*J. Physiol.* (1967), **191**, *pp.* 107–121
*With 8 text-figures*
*Printed in Great Britain*



# INFLUENCE OF THE IONIC ENVIRONMENT ON THE MEMBRANE POTENTIAL OF ADRENAL CHROMAFFIN CELLS AND ON THE DEPOLARIZING EFFECT OF ACETYLCHOLINE

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(*Received* 12 *December* 1966)

SUMMARY

1. Intracellular recordings were made from chromaffin cells isolated from adrenal medullae of gerbils to examine the effects, on membrane potential, of changes in the ionic environment that are known, from other experiments, to influence the rate of catecholamine secretion.

2. Depolarization in response to acetylcholine fell linearly with the logarithm of the extracellular sodium concentration over the range 154-3 mM and reached a value, in sodium-free medium, of about 30 % of the control value.

3. The depolarizing effect of acetylcholine in sodium-free media increased linearly with the logarithm of the extracellular calcium concentration over the range 1–117 mM.

It is concluded that depolarization in response to acetylcholine involves inward movement of both sodium and calcium ions.

4. Depolarization was also observed in response to the secretagogues, excess potassium and barium, both in sodium-rich and sodium-free media. The effect of barium was antagonized by calcium, and it is suggested that these two cations interact at the level of the plasma membrane.

5. Depolarization does not appear to be tightly coupled to secretion, for acetylcholine or excess potassium still depolarized the chromaffin cells when the environment was calcium-free or contained an excess of magnesium, conditions that inhibit secretion. Furthermore, although acetylcholine had some depolarizing effect in sodium-free media, the level to which the membrane potential fell was not below the control 'resting'

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potential since the cells in sodium-free medium were hyperpolarized; yet, secretory responses are augmented in such conditions.

6. It is proposed that depolarization in response to acetylcholine may be no more than the electrical sign of increased permeability to ions such as sodium and calcium, and that depolarization is not, in itself, a key event in stimulus–secretion coupling. The evidence is held to favour the view that movement of calcium into the chromaffin cells on exposure to acetylcholine is responsible for evoking secretion.

## INTRODUCTION

A fresh clue to the mode of action of acetylcholine at the adrenal medulla has recently been provided by studies made with intracellular micro-electrodes which have shown that acetylcholine depolarizes the chromaffin cells. This response is blocked by hexamethonium and by atropine, drugs that are known to antagonize acetylcholine's stimulant effect on secretion (Douglas, Kanno & Sampson, 1967). These findings offer evidence that acetylcholine acts on the plasma membrane of the chromaffin cell, and suggest that depolarization, or the movement of ions underlying this effect, is the event initiating secretion. The further observation that chromaffin cells are also depolarized by various other secretagogues with quite different chemical structures supports this view. The electrophysiological evidence thus complements that derived from earlier studies on the effects of ions on medullary secretion which have already prompted the conjecture that acetylcholine owes its stimulant effect on the chromaffin cell to some action increasing the permeability of the plasma membrane to cations in the extracellular environment (Douglas & Rubin, 1961, 1963, 1964*a*, *b*).

We have now made further observations of the transmembrane potentials of chromaffin cells in an attempt to learn which ions are involved in the depolarizing action of acetylcholine, and to examine the effect of changes in the ionic environment that are known to influence secretory activity. Our main purpose has been to assess the role of ion movement and membrane potential in stimulus–secretion coupling in the adrenal chromaffin cell.

## METHODS

The experiments were carried out on chromaffin cells isolated from adrenal medullae of young gerbils (*Meriones unguiculatus*) by cell dissociation techniques and maintained in growth medium in a feeder culture of hamster lung cells in a Sykes-Moore culture chamber. After about 8 hr incubation the growth medium was replaced with *control* (recording) *medium*, and the cells were allowed to equilibrate at room temperature (about 25° C). Chromaffin cells were then identified and impaled with KCl-filled micro-electrodes under

direct visual control with the assistance of an inverted microscope, and the potentials were displayed on a wide band ink-writing recorder. A number of cells (8–15) was impaled to establish the mean 'resting' potential of the population of chromaffin cells. Thereafter, the process was repeated (up to 5 times) after adding acetylcholine to the chamber or after substituting for the control medium a solution of different ionic composition. After replacing one medium with another, about 15 min was allowed to pass before beginning the next series of impalements. When acetylcholine was added to the chamber, however, recording was begun within 1 min of adding the drug. A full description of the procedure has been given in a previous report (Douglas *et al.* 1967).

