*J. Physiol.* (1976), **263**, *pp.* 417–439
*With 1 plate and 9 text-figures*
*Printed in Great Britain*



# ACTION POTENTIALS IN THE RAT CHROMAFFIN CELL AND EFFECTS OF ACETYLCHOLINE

BY B. L. BRANDT, S. HAGIWARA*, Y. KIDOKORO AND S. MIYAZAKI*

*From the Salk Institute, P.O. Box 1809, San Diego, California 92112, U.S.A. and the *Department of Physiology, School of Medicine, UCLA, Los Angeles, California 90024, U.S.A.*

(*Received 9 April 1976*)

SUMMARY

1. Electrophysiological properties of the rat chromaffin cell were studied using intracellular recording techniques.

2. The resting potential in the chromaffin cell was $-49 \pm 6$ mV (mean $\pm$ S.D., $n = 14$) in standard saline containing 10 mM-Ca whereas that in Na-free saline was $-63 \pm 9$ mV ($n = 17$). At rest, the membrane has a substantial Na permeability.

3. Action potentials were evoked by passing current through the recording electrode. In standard saline the major fraction of the action potential disappeared either upon omission of external Na ions from standard saline or addition of 1 $\mu$M tetrodotoxin (TTX). We conclude that action potentials in the chromaffin cell are due mainly to an increase in the permeability of the membrane to Na ions.

4. Small but significant regenerative action potentials were observed in Na-free saline, and when Ca in Na-free saline was replaced by Ba, prolonged action potentials occurred. We conclude that action potentials in the chromaffin cell also have a Ca component.

5. Iontophoretic application of acetylcholine (ACh) produced a transient membrane depolarization in standard saline.

6. Spontaneous action potentials were recorded extracellularly by microsuction electrodes. They occurred at a rate of 0·05–0·1/sec in almost all cells.

7. When the perfusion fluid contained $3 \times 10^{-7}$ M to $10^{-4}$ M ACh the spike frequency increased up to about 2/sec. This stimulatory effect of ACh was blocked by $10^{-7}$ M atropine but not by $10^{-3}$ M hexamethonium nor by $10^{-5}$ M-d-tubocurarine.

8. The importance of Ca entry during action potentials for catecholamine secretion is discussed.

The experiments were carried out at the Salk Institute.

## INTRODUCTION

In the adrenal chromaffin cell (Douglas & Rubin, 1961), as well as in other secretory cells (Douglas, 1968), Ca ions are indispensable in the external solution for hormone secretion. There are several observations which indicate that Ca ions enter the cell after appropriate stimulation. These are: (1) the rate of $^{45}Ca$ uptake is increased in the adrenal chromaffin cells responding to acetylcholine (ACh) (Douglas & Poisner, 1962); (2) in the mast cell the Ca ionophores A-23187 and X-537A initiate exocytosis in the presence of Ca ions in the external medium (Foreman, Mongar & Gomperts, 1973); (3) iontophoretic injection of Ca ions into the mast cell produces a visually observable extrusion of the granules (Kanno, Cochrane & Douglas, 1973).

The next question is just how does stimulation produce Ca entry to the cells. An elevation of the bath K concentration sufficient to depolarize the cell membrane increases the release of catecholamine from the chromaffin cell (Vogt, 1952) and external Ca ions are necessary for this stimulatory effect (Douglas & Rubin, 1961, 1963). In the chromaffin cell, high concentrations of ACh have been found to cause membrane depolarization (Douglas, Kanno & Sampson, 1967*a*). It is possible that an influx of Ca may well be due to a potential-dependent membrane permeability increase to Ca ions. However, Kanno & Douglas (1967) did not find any action potentials in gerbil chromaffin cells and therefore eliminated the possibility of action potential involvement in the stimulus–secretion coupling. Action potentials do seem to be involved in certain secretory cells. In a cell line of anterior pituitary cells ($GH_3$) which secrete growth hormone and prolactin continuously (Tashjian, Yasumura, Levine, Sato & Parker; 1968), Kidokoro (1975*c*) found spontaneous Ca action potentials. Their frequency increased when the external solution contained a small amount of thyrotropin releasing factor (TRF). TRF is known to increase prolactin secretion (Hinkle & Tashjian, 1974). This increase of action potential frequency did not occur when a similar but inactive TRF derivative was applied. It was proposed that Ca entry as a consequence of a Ca action potential underlies the hormone secretion (Kidokoro, 1975*c*). A similar suggestion has been made for insulin secretion by the pancreatic $\beta$ cell (Matthews & Sakamoto, 1975).

We decided to re-examine the membrane electrical properties of the chromaffin cells. Spontaneous action potentials whose frequency was changed by acetylcholine were found. The data suggest that Ca ions which enter the cell during action potentials are important for catecholamine secretion.

## METHODS

### *Isolation and identification of chromaffin cells*

Adrenal glands were removed aseptically from Fisher F/344 rats (Simonsen, Calif., U.S.A.) under ether anaesthesia. Each adrenal medulla was dissected free of the cortex and cut into about eight pieces with a pair of fine needles. On one occasion histological sections for light microscope were made from the decorticated adrenal gland and examination showed that essentially all of the cortex was removed. To dissociate the cells enzyme treatment was done as described by Vale & Grant (1975) except that Viokase treatment was omitted. Each adrenal medulla yielded about $10^5$ chromaffin cells. The dissociated cells from one animal were distributed into ten 60 mm plastic cultured dishes (Falcon, Calif., U.S.A.) containing 4 ml. modified Eagle medium (Vogt & Dulbecco, 1963) plus 10% foetal bovine serum. The chromaffin cells did not attach well to plastic tissue culture dishes. This presented no problem for extracellular recordings since unattached cells were used. However, cells to be used for intracellular electrode studies were usually plated on dishes containing confluent layers of other cells. Four different clonal cell lines with different electrophysiological properties, $BC_3H_1$ (Schubert, Harris, Devine & Heinemann, 1974), $A_7$, $A_9$ (Kimes & Brandt, 1976) and 3T3 (Nelson, Peacock & Minna, 1972) were used as attachment layers. The electrophysiological properties of the chromaffin cells were tested after one to seven days and were the same in all cases. On one occasion cells from adrenal medulla were plated on the plastic dish without underlying cells and kept for several days. Some chromaffin cells were attached to the culture dish and their electrophysiological properties were the same as the cells attached to underlying cells.

Chromaffin cells were distinguished from other cells in the culture by their distinctive appearance with phase contrast optics (refer to Douglas *et al.* 1967*a*). The cells were observed with a 40× water-immersion phase-contrast objective and 10× ocular lens (Carl Zeiss, West Germany) during both intra- and extracellular recording. See Kidokoro (1975*a*) for the details of the optical apparatus. Identification was easier when the cell was one of a cluster of two to five similar cells. The identification criteria were validated by positive bichromate staining of cells from which recording had been done. Cells were stained by a modification of the method of Hillarp & Hökfelt (1955). After electrophysiological recording was completed the cell was photographed *in situ* with the micro-electrode still in place. The saline was replaced with the staining solution (4% $K_2Cr_2O_7$, 4% formaldehyde, 0·2 M Trisma base) and the cells were left at room temperature for 2 hr. Then the bichromate solution was washed off of the cells. The identified cells were stained yellow or yellow-green with this staining procedure. Identification of cells used for extracellular recording was simpler because of the absence of a foreign cell layer. Phase contrast microscopy was used with the same criteria as described above for attached cells to identify chromaffin cells. No cells with the morphology expected of ganglion cells were seen in any of the chromaffin cell cultures. The chromaffin cell size was measured on microphotographs. The average diameter of nineteen round cells was $16 \pm 3{\cdot}5$ $\mu$m (mean ± S.D.).

