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THE EFFECT OF AMETHOCAINE ON ACETYLCHOLINE-INDUCED DEPOLARIZATION AND CATECHOLAMINE SECRETION IN THE ADRENAL CHROMAFFIN CELL

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Acetylcholine is the transmitter substance liberated by the secretomotor nerves to the chromaffin cells of the adrenal medulla and is thus the immediate stimulus to which these cells respond by secreting their hormones, the catecholamines. How acetylcholine evokes secretion is uncertain, but there are grounds for believing that its action is exerted on the plasma membrane of the chromaffin cells and results in the inward movement of commonly occurring cations. Thus it has been found, by recording from chromaffin cells with intracellular microelectrodes, that acetylcholine causes a fall in transmembrane potential and that this depolarizing effect increases as the extracellular concentrations of sodium and calcium are raised (Douglas, Kanno & Sampson, 1967a, b). For a variety of reasons it seems likely that of these various effects of acetylcholine it is the entry of calcium ions that is most closely related to the secretory response (Douglas, 1966a, b). For example, catecholamine secretion in response to acetylcholine is lost when calcium is omitted from the extracellular environment but persists when all sodium is omitted (Douglas & Rubin, 1961, 1963) and when, as a result, the depolarizing action of acetylcholine is greatly weakened (Douglas *et al.*, 1967b). The present experiments support this view. They show that the secretory response to acetylcholine is inhibited by amethocaine in a concentration that blocks inward calcium current while having little or no effect on inward sodium current and the resulting depolarization. A short account of some of the findings has appeared (Kanno & Douglas, 1967).

METHODS

All the experiments were carried out in gerbils (*Meriones unguiculatus*) weighing 40–60 g and anaesthetized with urethane (30%, w/v ; 0.5 ml./100 g body weight, intraperitoneally).

Perfusion of adrenal gland

The stomach and intestines were removed through a midline abdominal incision. The abdominal aorta was cannulated below the renal arteries and perfused with modified Locke solution of the

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following composition (mM); NaCl, 154; KCl, 5.6; $CaCl_2$, 2.2; $MgCl_2$, 1.0; Na_2HPO_4—NaH_2PO_4 buffer (pH 7.1), 3.0; glucose, 10. Ascorbic acid (10^{-5} g/ml.) was added to inhibit oxidation of catecholamines, and the solution was equilibrated with oxygen and maintained at about 28° C. The aorta was tied above the diaphragm, both splanchnic nerves were cut, and the vena cava was opened to allow free escape of fluid. Outflow from the adrenal gland was collected through a fine polyethylene cannula inserted into the lumbar vein and passed up to and tied into the adrenal vein. Small branches entering the adrenal vein from regions other than the adrenal gland were tied. The flow through the gland was about 0.15 ml./min. The samples of effluent were collected on ice and kept frozen for bioassay.

Bioassay

Catecholamines were assayed by the rat blood pressure method as follows: Rats weighing about 250 g were anaesthetized with urethane (30%, w/v; 0.3 ml./100 g body wt., intraperitoneally). Blood pressure was stabilized and sensitivity to catecholamines increased with pentolinium (2.5 mg/100 g/body wt., intraperitoneally) and a β-blocking agent, propranolol (0.2 mg/100 g body wt., intravenously) was given so that adrenaline gave pure pressor responses (Vanov & Vogt, 1963). Atropine (0.25 mg/100 g body wt., intravenously, repeated as necessary) was given to inhibit depressor responses to acetylcholine. Samples of perfusion fluid were assayed against standard dilutions of adrenaline made up in the same perfusion medium and their pressor activity was expressed in terms of adrenaline base. Adrenaline was found to be the predominant catecholamine in gerbil adrenal glands as it is in the adrenal glands of other rodents (von Euler, 1963): thus, in eight glands from four animals assayed by the method of Anton & Sayre (1962), adrenaline accounted for $91.8 \pm 2.8\%$ of the total.