*Solutions*

Solutions were based on a modified version of the tissue culture medium (F 10) described by Ham (1963). The modifications were as follows. $MgSO_4$ was replaced by $MgCl_2$, 1 mM; phosphate buffer was omitted; and the concentrations of other salts were (mM): NaCl, 143; KCl, 5·6; $CaCl_2$, 2·2; $NaHCO_3$, 14·3.

*Growth medium* consisted of 8 parts of the modified F 10 medium, 2 parts foetal calf serum, and 0·2 parts lactalbumin hydrolysate. All these were supplied by Grand Island Biologicals, Grand Island, New York. This growth medium was bubbled with 5% $CO_2$ in oxygen, and incubation was at 37° C.

*Control (recording) medium* was composed of 1 part foetal calf serum and 9 parts of the F 10 medium with further modifications as follows: $NaHCO_3$ was replaced by 3 mM $NaH_2PO_4$-$Na_2HPO_4$ buffer (pH 7·1) and NaCl was increased to 154 mM. Lactalbumin hydrolysate was omitted. This medium (and all the other media used when recording) was equilibrated with air at room temperature. In experiments where the effects of low concentrations of Na, K, and Ca, were studied, dialysed foetal calf serum was used. When Ba, or extra Ca or Mg, was added to the medium, tonicity was maintained by removing an osmotically equivalent amount of NaCl.

To test the effects of ions, the medium just described was modified in a number of ways. In *low-sodium media*, NaCl was replaced partly or wholly by osmotically equivalent amounts of sucrose. In *sodium-free medium*, NaCl was replaced with sucrose and the sodium phosphate buffer was replaced with potassium phosphate buffer. The total K concentration of this solution was maintained at 5·6 mM by reducing the concentration of KCl. Where Ba, or excess Ca or excess Mg, was added to this sodium-free medium, tonicity was adjusted by lowering the sucrose concentration, and tris (hydroxymethyl)aminomethane buffer (1·0 mM) was used in place of phosphate buffer. In sulphate medium, NaCl, KCl, $MgCl_2$, and $CaCl_2$ were replaced by (mM): $Na_2SO_4$, 77; $K_2SO_4$, 1·3; $MgSO_4$, 1·0; $CaSO_4$, 10. The high concentration of calcium sulphate was used to compensate, partially, for the low calcium ion concentration that exists in the presence of sulphate (Hill & Howarth, 1957).

## RESULTS

### *Ions and the depolarizing effect of acetylcholine*

*Sodium.* At the most thoroughly studied site of cholinergic transmission, the motor end-plate, the depolarizing effect of acetylcholine is mainly due to inward movement of sodium ions, and the depolarizing effect of acetylcholine diminishes as the concentration of sodium in the extracellular environment is lowered (Fatt & Katz, 1952; Takeuchi & Takeuchi, 1960). As shown in Fig. 1, the presence of sodium in the extracellular environment is also important for the depolarizing effect of acetylcholine on the adrenal chromaffin cell, for this effect was greatly

diminished when sodium was absent. The same figure illustrates two further points: first, that omission of sodium caused a rise in membrane potential; and, secondly, that the membrane potential during exposure to acetylcholine was not below the resting potential observed in the control (sodium-rich) medium. In Fig. 1, values from three experiments have been plotted individually to indicate the reproducibility of the results obtained from different populations (chambers) of chromaffin cells isolated on different occasions from different gerbils, and to illustrate the reversibility of the hyperpolarizing effect of sodium-free media.