### *Extracellular recording*

For extracellular spike recording a glass electrode which had an inner tip diameter of 2–5 $\mu$m was made using a microforge (De Fonbrune Establishments, Beaudouin, Paris, France) and filled with standard saline. The shaft of the electrode was connected to a syringe with a polyethylene tube filled with mineral oil. The metal

syringe (Gordon Instruments, Ind., U.S.A.) was modified by installing a finer plunger inside the original plunger. With this modification there were both a fine control and a coarse control of the pressure. To record extracellular spike potentials the tip of the electrode was first placed on the cell surface and a part of the cell was slowly sucked 5–10 $\mu$m into the electrode (see Pl. 1). The resistance of the electrode before suction ranged between 1 and 5 MΩ. Suction resulted in an increase of the resistance to 10–20 MΩ. Since the membrane resistance of the entire cell observed with an intracellular electrode was on the order of 400 MΩ, the above 10–20 MΩ resistance represented the leakage resistance between the electrode and the bath. The extracellular spike was recorded as the IR drop of the action current across this leakage resistance. The amplitude of the recorded spike could be as large as 3 mV but usually was kept between 0·2 and 1·0 mV so as to avoid excessive deformation of the cell. The extracellular spike potential recorded with a microsuction electrode was diphasic in standard saline. The positivity at the inside of the electrode was followed by the negativity (see record *A* 2 of Text-fig. 6). The initial positive deflexion represents the excitation of the cell membrane outside the electrode and the negative deflexion, that of the membrane inside the electrode. This can be demonstrated by the experiment shown in Fig. 6. Trace *B*1 shows a spike potential obtained during discharge of the cell in $10^{-5}$ M-ACh. When 1 $\mu$M-TTX was introduced into the external solution the immediate effect was the decrease of the positive phase. This corresponds to the fact that the membrane area outside the electrode is affected by TTX, while the membrane inside the electrode is protected from the TTX in the bath. Although the relative amplitude of the positive deflexion to the negative deflexion varied among different cases the sequence was always positive–negative.

Current pulses were applied through the microsuction electrode and resulting spike potentials were recorded by using a bridge circuit (M-4A, W.P. Instruments, Conn., U.S.A.). Most of the extracellular spike recordings were made with an AC coupled amplifier (time constant, 20 msec).

*Intracellular recording*

Glass micro-electrodes for intracellular recording were filled with 3 M-KCl and had resistances of 120–180 MΩ. Single electrodes were used for both passing current and measuring membrane potential by means of a high-input impedance preamplifier with a bridge circuit (M-4A, W.P. Instruments, Conn., U.S.A.). The bridge circuit was modified so that it could be balanced with the high-impedance electrodes used as long as the current being passed was less than 0·5 nA. The current passed through the electrode was measured by a conventional current–voltage conversion circuit having a feed-back resistance of 10 MΩ, inserted between an Ag–AgCl pellet in the bath and earth. The current monitoring circuit integral to the bridge circuit in the preamplifier was also used for some measurements. Both methods gave the same values. The input impedance of the preamplifier was $10^{10}$ Ω shunted by 1·5 pF, and the input offset current was less than $10^{-11}$ A. Thus the offset current induced error in the membrane potential of a cell with the average input resistance of 430 MΩ would be less than 4·3 mV.

The microscope on which the cells were placed and the micromanipulators (E. Leitz, West Germany) that held the micro-electrode and ACh micropipette were all mounted on an efficient vibration isolation table (Serva-Bench, Barry Wright Corp., Mass., U.S.A.). The combination of high resistance micro-electrodes and vibrationless mounting of the cells and electrodes helped to obtain stable penetrations with relatively large resting potentials and high input impedances. For further details of the electrophysiological methods, see Kidokoro (1975*a*).

*Iontophoretic application of ACh*

Glass micropipettes for iontophoretic application of ACh filled with 3 M-ACh and had resistances of 120–400 MΩ. ACh was delivered by outward current pulses of 2–15 nA in intensity and 4–10 msec in duration. To keep ACh from diffusing from the pipette, a backing current of about 3 nA was always applied. When the amplitude of the depolarization evoked by iontophoretically applied ACh was plotted against the total charge passed through the ACh pipette, the dose–response curve was almost linear over a certain range (Text-fig. 5*D*). The ACh sensitivity of a cell was defined as the slope of the linear portion of the dose–response curve (Kuffler & Yoshikami, 1975).

TABLE 1. Composition of salines used (mM)

| | Na | Ca | Ba | K | Tris | HEPES | Cl | Glucose |
|---|---|---|---|---|---|---|---|---|
| Normal saline | 157·5 | 1·8 | 0 | 5·6 | 0 | 5 | 161·2 | 38 |
| Standard saline | 145·1 | 10 | 0 | 5·6 | 0 | 5 | 168·2 | 38 |
| Na-free saline | 0 | 10 | 0 | 5·6 | 182·5 | 0 | 177·1 | 38 |
| Na-free, Ba saline | 0 | 0 | 10 | 5·6 | 182·5 | 0 | 177·1 | 38 |

pH was adjusted to 7·4.

*Solutions*

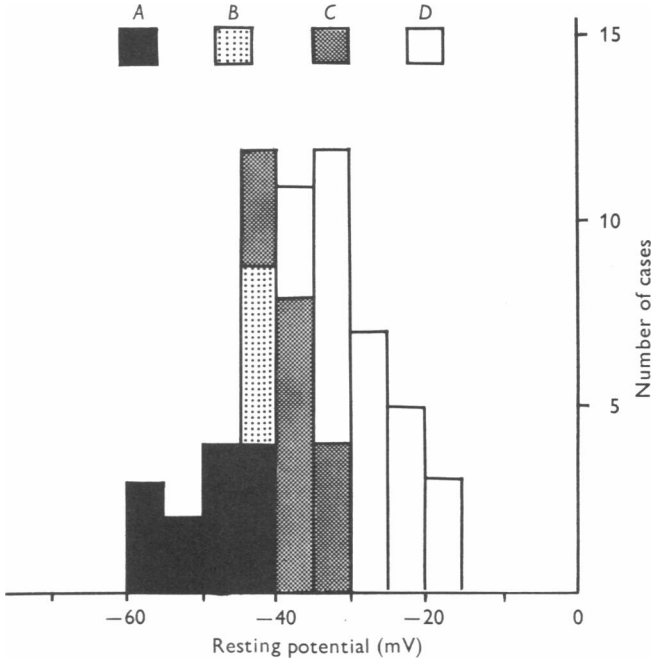
The compositions of solutions used are shown in Table 1. Most experiments were performed in standard solution (10 mM-Ca) because the resting potential recorded with an intracellular electrode tended to remain at its initial value longer in standard solution than in the normal solution (1·8 mM-Ca). Control experiments performed with extracellular microsuction electrodes showed that responses of the cells to applied current as well as to ACh were essentially the same in standard and normal saline, except that the frequency of spontaneous action potentials was higher in normal saline. ACh chloride, atropine sulphate, hexamethonium bromide, D-tubocurarine chloride and TTX (Sigma, Mo. U.S.A.) were dissolved in distilled water at high concentrations and small amounts of these solutions were diluted into standard solution for application to the cells. The test solution was introduced into the bath from an inlet tube placed at the water immersion objective lens of the microscope and the excess solution was removed by suction at the edge of the culture dish so that only a small amount of solution (about 0·5 ml.) was left around the objective lens forming a meniscus. With this arrangement the solution surrounding the cell under observation was altered in a short time even though the perfusion rate was slow (2·6–2·8 ml./min). All experiments were performed at room temperature (22–24° C).

## RESULTS

### *Resting membrane potentials*

The resting membrane potential of the chromaffin cell often diminished rapidly after impalement by a micropipette. This was probably due to injury of the cell by the electrode penetration. Text-fig. 1 shows the

distribution of resting potentials in cells impaled in standard saline. In about 30% of the cells the amplitude of the resting potential either remained at its initial value or decreased only slightly during the first minute.




Text-fig. 1. Histogram of the resting potentials in the chromaffin cells. The cells showed four types of behaviour in terms of excitability. The behaviour was also related to membrane potential as follows: area *A*, action potentials were evoked only by outward current; *B*, evoked both by outward current and by break of inward current; *C*, evoked only by break of inward current; *D*, no action potential.

