrane leaks caused by the micro-electrodes. If the appropriate correction is applied to the mean observed E_{ACh} ($-86{\cdot}7 \pm 4{\cdot}0$ mV), one obtains a 'true' E_{ACh} of -109 (± 5) mV, in excellent agreement with the probable E_K.

The changes in membrane potential and resistance which would be in accordance with the proposed scheme can be predicted as follows:

since
$$V = E_K T_K + E_{Na} T_{Na} \tag{1a}$$
$$= E_K(1 - T_{Na}) + E_{Na} T_{Na} \tag{7}$$
$$= E_K + T_{Na}(E_{Na} - E_K) \tag{8}$$
$$= E_K + R g_{Na}(E_{Na} - E_K). \tag{9}$$

If g_{Na} remains constant, a reduction in g_K causes a rise in R and a depolarization, such that
$$\delta V/\delta R = g_{Na}(E_{Na} - E_K). \tag{10}$$

Substituting the observed ratios $\Delta V/\Delta R$ for $\delta V/\delta R$ (cf. Table 1) one can calculate g_{Na} and the initial T_{Na} from the following equations:
$$g_{Na} = (\Delta V/\Delta R)/(E_{Na} - E_K) \tag{11}$$
and
$$T_{Na} = (\Delta V/\Delta R)R/(E_{Na} - E_K). \tag{12}$$

As might be expected, there was a negative correlation between $\Delta V/\Delta R$ and R ($r - 0{\cdot}357$, $n = 30$, $P < 0{\cdot}05$); and although the product $(\Delta V/\Delta R)R$ was far from constant in different cells (Fig. 12), it was independent of R (r 0·042) or V ($r - 0{\cdot}029$).

This is not surprising, since R must be determined mainly by the size of the cells and V mainly by the efficiency of intracellular recording, neither of these factors being obviously related to T_{Na}.

The average initial T_{Na} can be estimated at 0·207 (S.E. 0·0254) from a mean $(\Delta V/\Delta R)R$ of 31·0 mV (Table 1) and 150 mV for $E_{Na}-E_K$ – the latter is obtained by taking E_K as -105 mV (see above) and E_{Na} as $+45$ mV (calculated from the values of 160 mM for $[Na]_o$ and 32 mM for

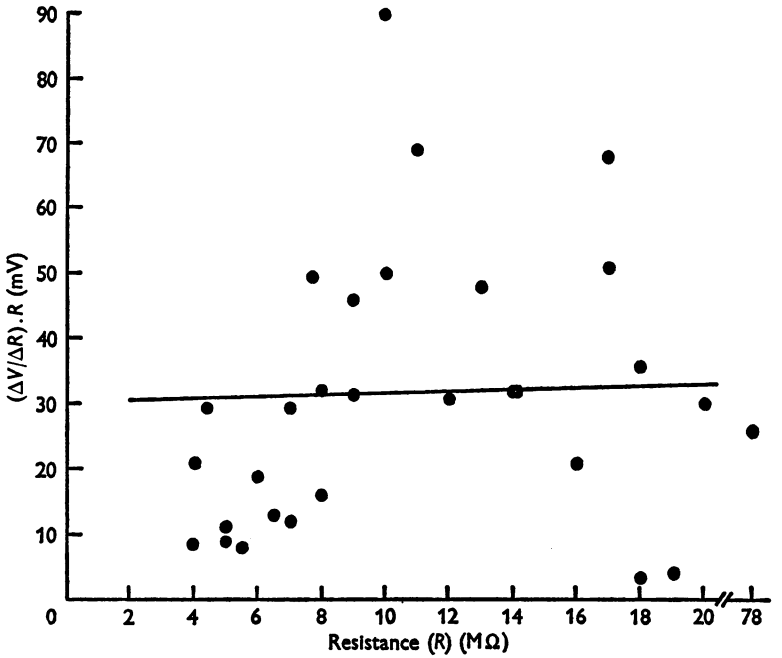

Fig. 12. Thirty values of $(\Delta V/\Delta R).R$ calculated from data obtained in thirteen different neurones, before, during and after release of ACh, and plotted against corresponding value of resistance (R). Line of best fit was given by $y = 0{\cdot}0652x + 30{\cdot}13$; S.E. of regression coefficient was 0·297.

$[Na]_i$ given by Ames *et al.* (1965) and Bourke & Tower, 1966). If these values of T_{Na}, E_K and E_{Na} are inserted in eqn. (2), the resting potential (V) comes to $-74{\cdot}1$ mV (with a $\pm$S.E. range of -70 to -78 mV), which is in good agreement with expectation. One can reasonably conclude that the results are consistent with the hypothesis that ACh acts by selectively reducing g_K.

Changes in g_{Cl}

A reduction in g_{Cl} could also explain a depolarization associated with a rise in resistance. The strongest argument against this possibility is the demonstration that E_{ACh} is not at all sensitive to intracellular injections of Cl^- (or other relevant anions),

so that E_{IPSP}, which probably reflects E_{Cl} (Kelly *et al.* 1969), can be made to differ from E_{ACh} by 20–30 mV (Table 1; see also Table 3 in Godfraind *et al.* 1971).

The failure to observe during applications of ACh any depression of the conductance increase associated with IPSPs, or any clear change in E_{IPSP} confirms other evidence that neither ACh nor K^+ play a major role in the inhibitory process (Krnjević *et al.* 1966; Kelly *et al.* 1969; Krnjević *et al.* 1971*b*). The possibly significant subsequent negative shift in E_{IPSP} may result from an increased activity of the Cl^- pump, triggered by the depolarizing effect of ACh.