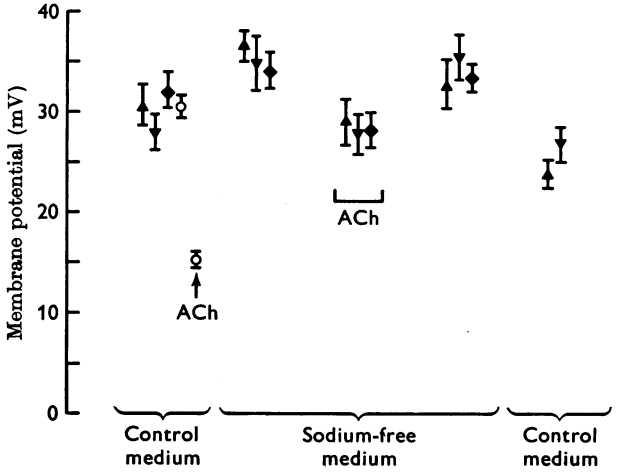


Fig. 1. The effects of sodium-free medium on the membrane potential (mean ± S.E.) of chromaffin cells and on depolarization in response to acetylcholine. Three experiments, each carried out on a different population of cells, are depicted by the three filled symbols. The results are plotted from left to right in the sequence they were obtained during the experiment. In each experiment membrane potentials were first recorded from the cells in control medium. Then this medium was replaced with sodium-free medium and potentials were recorded before, during, and after exposure to ACh ($10^{-4}$ g/ml.). Finally, in two of the experiments, measurements were again made in control medium. For comparison, the effect of ACh on cells in control medium (154 mM-Na) is shown by the open circles which represent mean values obtained from two populations of cells.

In a further series of experiments, the effects of intermediate concentrations of sodium were examined. As shown in Fig. 2, the depolarization produced by acetylcholine increased in an approximately linear fashion with the logarithm of the sodium concentration in the incubation medium over the range 3–154 mM.

Two experiments carried out with sulphate medium (where chloride was replaced with the impermeant anion, $SO_4$) gave somewhat similar results. In the first experiment, where the sodium concentration was 154 mM,

the membrane potential fell from $29{\cdot}3 \pm 1{\cdot}0$ to $13{\cdot}0 \pm 0{\cdot}4$ mV. In the second experiment, where 100 mM sodium was present, the control potential was $29{\cdot}7 \pm 1{\cdot}9$ mV and this fell to $20{\cdot}0 \pm 2{\cdot}0$ mV on exposure to acetylcholine. These results may be compared with those illustrated in Fig. 2 which were obtained in the control medium rich in chloride.

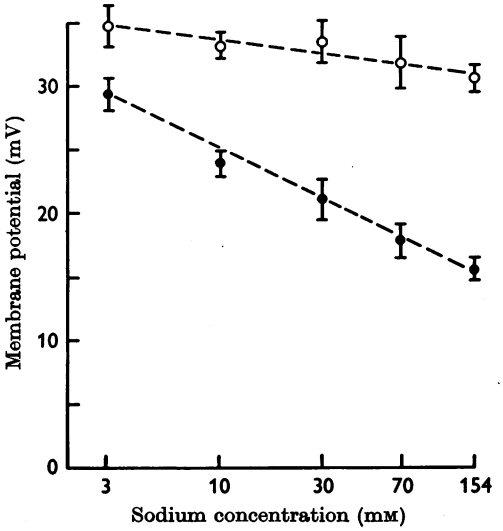


Fig. 2. The relation between the sodium concentration in the medium and depolarization in response to acetylcholine. The points represent the membrane potentials of five populations of cells in media containing five concentrations of sodium. The filled circles (●) represent values recorded in the presence of ACh ($10^{-4}$ g/ml.). The open circles (○) immediately above represent the corresponding potentials from the same populations of cells before adding ACh.

*Calcium.* Since the depolarizing effect of acetylcholine was not abolished in sodium-free medium, it was apparent that some other ion or ions must be involved. Calcium seems to be important, for depolarization in response to acetylcholine increased linearly with the logarithm of the extracellular calcium concentration over the range 1–117 mM (Fig. 3). The experiments illustrated in this figure were conducted in sodium-free media to avoid the masking effect of the greater depolarizing current carried by sodium ions. The contribution of calcium to depolarization was not so easily demonstrated when the medium was rich in sodium. Nevertheless, in each of two experiments where the calcium concentration of the conventional medium was raised to 10 mM or to 17·6 mM, the fall in membrane potential observed on exposure to acetylcholine ($10^{-4}$ g/ml.) was greater than the corresponding depolarization observed in control experiments. In the two experiments with excess calcium, membrane potentials fell to $12{\cdot}7 \pm 0{\cdot}7$

and $12{\cdot}6 \pm 0{\cdot}9$ mV respectively from corresponding values of $34{\cdot}3 \pm 2{\cdot}0$ and $36{\cdot}1 \pm 1{\cdot}1$ mV. In the control experiments carried out on two populations of cells, where the calcium concentration was 2·2 mM, mean membrane potential fell to $15{\cdot}7 \pm 0{\cdot}9$ mV from $30{\cdot}6 \pm 1{\cdot}1$ mV.