The excitability of the cell membrane was examined in sixty-one cells by applying current pulses through the recording pipette. The responses of the cell membrane to current pulses could be classified into four categories related to the resting potential. *A*, action potentials were produced by an outward current pulse only. *B*, they were produced both by outward current and by termination of an inward current pulse. *C*, they were produced only by the break of inward current and no all-or-none responses were obtained with the outward current. *D*, no sign of a regenerative response was found. Several of these responses were occasionally encountered during impalement of one cell. That is, in a cell which initially had a resting potential more negative than −50 mV action potentials were evoked only with outward current, but as the

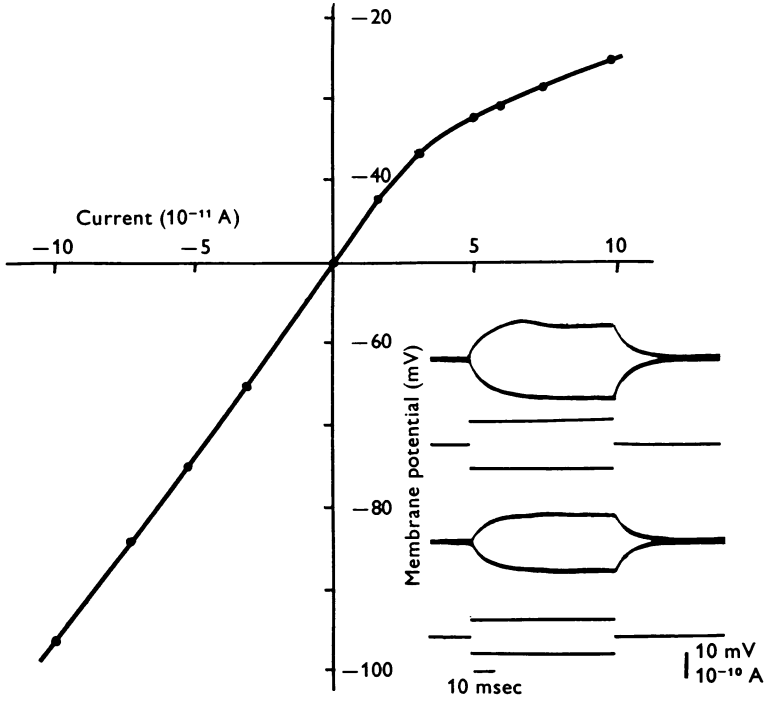
resting potential gradually decreased the action potentials soon were evoked only with anodal break stimulation and finally after 10–20 min the cell did not show any sign of a regenerative response. Therefore we conclude that these varieties of excitability among chromaffin cells were due to damage and not due to the presence of several cell types with different properties. As will be described below, the excitability of the chromaffin cell can be examined also by observing the extracellular spike potentials. With this technique practically all the cells responded with all-or-none spikes to an outward current pulse. Anodal break responses were seen only when the cell showed spontaneous discharges of relatively high frequency and this was found rarely in standard saline. These extracellular observations therefore support the conclusion that categories *B*, *C* and *D* of the cell responses to a current pulse obtained with an intracellular electrode probably represent artifacts due to injury caused by insertion of the electrode. This leads to the conclusion that the true resting potential of the chromaffin cell in standard saline is somewhat more negative than $-45$ mV. The average resting potential of the cells in category *A* was $-49 \pm 6$ mV (mean $\pm$ S.D., $n = 14$). This is considerably more negative than $-29$ mV reported by Douglas *et al.* (1967*a*).

In Na-free saline the average value of the membrane potential for cells which showed regenerative responses with depolarization was $-63 \pm 9$ mV ($n = 17$). This value is significantly more negative than that in standard saline. In a few cases the external solution was changed from Na-free saline to standard saline while the electrode was kept inside the cell. The resting potential was $-60$ to $-70$ mV in Na-free solution in all of these cases and became 10–20 mV more positive. Therefore we conclude that the Na permeability of the resting chromaffin cell membrane is more significant than it is in the membrane of the giant axon of the squid (Hodgkin & Katz, 1949) and of the frog muscle cell membrane (Boyle & Conway, 1941). A similar conclusion has been reached by Matthews & Petersen (1973) for pancreatic acinar cells.

*Membrane resistance and capacitance*

The steady-state relation between the current and voltage obtained at the end of an 80 msec current pulse in standard saline is shown in Text-fig. 2. The relation was linear when the membrane potential was more negative than $-35$ mV. When the membrane potential was made more positive than $-35$ mV the slope resistance decreased. In other words, the chromaffin cell membrane shows an outward rectification in this range but has no inward rectification in the range more negative

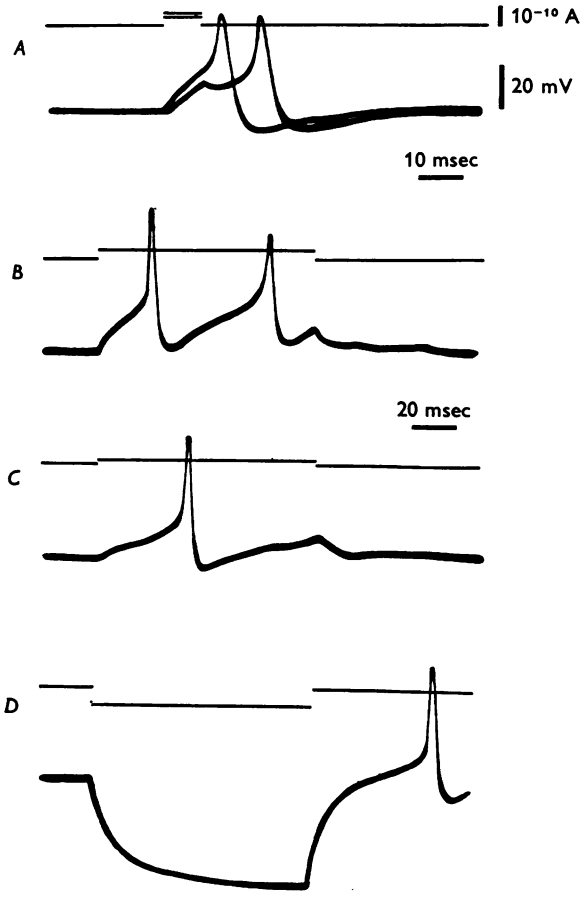
than the resting potential. The input resistance was estimated from the linear portion of the current–voltage relation in the cells which showed action potentials only with outward current in standard saline and the average value was $430 \pm 70$ MΩ ($n = 6$). The time constant calculated from the exponential time course of the potential change (see sample records in Text-fig. 2) produced by a small current pulse ranged from




Text-fig. 2. Steady-state current–voltage in standard saline. Membrane potential changes plotted at the end of 80 msec current pulses were measured. Outward current is positive on the abscissa. Resting potential: −50 mV. Inset: sample records showing time course of the potential change.

6 to 10 msec. The membrane capacity obtained from the membrane resistance and the time constant was $1{\cdot}9 \pm 0{\cdot}3 \times 10^{-11}$ F ($n = 5$). There were considerable variations in the membrane resistance and capacitance from cell to cell. This is due partly to the variation in size of the impaled cells. Often recordings were made from a cell which was one of a cluster of two to five cells. If there were a significant degree of electrical coupling among those cells the variations in the total membrane resistance and capacitance would be expected to be even larger than were seen. Therefore

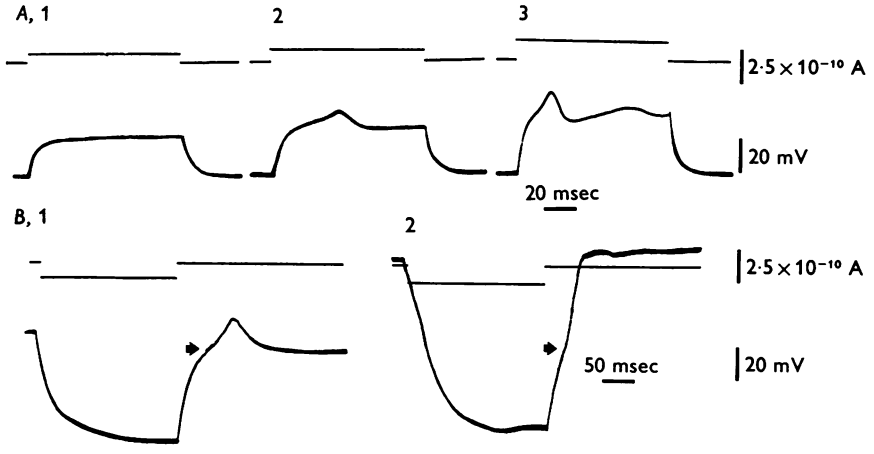
it is unlikely that there is a strong coupling among cells in a cluster. This conclusion was confirmed by applying ACh iontophoretically to adjacent cells of a cluster; responses were found only when ACh was applied to the impaled cell. Some of the variation is likely due to damage to the