Anomalous rectification

The observed depolarizing action of ACh could also be explained by an indirect mechanism involving anomalous rectification (Katz, 1949; Adrian & Freygang, 1962; Nelson & Frank, 1967): if the membrane resistance of cortical neurones tended to rise with depolarization, and if ACh acted only on remote dendrites – by a conventional depolarizing mechanism, through an increase in ionic permeability, but operating too far away to change the input resistance measured at the soma (cf. Smith, Wuerker & Frank, 1967) – then one might record a somatic depolarization associated with an increase in resistance. There are several arguments against this possibility. The first is that cortical neurones do not show any significant anomalous rectification at potentials near the resting level. There is little evidence of any increase in slopes of control voltage–current lines with depolarization (cf. Fig. 9, and also Lux & Pollen, 1966, Koike *et al.* 1968; Dreifuss *et al.* 1969; Godfraind *et al.* 1971). Anomalous rectification has only been detected in cortical neurones during strong hyperpolarization (Takahashi, 1965; Purpura, Prelević & Santini, 1968). Moreover, if the increase in resistance was solely a consequence of depolarization, one would expect ΔR to be clearly related to ΔV: in fact quite large changes in resistances were sometimes recorded in association with only small depolarizations (cf. Fig. 8), and in general there was no correlation between pairs of values of ΔR and ΔV (r 0·0702, n 30). Further evidence is the mainly perisomatic distribution of cortical acetylcholinesterase (Krnjević & Silver, 1965), which is more consistent with a somatic rather than a remote dendritic site of action of ACh.

Action of ACh on different types of cells

Several facts suggest that unlike Ba^{2+}, ACh does not simply compete with K^+ at sites of K^+ movements through the membrane: the blocking effect of atropine and hyoscine, the marked discrepancy between the hydrated dimensions of ACh^+ and K^+, and some significant differences between the depolarizing action of ACh and Ba^{2+} (Krnjević *et al.* 1971*b*). Presumably ACh interacts with specific membrane receptors which can influence the passive fluxes of K^+.

We do not have enough information to be able to say categorically that ACh acts only on 'ACh-excitable' neurones. The cells which are not excited by ACh might have a very low resting T_{Na}, or they might accommodate too rapidly to be excited by a reduction in g_K. However, the regular absence of responses to ACh in superficial cells, and the fact that the latter do not show even a potentiation of discharges evoked in other ways (cf. Fig. 3), are more consistent with a relative or absolute lack of specific ACh-

receptors (cf. Krnjević & Silver, 1965) rather than a merely quantitative difference in membrane parameters.

It was suggested previously that other muscarinic excitatory actions of ACh may operate by a similar mechanism (Godfraind *et al.* 1970; Krnjević *et al.* 1970). This idea has recently been given strong support by evidence that the slow muscarinic excitation of sympathetic ganglia, which is also associated with a rise in membrane resistance (Kobayashi & Libet, 1968, 1970), is caused by a reduction in g_K (Weight & Votava, 1970).

There is little conclusive evidence about changes in permeability at other muscarinic junctions. The demonstration by Durbin & Jenkinson (1961*a*) that carbachol increases fluxes of K^+ in depolarized smooth muscle could be explained by the 'squeezing out' effect of the accompanying contraction; this possibility is supported by these authors' observation that the removal of external Ca^{2+}, which prevents the contraction, can also abolish the increase in K^+ flux (Durbin & Jenkinson, 1961*b*). The effects of ACh on the spike configuration in the same muscle (Bülbring, 1957) are comparable with some of our observations on cortical neurones.

The clear increase in the membrane resistance of several 'neuroglia' which confirms previous observations (Krnjević & Schwartz, 1967), is of some interest. This is not inconsistent with the notion that ACh acts on specific receptors, since neuroglia show a substantial amount of cholinesterase activity (Koelle, 1954; Gerebtzoff, 1959). As might be expected in view of the low probable g_{Na} of the neuroglial membrane (Kuffler & Nicholls, 1966; Dennis & Gerschenfeld, 1969), a depolarizing effect was seen less frequently than a rise in resistance. These, and previous observations on red blood cells (Holland & Greig, 1950), raise the intriguing possibility that cholinesterase may itself be the K^+ carrier or ionophore, and that it may control K^+ movements in a wide variety of cell membranes.

Significance of excitatory action of ACh on cortical neurones

Two aspects of this excitatory mechanism are of some importance. First, its effectiveness is largely dependent on the magnitude of the prevailing T_{Na}. A cell that is initially totally impermeable to Na^+ cannot be depolarized by this mechanism. Hence, the excitatory action of ACh (and therefore of any cholinergic input) on cortical neurones can be expected to vary according to the condition of the cells, and particularly the amount of background excitatory input. This factor may help to explain the striking variations in the numbers of cells readily excitable by ACh in different preparations (Krnjević & Phillis, 1963*b*; Crawford & Curtis, 1966). The second important feature is the marked enhancement of the excitatory action of other inputs, with a particularly striking prolongation

of evoked discharges. As pointed out before (Krnjević, 1969), these unusual properties of ACh may throw some light on its role in cortical function. Ascending cholinergic pathways, by a discrete or diffuse liberation of ACh, may greatly change both the general responsiveness and the pattern of firing of small or large groups of cortical neurones; a prolongation of discharges may be of particular importance in the development of memory traces (Gerard, 1955) and conscious processes (Libet, 1965; Libet, Alberts, Wright & Feinstein, 1967).

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