The effect on membrane potential of completely removing calcium from the medium is of interest in the light of evidence that such calcium

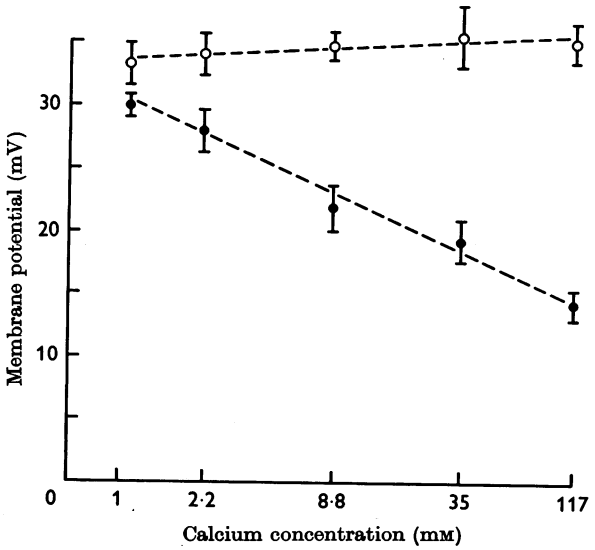


Fig. 3. The relation between the calcium concentration in the medium and depolarization in response to acetylcholine. The points represent the membrane potentials of five populations of cells in sodium-free media containing five concentrations of calcium. The filled circles (●) represent the values recorded in the presence of ACh ($10^{-4}$ g/ml.). The open circles (○) immediately above represent the corresponding potentials from the same populations of cells before adding ACh.

deprivation prevents acetylcholine (and other secretagogues) from evoking catecholamine secretion (Douglas & Rubin, 1961, 1963; Poisner & Douglas, 1966). In three experiments acetylcholine was tested about 15 min after replacing the standard medium with calcium-free medium. In each instance, acetylcholine had a clear depolarizing effect: the potentials fell from control values of $25{\cdot}3 \pm 1{\cdot}6$, $19{\cdot}9 \pm 2{\cdot}1$ and $25{\cdot}3 \pm 2{\cdot}1$ mV to $7{\cdot}8 \pm 0{\cdot}3$, $11{\cdot}3 \pm 1{\cdot}7$ and $10{\cdot}8 \pm 0{\cdot}8$ mV respectively. However, it was evident that the membrane potential observed in the calcium-free medium before exposure to ACh was lower than the value recorded immediately beforehand in the conventional medium. Thus the mean potentials in the same three experiments recorded before removal of calcium were $29{\cdot}8 \pm 1{\cdot}6$, $26{\cdot}4 \pm 1{\cdot}6$ and $30{\cdot}1 \pm 1{\cdot}8$ mV, respectively. Removal of calcium was thus, in itself,

sufficient to cause some depolarization. Within the time limits we have studied (up to 80 min) this effect of removal of calcium was reversible (Fig. 4).

Depolarization on removal of calcium also occurred in cells incubated in sodium-free medium. In two experiments, where the mean membrane potentials in sodium-free medium containing calcium were $32{\cdot}3 \pm 2{\cdot}4$ and $31{\cdot}3 \pm 0{\cdot}7$ mV, the corresponding potentials had fallen to $21{\cdot}4 \pm 0{\cdot}1$ and $18{\cdot}5 \pm 0{\cdot}7$ mV, respectively, some 30 min after removing calcium.

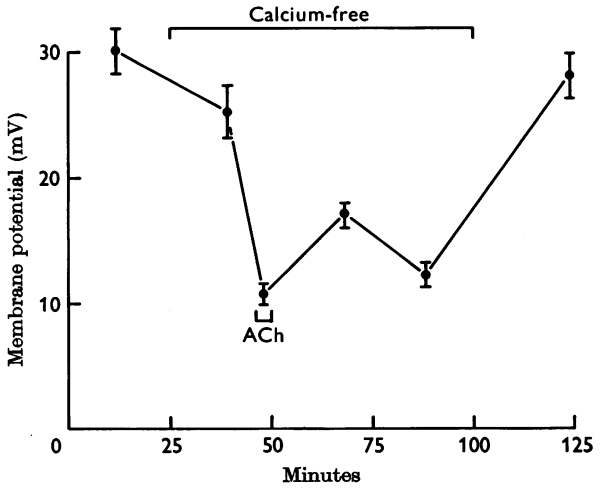


Fig. 4. The effects of removing calcium from the control medium on membrane potential and on the response to acetylcholine. The values were obtained sequentially from the same population of chromaffin cells, first in control medium, then in calcium-free medium, before, during, and after exposure to ACh ($10^{-4}$ g/ml.), and finally in control medium. The abscissa represents the time from beginning to record.