Text-fig. 3. Intracellular records of action potentials in standard saline. *A*, regenerative responses evoked by short current outward pulses; *B*, *C*, action potentials produced by long current pulses. *D*, an action potential elicited at termination of an inward current. Resting potential, −45 mV.

cell membrane accompanying the electrode impalement. If the average cell diameter is taken as 16 $\mu$m, then 430 M$\Omega$ and $1{\cdot}9 \times 10^{-11}$ F correspond to 3·5 k$\Omega \cdot$cm$^2$ and 2·3 $\mu$F/cm$^2$ respectively. The calculated membrane resistance and capacitance are of the same order of magnitude as the values for other neuronal and muscle membranes (Hubbard, Llinas & Quastel, 1969). Thus the unusually high input impedance of these cells

can be accounted for completely by their small size. The input resistance of the cell in Na-free medium was observed also from the linear portion of the current–voltage relation. Values tended to be higher (average $550 \pm 130$ MΩ, $n = 8$), probably due to the significant Na permeability in the resting membrane of the chromaffin cell. This is in agreement with the resting potential data.




Text-fig. 4. *A*, depolarizing responses in Na-free saline. The current intensity was increased threefold. A graded response appears, associated with an inflexion at the foot. Resting potential, −70 mV. *B*, 1: a small anode break response in Na-free saline. The membrane was preliminarily hyperpolarized to −107 mV from the resting potential of −43 mV. 2: a prolonged anode break response in the same cell after introduction of Ba saline. Resting potential +3 mV and conditioning hyperpolarization −96 mV. Note that the potential level after the pulse is more positive than the original resting potential. Arrows indicate the inflexion reflecting the critical membrane potential for action potential initiation.

*Action potentials*

Some action potentials produced by an outward current pulse are shown in records *A–C* of Text-fig. 3. The membrane potential level for initiation of an action potential was about −35 mV in standard solution. The maximum rate of rise ranged between 50 and 70 V/sec. When a current of suprathreshold intensity was applied prolonged repetitive action potentials appeared as shown in Text-fig. 3*B*. The resting membrane potential of this cell, −45 mV, was in the range (−40 to −45 mV) in which anode break action potentials were usually present. The overshoot of the action potential in the absence of stimulus currents was usually +10 to +15 mV. Since the observed overshoot could have been attenuated by the leakage of the membrane due to impalement the actual value of

the overshoot may be significantly greater. Further evidence suggesting significant membrane damage by impalement is the finding that the spontaneous action potentials observed with extracellular recording (see below) were not seen with intracellular recording. This may be explained by the greater damage caused by intracellular electrodes than by extracellular electrodes.

Replacement of the Na ions in the bath with Tris caused the major fraction of the action potential to disappear (Text-fig. 4 *A*). In spite of the large negative resting potential of the cell whose responses are illustrated, only graded responses to the outward current pulses were found (Text-fig. 4 *A* 2, *A* 3). Moreover when a cell was impaled in Na-free saline and then the external solution was quickly perfused with standard saline, the resting potential became less negative to about $-45$ mV but action potentials became larger and faster. This indicates that the major component of the action potential in the chromaffin cell is produced by an increase in the Na permeability of the membrane. Since Mn ions are known to block Ca action potentials (Hagiwara & Nakajima, 1966), a few experiments were performed in standard solution in which 10 mM-$CaCl_2$ was replaced with 2 mM-$MnCl_2$ plus 12 mM-NaCl. All-or-none action potentials with a normal rate of rise were seen in the Ca-free Mn medium. This result shows that Na alone can support the all-or-none action potential.

After removal of the external Na ions a small but significant graded response with an inflexion at the foot remained (Text-fig. 4 *A*). A similar regenerative response was seen after cessation of inward current when the resting potential was $-43$ mV (Text-fig. 4 *B* 1). The response is likely due to an increase of the membrane permeability to Ca ions. In other tissues such as in the amphioxus muscle cell (Hagiwara & Kidokoro, 1971) a similar small graded response becomes an all-or-none response when the K conductance is suppressed by either procaine or tetraethylammonium (TEA) ions. This was examined for the chromaffin cell without success since no cells showed a resting potential more negative than $-30$ mV in the presence of either 7 mM procaine or 10 mM-TEA-Cl.

In most tissues Ba can substitute Ca for the Ca-dependent action potential, and often the amplitude and duration of the action potential become greater in Ba at equimolar substitution (Fatt & Ginsborg, 1958; Hagiwara & Naka, 1964; Kidokoro, 1975*b*). In several experiments a chromaffin cell was first impaled in Na-free Ca saline and then Na-free Ba saline was introduced into the dish while the electrode was inside the cell. The amplitude of the resting potential rapidly decreased upon introduction of Ba saline. A similar decrease of the resting potential with Ba ions has been observed by Douglas *et al.* (1967*b*). During the

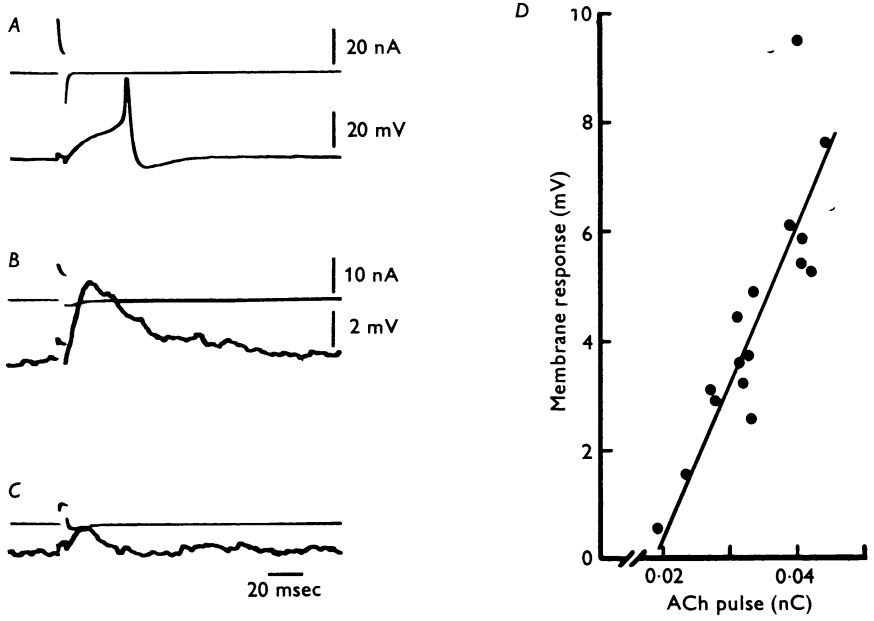
course of depolarization spontaneous action potentials were often observed. The action potentials had a short duration at first and then the duration gradually increased. This was followed by an abrupt depolarization to almost zero level. Records 1 and 2 of Text-fig. 4*B* were obtained from the same cell before and after application of Ba saline. The resting potential was −43 mV in Ca saline and a small action potential was seen after termination of the inward current (record *B*1). In Ba saline the resting potential shifted to a small positive value. The termination of the inward current pulse initiated a long-lasting anode-break response (record *B*2) and then the membrane potential became more positive than the original potential level. Over a period of several seconds the membrane potential returned slowly to the original level. The positive potential observed following the termination of the anodal pulse (Text-fig. 4 *B*2) probably represents the plateau phase of the action potential. The abrupt positive shift in the resting potential upon the application of the Ba solution could be due to the spontaneous initiation of a prolonged action potential as has been reported in frog neurones after application of Ba ions (Tasaki, 1959). The powerful stimulatory effect of Ba ions on catecholamine release (Douglas & Rubin, 1964) could be explained by the entry of Ba ions subsequent to an increase in the membrane conductance to Ba ions. It is possible that a decrease of K conductance with depolarization in Ba saline might also cause the abrupt positive shift in the resting potential. This is less likely since we did not observe inward rectification in the chromaffin cell as described previously. The threshold membrane potential for initiation of the response as determined by the inflexion (indicated by an arrow in Text-fig. 4 *B*) on the rising phase of the potential change was similar in Ca and Ba media. This suggests that Ba can substitute for Ca for the response of the chromaffin cell in Na-free media in the way found in other tissues producing Ca dependent action potentials.