Intracellular recording from isolated chromaffin cells

Cells were isolated from adrenal medullae of gerbils by tissue disaggregation techniques and were incubated in modified F 10 medium in Sykes-Moore culture chambers for about 6–8 hr. The chambers were then placed over an inverted microscope and chromaffin cells were identified and impaled under direct visual control with KCl-filled micro-electrodes connected, through a pre-amplifier, to an inkwriting recorder. Mean potentials from the chromaffin cell populations in the various experimental conditions were established by impaling 8–10 cells. The standard recording solution was phosphate buffered modified F 10 medium which contains the same concentration of salts as the modified Locke solution described above. In some experiments various amounts of sodium were omitted and replaced with osmotically equivalent amounts of sucrose. In others, sodium was omitted entirely and the calcium concentration was varied. In these latter experiments tonicity was again maintained, where necessary, by adding suitable amounts of sucrose. A full description of the experimental procedures, including the composition of the various media used, will be found in previous reports (Douglas *et al.*, 1967a, b).

RESULTS

Inhibitory effect of amethocaine on catecholamine secretion

Various local anaesthetics, including amethocaine, have been reported to suppress the secretory response of adrenal medullae of different species to several stimulant substances, among them the physiological secretagogue acetylcholine (Eichholtz & Roesch, 1949; Lecomte, 1953; Philippu & Schümann, 1962). The following experiments show that amethocaine also has such an inhibitory effect on gerbil adrenal glands stimulated with acetylcholine. In each experiment, the adrenal gland was perfused for three successive periods of 20 min, first with Locke solution, then with Locke solution containing amethocaine (0.33 mM) and once more with Locke solution. During the last 5 min of each of the three periods, acetylcholine (0.55 mM) was present in the perfusion medium. Each response to acetylcholine in the presence of amethocaine was thus bracketed by two

TABLE 1

THE INHIBITORY EFFECT OF AMETHOCAINE ON CATECHOLAMINE SECRETION EVOKED BY ACETYLCHOLINE FROM THE GERBIL ADRENAL GLAND

Catecholamine efflux (ng/5 min) in response to acetylcholine (0·55 mM)		
1st response (in Locke solution)	2nd response (in Locke solution +amethocaine (0·33 mM)	3rd response (in Locke solution)
450	14	150
204	28	100
135	40	75
90	9	36

control responses to acetylcholine in the absence of amethocaine. As can be seen from Table 1 amethocaine greatly reduced the response to acetylcholine in each of four experiments.

Effect of amethocaine on membrane potentials of chromaffin cells

Previous experiments have shown that depolarization of chromaffin cells in response to acetylcholine is linearly related to the logarithm of the concentration of sodium in the bathing medium over a wide range, and that depolarization becomes small when no sodium is

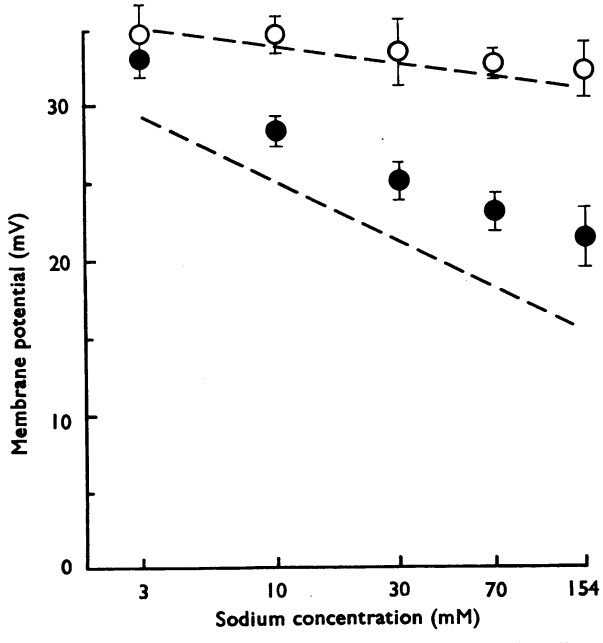

Fig. 1. Effect of amethocaine on the depolarizing response to acetylcholine in media containing various concentrations of sodium. Five experiments are represented, each carried out on a different chamber of chromaffin cells. The open circles (○) represent the control membrane potentials (mean ± S.E.) in the presence of amethocaine (0.33 mM) before adding ACh. The solid circles (●) represent the membrane potentials from the same populations of cells on the addition of ACh (0.55 mM). The interrupted lines represent the corresponding control "resting" potentials and the responses to the same concentration of ACh in the absence of amethocaine; they are taken from a previous paper by Douglas *et al.* (1967b).