*Magnesium.* Since calcium was clearly involved in the depolarizing response to acetylcholine, it was of interest to examine the effect of magnesium. The first experiments were again performed in sodium-free media. The result was quite different from that observed in the comparable experiments with calcium. Thus, excess magnesium tended to lower membrane potential rather than raise it, and the depolarization in response to acetylcholine did not increase as the magnesium concentration of the medium was increased, but remained about the same. This result, shown in Fig. 5, may be contrasted with that obtained with calcium (Fig. 3).

When excess magnesium was tested in control medium, rich in sodium, results were somewhat similar. Slight depolarization was observed with 10 mM-Mg, and more marked depolarization with 20 mM-Mg. Acetyl-

choline given in the presence of 10 mM-Mg lowered membrane potential by about the same amount as it did in control experiments where the concentration of Mg was 1·0 mM (Table 1).

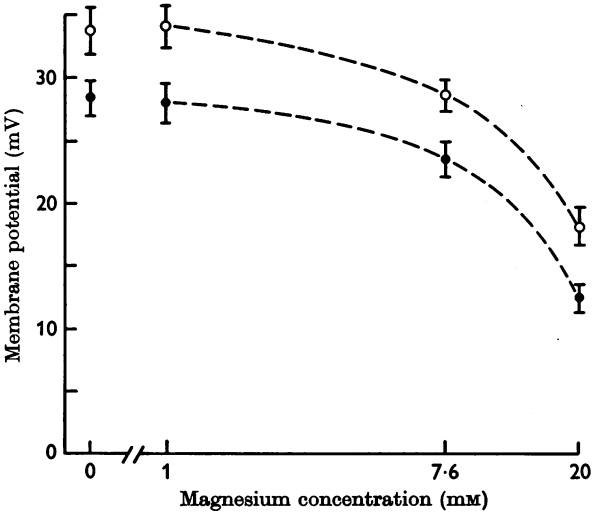


Fig. 5. The effects of different concentrations of magnesium in the medium on membrane potential and on depolarization in response to acetylcholine. The points represent the membrane potentials of four populations of cells in sodium-free media containing four concentrations of magnesium. The filled circles (●) represent values recorded in the presence of ACh ($10^{-4}$ g/ml.). The open circles (○) immediately above represent the corresponding potentials from the same populations of cells before adding ACh.

TABLE 1. Effects of excess magnesium on membrane potentials of chromaffin cells and on depolarization in response to ACh

| Membrane potential (mV ± S.E.) in control medium (1 mM-Mg) | Mg concentration (mM) in test medium | Membrane potential (mV ± S.E.) in test medium | |
|---|---|---|---|
| | | Before ACh | During ACh |
| 29·8 ± 1·3 | 10 | 27·2 ± 0·7 | 12·5 ± 1·3 |
| 28·8 ± 1·4 | 20 | 17·1 ± 1·4 | — |
| 31·0 ± 1·3 | 20 | 18·3 ± 1·3 | — |

From previous studies it is known that chromaffin cells that have been exposed to a calcium-free environment secrete violently for a brief period when calcium is reintroduced, and that this effect may be prevented by having magnesium present throughout the period of calcium deprivation (Douglas & Rubin, 1963). The present experiments showed that magnesium did not prevent cells from depolarizing in calcium-free media. Magnesium (1 mM) was present in all the experiments performed with calcium-free

media which have been described above. In a further experiment, a higher concentration of magnesium (10 mM) also failed to prevent depolarization: mean membrane potential in this experiment fell from $30{\cdot}8 \pm 3{\cdot}2$ to $19{\cdot}9 \pm 0{\cdot}3$ mV within 30 min of withdrawing calcium.