*Responses to iontophoretically applied ACh*

Iontophoretic application of ACh to the membrane of the chromaffin cell produced a transient depolarization (Text-fig. 5*A*, *B*, *C*). The time to peak ranged from 10 to 20 msec; and the half-decay time ranged from 40 to 70 msec. Action potentials were evoked when the depolarization was sufficiently large (Text-fig. 5*A*). The ACh sensitivities measured in standard saline as described in the Methods are shown in Table 2. Considering the large input resistance (about 400 MΩ) of the chromaffin cells, the sensitivities are low compared to the 5000 mV/nC found at the end-plate region of a frog muscle cell with 1 MΩ input resistance (Kuffler & Yoshikami, 1975) and the 258 mV/nC with 20–50 MΩ input resistance

in the frog heart parasympathetic neurones after denervation (Harris, Kuffler & Dennis, 1971; Kuffler, Dennis & Harris, 1971).

Douglas *et al.* (1967*b*) reported that ACh depolarized chromaffin cells in sucrose substituted Na-free saline and they attributed this to an inward Ca influx. ACh responses were recorded in Na-free saline in thirteen cells which had resting potentials between −35 and −64 mV




Text-fig. 5. *A–C*, depolarizations (in standard saline) induced by iontophoretically applied ACh. The intensity of outward current through an ACh electrode (upper trace) was increased from *C* to *A*. Resting potential, −40 mV. An action potential is superimposed on the depolarization in *A*, where gains of both potential and current traces are lower than in *B* and *C*. *D*, relation between iontophoretically applied ACh (nC) and the amplitude of depolarization (mV).

and input resistances between 240 and 670 MΩ. We did not observe depolarization with application of ACh except for one cell which showed a small depolarization that had a similar time course to the ACh response observed in standard saline and an ACh sensitivity of 6 mV/nC. In isotonic Ca saline two cells were examined but no obvious depolarization was seen with application of ACh. We conclude that the Ca influx caused by ACh is small compared with that of Na.

*Extracellular recording of chromaffin cell activity*

Spontaneous action potentials occurring at a rate of 0·05–0·1/sec were observed extracellularly in most of the chromaffin cells (more than 90% of the cells tested). The intervals between the spikes were usually irregular and occasional bursts were seen.

TABLE 2. ACl response

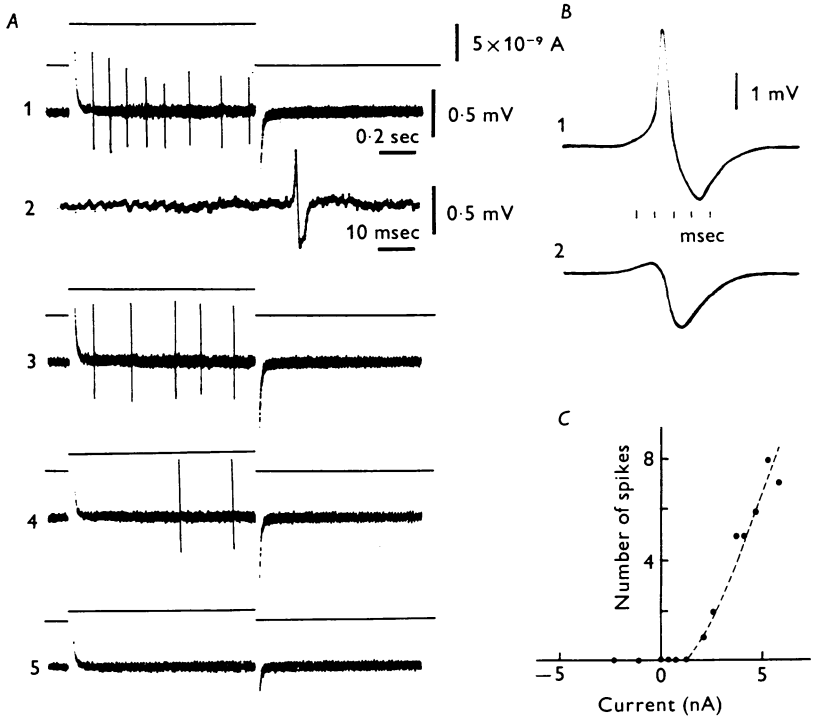
| Cell no. | Resting potential (mV) | Input resistance (MΩ) | Sensitivity (mV/nC) |
|---|---|---|---|
| 1 | −28 | 290 | 210 |
| 2 | −50 | 170 | 210 |
| 3 | −40 | 410 | 510 |
| 4 | −35 | 190 | 290 |
| 5 | −45 | 1040 | 430 |
| 6 | −37 | 220 | 240 |
| 7 | −40 | 360 | 490 |
| 8 | −30 | 250 | 420 |
| | −28 to −50 | 170 to 1040 | 210 to 510 |

Outward current pulses applied through the extracellular electrode initiated spike potentials by depolarizing the portion of the cell membrane outside the electrode. The number of spikes produced during the current pulse increased with increasing intensity as shown by Text-fig. 6*A* and *C*. When the intensity of the current was high, as in the case of record *A* 1 of Text-fig. 6, the amplitude of the positive phase and also the spike frequency decreased with time. This is probably due to the inactivation of action potentials caused by sustained membrane depolarization. The termination of inward current pulses did not usually produce spike potentials with extracellular recording. In other words the uninjured chromaffin cell usually does not show an anode break response. On a few occasions, spontaneous discharges of a relatively high frequency (0·5–1/sec) were observed in cells in standard saline. In those cells an inward current pulse suppressed discharge during the pulse and a train of spikes occurred at the termination of the pulse.

*Effects of ACh and its antagonists*

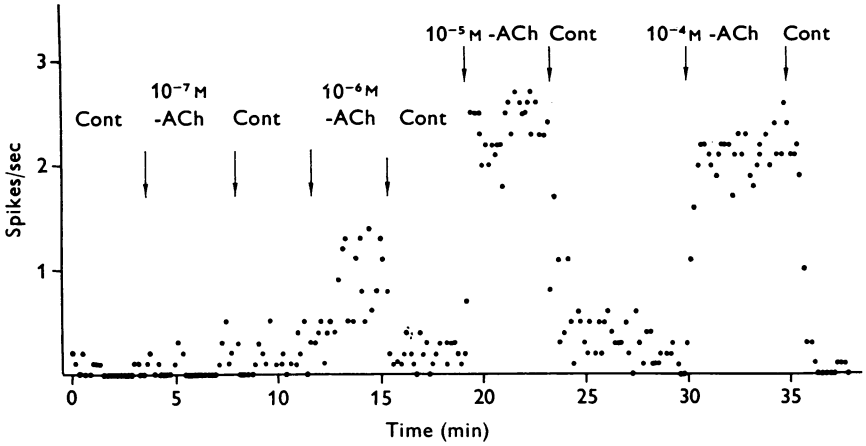
The membrane of the chromaffin cell is depolarized by ACh (Douglas *et al.* 1967*a*; see also above) and this leads to catecholamine release by the cell (Douglas & Poisner, 1962). Standard saline containing varying concentrations of ACh was applied to the cell from which extracellular spikes were recorded. As described above, most cells showed a low

frequency (0·05–0·1/sec) spontaneous discharge in the absence of ACh. A noticeable increase of the frequency was found at $3 \times 10^{-7}$ M-ACh. The frequency increased with increasing ACh concentration. Text-fig. 7 shows the effects of various ACh concentrations on the average number of