*The effects of the stimulant cations, barium and potassium*

*Barium* is a powerful medullary secretagogue (Douglas & Rubin, 1964*a*), and in the present experiments barium was found to depolarize the chromaffin cells. The effect of adding 10 mM barium is shown in Fig. 6. In other

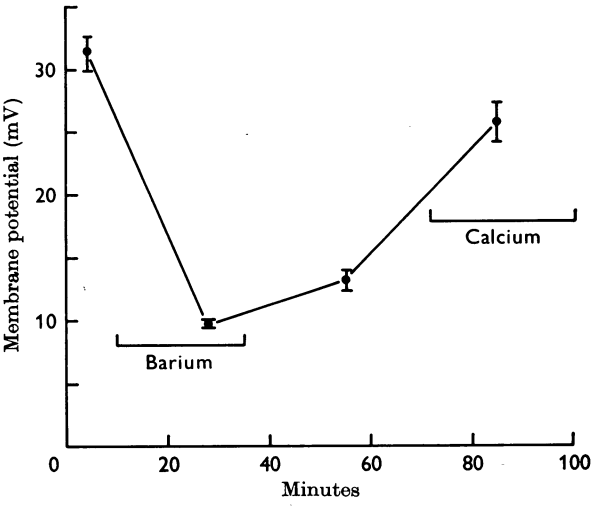


Fig. 6. The depolarizing effect of barium. The values were obtained sequentially from the same population of cells in control medium with or without the addition of 10 mM barium or 10 mM calcium as indicated.

experiments lower concentrations of barium (2 and 5 mM) were used and caused smaller depolarizations. The return of membrane potential to control levels on removing barium occurred slowly, but seemed to be accelerated by an increase in the calcium concentration of the medium.

The depolarizing effect of barium was not dependent on extracellular sodium, for the effect was also observed in experiments where the cells were in sodium-free medium. In such conditions, it is known that barium retains its stimulant effect on secretion, and that stimulation can be antagonized by adding calcium (Douglas & Rubin, 1964*a*). As shown in Fig. 7, an increase in the calcium concentration of the medium antagonized the depolarizing effect of barium.

*Potassium* in excess also provides a strong stimulus for medullary secretion (Vogt, 1952; Douglas & Rubin, 1961, 1963). As shown in Fig. 8,

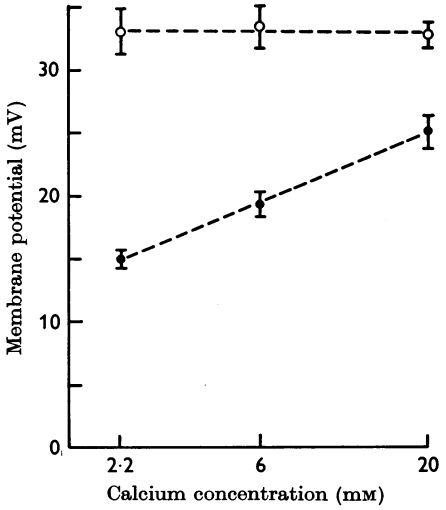


Fig. 7. The relation between the calcium concentration in the medium and depolarization in response to barium. The points represent the membrane potentials of three populations of cells in sodium-free media containing three concentrations of calcium. The filled circles (●) represent values recorded in the presence of barium (5 mM). The open circles (○) immediately above show the corresponding potentials from the same populations of cells before adding barium.

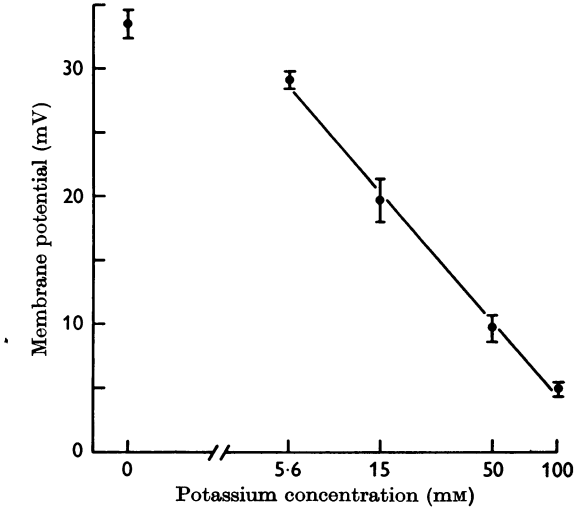


Fig. 8. The relation between the potassium concentration of the medium and membrane potential. The five values were obtained from five different populations of cells in media in which different amounts of NaCl were exchanged for KCl.