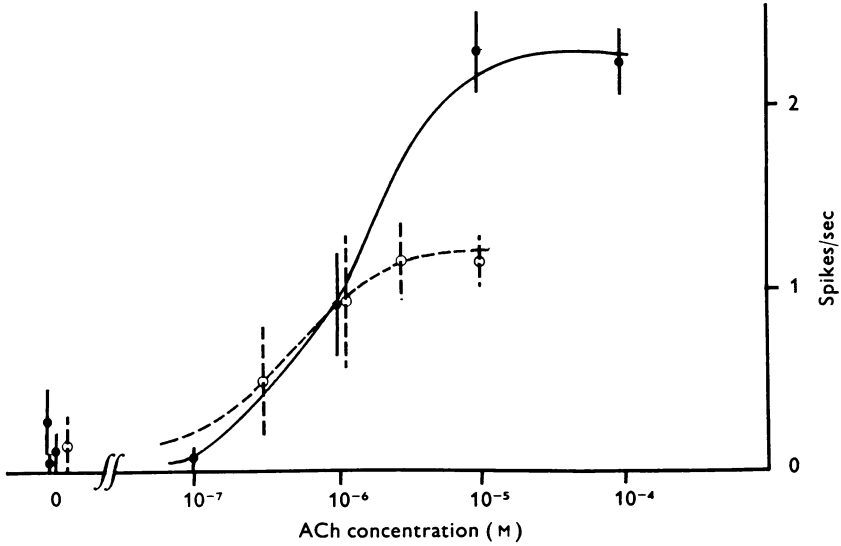



Text-fig. 6. *A*, extracellular records of spikes (in standard saline) induced by outward current (upper trace) through a microsuction electrode. The current intensity was increased from 4 to 1. One of the spikes during application of the current is shown on a faster time base in 2. AC recordings with time constant 20 msec. *B*, expanded records showing the shape of the spike recorded extracellularly using a microsuction electrode. 1, standard saline; 2, during application of 1 $\mu$M-TTX. *C*, relation between amounts of stimulus currents through the microsuction electrode and number of spikes during the 1 sec current pulse. Outward current is positive on the abscissa.

spikes per second obtained in every successive 10 sec interval. The frequency of spike discharge did not show any appreciable decline for at least 3–4 min in the ACh solutions (concentrations up to $10^{-4}$ M) used in this experiment. When the ACh solution was replaced with standard saline, the frequency usually returned to the original level. The




Text-fig. 7. Sequential plots of spike frequency in standard solution (cont) and in solutions containing various concentrations of ACh spike frequencies measured over 10 sec intervals. Solution changes are indicated by an arrow.




Text-fig. 8. Relation between ACh concentration and average frequency of spikes in ACh solution. Spike frequencies measured over 10 sec intervals. Responses in two different cells (continuous line and interrupted line) are plotted. A bar represents a standard deviation. The curves were drawn by eye.

spontaneous discharge was often suppressed for several minutes following return to standard saline from highly concentrated (such as $10^{-4}$ M) ACh solutions. The relationships between the average frequency and the ACh concentration obtained in two cells are shown in Text-fig. 8. They are S-shaped and indicate that the effect saturates at about $10^{-5}$ M. At the saturation level the frequency of spikes is low and ranges between 1·2 and 2·5/sec. Experiments with intra- as well as extracellular electrical stimulation indicate that the chromaffin cell is capable of producing spike potentials at much higher frequencies (at least 20/sec). Thus, if the increase of the spike frequency by ACh is due to its depolarizing effect, the depolarization seems to be small even at the saturation level of the ACh effect.

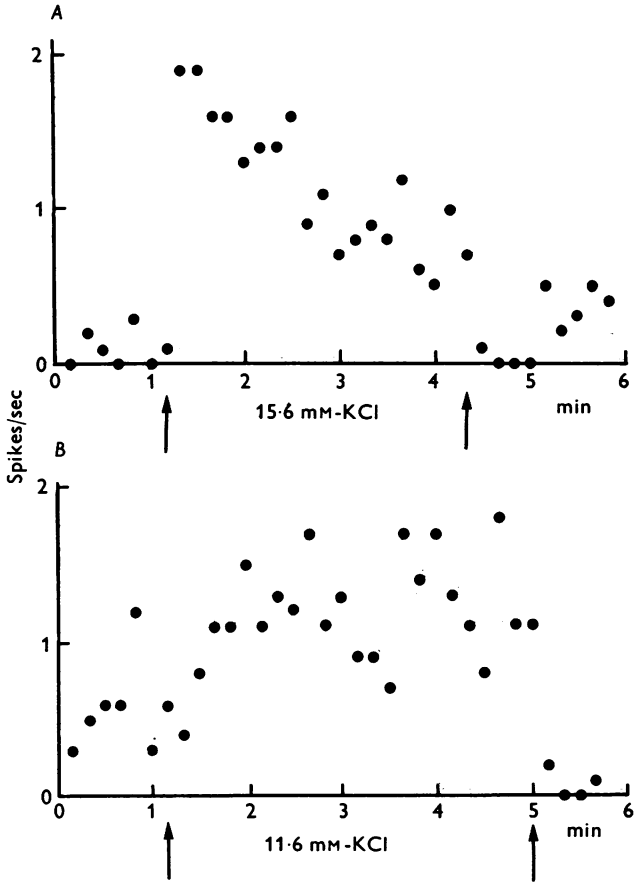
The addition of $10^{-5}$ and $10^{-7}$ M atropine to $10^{-5}$ M-ACh solution brought the increased discharge frequency by ACh back to the control level in standard saline (tested in three cells). Subsequent application of atropine-free ACh solution increased the spike frequency to the high level only after 10–20 min of washing with standard saline. In contrast to atropine, the blocking effect of hexamethonium was not marked (tested in three cells). $10^{-3}$ M hexamethonium in addition to $10^{-5}$ M-ACh resulted in a slight decrease in discharge frequency compared with $10^{-5}$ M-ACh alone and no significant effect was seen with $10^{-4}$ M hexamethonium. No significant effect of $10^{-5}$ M D-tubocurarine was found when added to $10^{-5}$ M-ACh (tested in one cell).

*Effects of increasing KCl*

The results reported above with iontophoretically applied ACh show that ACh depolarizes the cell membrane as has been shown by Douglas *et al.* (1967*a*). The increase in the spontaneous spike frequency may occur as a result of depolarization. An analysis of the spike frequency suggests that the depolarization caused by ACh in the concentration range between $3 \times 10^{-7}$ and $10^{-4}$ M is not large. In fact the resting potential of the cells in standard saline containing $10^{-5}$ M-ACh was $-48 \pm 4$ mV ($n = 5$) which was not significantly different from that in the control. However the measurements of the resting membrane potential in the chromaffin cell may not be precise enough to detect the small amount of depolarization. In order to estimate the amount of depolarization caused by ACh the effect of excessive KCl which is known to depolarize the cell membrane (Douglas *et al.* 1967*b*) was tested.

In cells perfused with fluid containing 15·6 mM-KCl the frequency of the action potentials increased and then gradually decreased, in some cases almost to the control level, after about 30 sec even though the same solution was kept perfused (Fig. 9, tested in four cells). The peak

frequency was about 2/sec which is similar to that induced by $10^{-5}$ M-ACh. However in the case of $10^{-5}$ M-ACh the peak frequency was maintained as long as the perfusion fluid contained ACh. With a lower concentration




Text-fig. 9. Changes in spike frequency, measured every 10 sec, in the presence of excessive KCl (between two arrows).

of KCl (less than 11·6 mM) the increased frequency was maintained (tested in five cells). The frequency was about 1/sec when the K concentration was 11·6 mM and was smaller than that induced by $10^{-5}$ M-ACh in the same cell. The spike frequency increase was smaller when smaller concentrations of KCl were applied. Therefore it was not possible to mimic the effect of $10^{-5}$ M-ACh with raised KCl.

## DISCUSSION

We conclude that the action potential in the chromaffin cell has both Na and Ca components. The evidence for the Na component is based on the following observations. (1) The removal of external Na decreases the amplitude of the action potentials as well as the maximum rate of rise significantly, even when the resting potential became more negative. When cells were first penetrated in Na-free saline and then perfused with standard saline, the resting potential became less negative but the action potential became larger and the maximum rate of rise increased. (2) Action potentials were observed in the saline in which Ca ions were replaced by Mn ions. (3) Action potentials were blocked reversibly by 1 $\mu$M TTX. The presence of the Ca component is concluded (1) since the action potentials were reduced in size but did definitely occur in Na-free saline and (2) since action potentials with long time courses were observed in Na-free, Ba saline.

Recently Biales, Dichter & Tischler (1975) studied human and gerbil chromaffin cells; they found that the cells were capable of producing action potentials and that these action potentials were inhibited by TTX.

Spontaneous action potentials occurring at frequencies of 0·05–0·1/sec were observed in standard saline. ACh ($3 \times 10^{-7}$ M or more) increased the spontaneous spike frequency. The effect was maximal at about $10^{-5}$ M-ACh. In the experiments by Douglas *et al.* (1967*a*) the threshold concentration of ACh for depolarization was about $1{\cdot}7 \times 10^{-5}$ M and that for maximal effect about $5{\cdot}5 \times 10^{-3}$ M. They concluded that the depolarization caused by ACh at concentrations less than $10^{-5}$ M is small. We have reached the same conclusion by observing the resting membrane potential in $10^{-5}$ M-ACh and the extracellularly recorded spike frequency under various conditions.

There are two types of chromaffin cells in the adrenal medulla which can be stained differently. The cell which contains adrenaline is predominant in the rat adrenal medulla. The other type contains noradrenaline (Wood, 1963). Muscarine causes adrenaline release preferentially and this effect is blocked by small amounts of atropine but not by hexamethonium in the cat (Douglas & Poisner, 1965). In our experiments $10^{-7}$ M atropine blocked the effect of $10^{-5}$ M-ACh on the spike frequency but $10^{-3}$ M hexamethonium did not. Since adrenaline containing cells are predominant in the rat adrenal medulla, it is likely that we recorded from these cells. Therefore there is a good correlation between the pharmacology of the ACh-stimulated increase in the action potential frequency in the rat adrenaline containing cells and that of adrenaline secretion in the cat.

The ACh-stimulated release of catecholamine from chromaffin cells previously depolarized in isotonic potassium sulphate led Douglas & Rubin (1963) to conclude that some mechanism other than membrane potential dependent Ca influx is involved in the effect of ACh. The end-plate membrane of a frog skeletal muscle has been shown to become permeable to Ca ions during ACh action (Takeuchi, 1963) and so Ca ions may enter the cell through the ACh channel. Further, Douglas *et al.* (1967*b*) found a significant depolarization of the chromaffin cell in Na-free Ca solution at $10^{-4}$ M-ACh. Our results show, however, that in Na-free Ca saline iontophoretically applied ACh did not cause any significant depolarization even though a significant depolarization was always found in standard solution. Although this does not exclude the existence of an increase in Ca permeability of ACh channels in the chromaffin cell, we are inclined to consider that Ca influx through the ACh channel is small, at least for the effective range of ACh concentration ($10^{-7}$ to $10^{-5}$ M).

Catecholamines are secreted spontaneously when the adrenal medulla is perfused with *Locke* solution (Douglas & Rubin, 1961). This could be accounted for by the spontaneous firing of chromaffin cells which we have found. The relation between ACh concentration and catecholamine secretion in the dissociated guinea-pig chromaffin cell has been examined recently (Hochman & Perlman, 1976). The stimulation of catecholamine secretion was evident at $10^{-6}$ M-ACh, was half-maximal at about $10^{-4}$ M, and was maximal at $10^{-3}$ M. These concentrations are somewhat higher than those found to produce an increase in spike frequency ($3 \times 10^{-7}$ to $10^{-5}$ M) in the rat. On the other hand, Kimura (1974) has found that the release of catecholamine from the rat adrenal medulla becomes significant at $5 \times 10^{-7}$ M-ACh and that the effect approaches saturation at $5 \times 10^{-5}$ M. These concentrations agree well with those affecting frequency of action potentials.

Finally, it is possible that the entry of Ca ions into the cells during action potentials plays a significant role in the release of catecholamine under normal physiological conditions.

We thank Dr Charles Edwards for criticism. We also thank Dr Tomio Kanno for informing us of the paper by Kimura. This research was supported by NIH Grant 09012 to S.H. and NIH Grant NS 11918-01 to Y.K. Y.K. is supported by an NIH Career Development Award, NS 00102-01. B.L.B. was supported by NIH Grant A100430 to Dr Melvin Cohn.

## REFERENCES


BIALES, B., DICHTER, M. & TISCHLER, A. (1975). Electrical excitability of cultured adrenal medulla. *Neuroscience Abstracts, 5th Annual Meeting of Society of Neuroscience*, New York City, p. 460.

BOYLE, P. J. & CONWAY, E. J. (1941). Potassium accumulation in muscle and associated changes. *J. Physiol.* **100**, 1–63.

DOUGLAS, W. W. (1968). Stimulus–secretion coupling: the concept and clues from chromaffin and other cells. *Br. J. Pharmac. Chemother.* **34**, 451–474.

DOUGLAS, W. W., KANNO, T. & SAMPSON, S. R. (1967*a*). Effects of acetylcholine and other medullary secretagogues and antagonists on the membrane potential of adrenal chromaffin cells: an analysis employing techniques of tissue culture. *J. Physiol.* **188**, 107–120.

DOUGLAS, W. W., KANNO, T. & SAMPSON, S. R. (1967*b*). Influence of the ionic environment on the membrane potential of adrenal chromaffin cells and on the depolarizing effect of acetylcholine. *J. Physiol.* **191**, 107–121.

DOUGLAS, W. W. & POISNER, A. M. (1962). On the mode of action of acetylcholine in evoking adrenal medullary secretion: increased uptake of calcium during the secretory response. *J. Physiol.* **162**, 385–392.

DOUGLAS, W. W. & POISNER, A. M. (1965). Preferential release of adrenaline from the adrenal medulla by muscarine and pilocarpine. *Nature, Lond.* **208**, 1102–1103.

DOUGLAS, W. W. & RUBIN, R. P. (1961). The role of calcium in the secretory response of the adrenal medulla to acetylcholine. *J. Physiol.* **159**, 40–57.

DOUGLAS, W. W. & RUBIN, R. P. (1963). The mechanism of catecholamine release from the adrenal medulla and the role of calcium in stimulus–secretion coupling. *J. Physiol.* **167**, 288–310.

DOUGLAS, W. W. & RUBIN, R. P. (1964). Stimulant action of barium on the adrenal medulla. *Nature, Lond.* **203**, 305–307.

FATT, P. & GINSBORG, B. L. (1958). The ionic requirements for the production of action potentials in crustacean muscle fibres. *J. Physiol.* **142**, 516–543.

FOREMAN, J. C., MONGAR, J. L. & GOMPERTS, B. D. (1973). Calcium ionophores and movement of calcium ions following the physiological stimulus to a secretory process. *Nature, Lond.* **245**, 249–251.

HAGIWARA, S. & KIDOKORO, Y. (1971). Na and Ca components of action potential in amphioxus muscle cells. *J. Physiol.* **219**, 217–232.

HAGIWARA, S. & NAKA, K. (1964). The initiation of spike potential in barnacle muscle fibres under low intracellular $Ca^{2+}$. *J. gen. Physiol.* **48**, 141–162.

HAGIWARA, S. & NAKAJIMA, S. (1966). Differences in Na and Ca spikes as examined by application of tetrodotoxin, procaine and manganese ions. *J. gen. Physiol.* **49**, 793–706.

HARRIS, A. J., KUFFLER, S. W. & DENNIS, M. J. (1971). Differential chemosensitivity of synaptic and extrasynaptic areas on the neuronal surface membrane in parasympathetic neurones of the frog, tested by microapplication of acetylcholine. *Proc. R. Soc.* B **177**, 541–553.