the membrane potential of the chromaffin cells fell progressively as the potassium concentration of the medium was raised by replacing sodium chloride with potassium chloride. Excess potassium, as would be expected on general grounds, also depolarized chromaffin cells in the absence of calcium. In two experiments, the membrane potentials in the presence of excess K (50 and 40 mM) in Ca-free medium were $10{\cdot}1 \pm 0{\cdot}7$ and $13{\cdot}4 \pm 0{\cdot}8$ mV compared with the corresponding control values in the same experiments of $28{\cdot}6 \pm 1{\cdot}1$ and $31{\cdot}8 \pm 1{\cdot}7$ mV.

## DISCUSSION

*Effect of ions on resting potential.* Although it was not the purpose of the present experiments to define the ionic mechanisms controlling the resting potential in the adrenal chromaffin cell, some of our results, made in the course of studying the effects of ionic changes known to influence secretion, do provide clues to possible factors involved. Since membrane potential varied approximately with the logarithm of the extracellular potassium concentration over a wide range, it appears likely that the ratio $[K_i]:[K_o]$ is a major determinant of membrane potential in the chromaffin cell. However, since removal of sodium from the extracellular environment raised the potential by several millivolts, sodium also seems to contribute. The hyperpolarizing effect of low sodium suggests that the chromaffin cell membrane, like the membrane of mammalian C fibres (Armett & Ritchie, 1963*a*), may be more permeable to sodium than are, for example, the membranes of squid axons and skeletal muscles. This might help to explain why the membrane potential of the chromaffin cell is comparatively low, and why the slope of the curve relating the extracellular potassium concentration to membrane potential (20 mV/10-fold increase in $[K_o]$) is not so steep as for skeletal muscle or squid axon, but closer to that found for mammalian C fibres (Armett & Ritchie, 1963*a*), for in these experiments the concentration of sodium was lowered as the concentration of potassium was raised. But it is unprofitable to pursue these questions further in the absence of information about the concentrations of sodium, potassium and other ions in the chromaffin cells, and without evidence of the permeability of the chromaffin cell membrane to the various ions. The trend toward hyperpolarization on exposure to excess calcium may be due to a reduction in the permeability of the membrane to sodium; and the depolarizing effect of calcium omission may be caused by a loss of intracellular potassium resulting from an increase in the permeability of the plasma membrane as bound calcium is removed from it. Such explanations have been advanced to account for similar phenomena observed in many other cells exposed to high or low levels of calcium (Brink, 1954; Shanes,

1958; Cerf, 1963). Since depolarization in response to barium was antagonized by calcium, it is possible that barium acts by displacing calcium bound to the membrane of the chromaffin cell and in this way increases membrane permeability as was suggested previously (Douglas & Rubin, 1964*a*). A similar conjecture may be made in respect to the weaker depolarizing effect of magnesium. Another possibility is that these ions penetrate the membrane more readily than calcium and themselves carry inward depolarizing currents (Mullins, 1961; Nishi, Soeda & Koketsu, 1965).

*Effect of ions on depolarization in response to acetylcholine.* Since a linear relation was found between depolarization in response to acetylcholine and the logarithm of the extracellular sodium concentration over a wide range, and since the effect of acetylcholine was much reduced in sodium-free media, it seems that most of the depolarizing current on exposure to acetylcholine is carried by inward movement of sodium ions. Calcium, however, also contributes to the depolarizing current for the residual effect of acetylcholine observed in sodium-free media increased as the calcium concentration was raised, and in these circumstances depolarization was linearly related to the logarithm of the external calcium concentration over the range 1–117 mM. Moreover, depolarization in response to acetylcholine in media containing sodium was increased when the calcium concentration was raised. These results provide an explanation, at the cellular level, for the observation that acetylcholine increases $^{45}Ca$ uptake in the intact adrenal medulla (Douglas & Poisner, 1962). What other ions participate in the response is at present uncertain. It was difficult to assess the contribution made by the magnesium since this ion itself has a depolarizing effect. It seems unlikely, however, that chloride is important, since the depolarizing effect of acetylcholine persisted when cells were bathed in solutions in which chloride had been replaced by the impermeant anion, sulphate.