HILLARP, N. A. & HÖKFELT, B. (1955). Histochemical demonstration of noradrenaline and adrenaline in the adrenal medulla. *J. Histochem. Cytochem.* **3**, 1–5.

HINKLE, P. M. & TASHJIAN, A. H., JR (1974). Interaction of thyrotropin-releasing hormone with pituitary cells in culture. In *Hormones and Cancer*, ed. MCKERNS, K. W., pp. 203–227. Oxford: Academic Press.

HOCHMAN, J. & PERLMAN, R. L. (1976). Catecholamine secretion by isolated adrenal cells. *Biochim. biophys. Acta* **421**, 168–175.

HODGKIN, A. L. & KATZ, B. (1949). The effect of sodium ions on the electrical activity of the giant axon of the squid. *J. Physiol.* **108**, 37–77.

HUBBARD, J. I., LLINAS, R. & QUASTEL, D. M. J. (1969). *Electrophysiological Analysis of Synaptic Transmission*, p. 93. London: Edward Arnold.

KANNO, T., COCHRANE, D. E. & DOUGLAS, W. W. (1973). Exocytosis (secretory granule extrusion) induced by injection of calcium into mast cells. *Can. Jnl Physiol. & Pharmacol.* **51**, 1001–1004.

KANNO, T. & DOUGLAS, W. W. (1967). Effect of rapid application of acetylcholine or depolarizing current on transmembrane potentials of adrenal chromaffin cells. *Proc. Can. Fedn Biol. Socs* **1**, 39.

KIDOKORO, Y. (1975*a*). Developmental changes of membrane electrical properties in a rat skeletal muscle cell line. *J. Physiol.* **244**, 129–143.

KIDOKORO, Y. (1975*b*). Sodium and calcium components of the action potential in a developing skeletal muscle cell line. *J. Physiol.* **244**, 145–159.

KIDOKORO, Y. (1975*c*). Spontaneous Ca action potentials in a clonal pituitary cell line and their relation to prolactin secretion. *Nature, Lond.* **258**, 741–742.

KIMES, B. W. & BRANDT, B. L. (1976). Characterization of two putative smooth muscle cell lines from rat thoracic aorta. *Expl Cell Res.* (in the Press).

KIMURA, K. (1974). A method for perfusing the rat adrenal medulla and the effect of acetylcholine on catecholamine release (in Japanese). *Scient. Rep. Nayoro Women's Junior Coll.* **7**, 11–18.

KUFFLER, S. W., DENNIS, M. J. & HARRIS, A. J. (1971). The development of chemosensitivity in the extrasynaptic area of the neuronal surface after denervation of parasympathetic ganglion cells in the heart of the frog. *Proc. R. Soc.* B **177**, 555–563.

KUFFLER, S. & YOSHIKAMI, D. (1975). The distribution of acetylcholine sensitivity at the post-synaptic membrane of vertebrate skeletal twitch muscles: iontophoretic mapping in the micron range. *J. Physiol.* **244**, 703–730.

MATTHEWS, E. K. & SAKAMOTO, Y. (1975). Electrical characteristics of pancreatic islet cells. *J. Physiol.* **246**, 421–437.

MATTHEWS, E. K. & PETERSEN, O. H. (1973). Pancreatic acinar cells: ionic dependence of the membrane potential and acetylcholine-induced depolarization. *J. Physiol.* **231**, 283–295.

NELSON, P. G., PEACOCK, J. & MINNA, J. (1972). An active electrical response in fibroblasts. *J. gen. Physiol.* **60**, 58–71.

SCHUBERT, D., HARRIS, A. J., DEVINE, C. E. & HEINEMANN, S. (1974). Characterization of a unique muscle cell line. *J. cell Biol.* **61**, 398–413.

TAKEUCHI, N. (1963). Effects of calcium on the conductance change of the end-plate membrane during the action of transmitter. *J. Physiol.* **167**, 141–155.

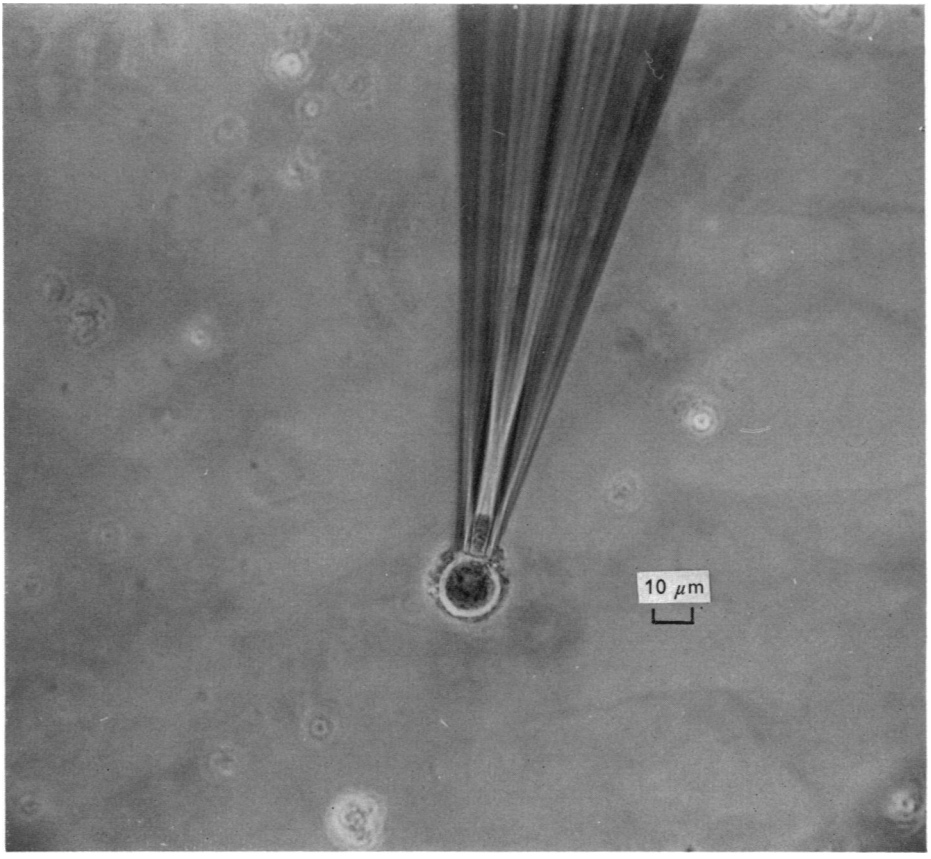
TASAKI, I. (1959). Resting and action potentials of reversed polarity in frog nerve cells. *Nature, Lond.* **184**, 1574–1575.

TASHJIAN, A. H., JR., YASUMURA, Y., LEVINE, L., SATO, G. H. & PARKER, M. L. (1968). Establishment of clonal strains of rat pituitary tumor cells that secrete growth hormone. *Endocrinology* **82**, 342–352.

VALE, W. & GRANT, G. (1975). *In vitro* pituitary hormone secretion assay for hypophysiotropic substances. In *Methods in Enzymology*, vol. 37, *Hormone Action*, part B, *Peptide Hormones*, ed. O'MALLEY, B. W. & HARDMAN, J. G., pp. 82–93. Oxford: Academic Press.

VOGT, M. (1952). The secretion of the denervated adrenal medulla of the cat. *Br. J. Pharmac. Chemother.* **7**, 325–330.

VOGT, M. & DULBECCO, R. (1963). Steps in the neoplastic transformation of hamster embryo cells by polyoma virus. *Proc. natn. Acad. Sci. U.S.A.* **49**, 171–179.


10 μm

WOOD, J. G. (1963). Identification of and observations on epinephrine and norepinephrine containing cells in the adrenal medulla. *Am. J. Anat.* **112**, 285–304.

## EXPLANATION OF PLATE

Photograph showing the microsuction electrode arrangement for extracellular spike recording. A part of a chromaffin cell was sucked into the electrode. A 40 × water-immersion phase-contrast objective (Carl Zeiss, West Germany) was used. The bar indicates 10 $\mu$m.