The effect of acetylcholine on the plasma membrane of the adrenal chromaffin cell seems to resemble its effects on the motor end-plate (Del Castillo & Katz, 1956; Nastuk, 1959; Takeuchi & Takeuchi, 1960; Takeuchi, 1963) and mammalian C fibres (Armett & Ritchie, 1963*b*) where the main event seems to be an increase in the permeability to commonly occurring cations.

*Membrane potential and secretion.* The manipulations of the ionic environment whose effects we have studied on membrane potential were selected because their effects on the secretory activity of chromaffin cells is known from work on perfused cats' adrenal glands (Douglas & Rubin, 1961, 1963, 1964*a*, *b*).

Barium and excess potassium are powerful direct medullary secreta-

gogues (Douglas & Rubin, 1961, 1964*a*, Vogt, 1952), and the finding that each depolarizes the chromaffin cells, as do all the other secretagogues we have tested (acetylcholine, pilocarpine, nicotine, histamine, 5-hydroxytryptamine, angiotensin, and bradykinin; Douglas *et al.* 1967), seems to lend further weight to the view that depolarization may be a key event in stimulus–secretion coupling. However, other evidence indicates that depolarization can occur without secretion. All the secretagogues mentioned fail to evoke secretion when calcium is absent from the extracellular environment (Douglas & Rubin, 1961; Poisner & Douglas, 1966), yet in our experiments the removal of calcium from the medium did not prevent depolarization in response to acetylcholine or potassium. Nor was depolarization prevented by magnesium which has a powerful inhibitory effect on catecholamine secretion (Douglas & Rubin, 1963). Moreover, it seems likely that intense secretion can occur while membrane potential is close to control 'resting' levels. Thus, the membrane potential of chromaffin cells exposed to acetylcholine in a sodium-free medium was close to the control 'resting' level, yet the secretory response to acetylcholine in such circumstances is potentiated (Douglas & Rubin, 1963). These various pieces of evidence indicate that membrane potential and secretory activity can be dissociated.

One possibility is that depolarization in response to acetylcholine (and other secretagogues) may be merely an electrical sign of an increased permeability of the chromaffin cell plasma membrane to ions such as sodium and calcium, and that secretion is due to the inward movement of these ions. Entry of sodium is clearly not important, since the secretory response to acetylcholine persists in sodium-free environments. But entry of calcium may well be the key event. Douglas & Rubin (1961) found that the secretory response to acetylcholine increases as the extracellular concentration of calcium is increased over a wide range and that calcium itself evokes secretion in circumstances where it might be supposed to penetrate the plasma membrane, and they suggested that inward movement of calcium could provide the stimulus for catecholamine release. Additional evidence has since been obtained for a calcium-activated link in stimulus–secretion coupling (Douglas & Rubin, 1963, 1964*a*, *b*; Poisner & Douglas, 1966).

Although our results indicate that entry of calcium into the chromaffin cells is a direct response to acetylcholine, inward movement of calcium may be facilitated by the strong depolarizing effect of the sodium current. Depolarization, as our experiments show, occurs in response to excess potassium and excess potassium promotes $^{45}Ca$ uptake in the adrenal medulla (Douglas & Poisner, 1961), The results obtained on sodium-free medium do not necessarily contradict this view. For example, there may

normally be competition between sodium and calcium at some stage (perhaps for entry into the cell as has been proposed to occur in muscle: Lüttgau & Niedergerke, 1958) and the effect on such a process of removing sodium might outweigh the effect of the reduced depolarization.

Finally, it seems appropriate to point out that the effects observed in these and the earlier experiments (Douglas, Kanno & Sampson, 1966, 1967) have extended the already striking parallel between the events involved in stimulus–secretion coupling in the chromaffin cell and excitation–contraction coupling in muscles to which attention was drawn earlier (Douglas & Rubin, 1961) and which has been stressed as further evidence has accumulated (see Douglas, 1965). Thus it seems: (*a*) that at both sites the key event is an interaction between acetylcholine and the plasma membrane of the receptive cell which can be prevented by familiar pharmacological antagonists; (*b*) that this interaction produces an influx of commonly occurring cations; (*c*) that this in turn causes depolarization; and (*d*) that calcium influx, whether directly evoked by acetylcholine or in response to depolarization, is tightly linked to the functional response.

We thank Dr E. Robbins and Miss Anita Micali for kindly supplying us with hamster lung cells. The work was supported by U.S.P.H.S. grants 5RO1-B-4006 and MH-6418-09.

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