present. From this it has been concluded that the depolarizing effect of acetylcholine is mainly due to inward sodium current (Douglas *et al.*, 1967b). When experiments of this sort were repeated in the presence of amethocaine (0.33 mM) a similar relation was obtained, but the depolarizing effect of acetylcholine was less (Fig. 1). One interpretation of the results depicted in Fig. 1 is that amethocaine causes an approximately parallel shift, of about 5 mV, in the curve relating the fall in potential on exposure to acetylcholine to the extracellular sodium concentration. Since there is evidence that inward calcium current accounts for about 5 mV of the depolarization in response to acetylcholine in such conditions (Douglas *et al.*, 1967b) the effect of amethocaine observed in these experiments might be due to block of inward calcium current. Support for this view was obtained from experiments on cells bathed with sodium-free media containing different concentrations of calcium. In the absence of amethocaine depolarization in response to acetylcholine increases approximately as the logarithm of the extracellular calcium concentration and thus seems to be due mainly to inward calcium current (Douglas *et al.*, 1967b). In the presence of amethocaine, however, acetylcholine was completely without depolarizing effect in such conditions, even when the calcium concentration of the medium was raised to 117 mM when in the absence of amethocaine the depolarizing effect of acetylcholine is large (Fig. 2). To corroborate that the partial blocking effect of amethocaine observed in

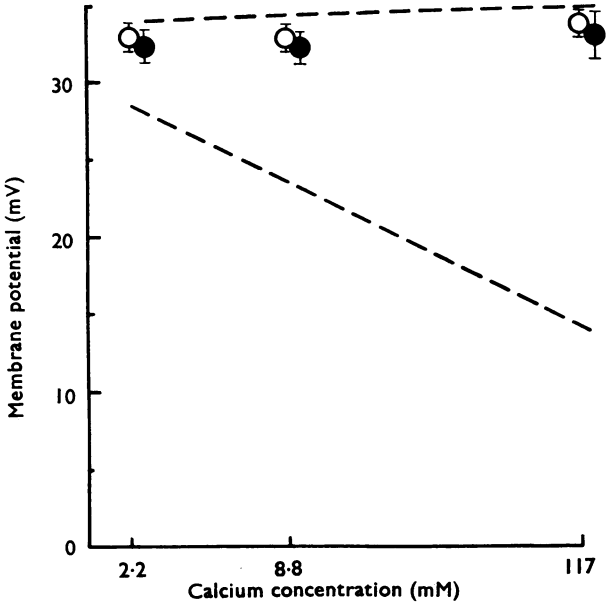

Fig. 2. Effect of amethocaine on the depolarizing response to acetylcholine in sodium-free media containing various concentrations of calcium. Three experiments are represented, each carried out on a different chamber of chromaffin cells. As in Fig. 1, the symbols represent mean membrane potentials of cells exposed to amethocaine (0.33 mM) alone (○), and amethocaine plus 0.55 mM acetylcholine (●). The latter have been drawn to the right of the former in the interest of clarity, but the calcium concentrations were the same as for the amethocaine controls. The interrupted lines represent the corresponding control potentials and responses to the same concentration of ACh in the absence of amethocaine: they are taken from a previous paper by Douglas *et al.* (1967b).

media of conventional ionic composition due entirely to interference with inward calcium current it would be desirable to perform comparable experiments in which sodium is present but no calcium. Unfortuately, experiments of this sort are unsatisfactory, since the chromaffin cells depolarize slowly when calcium is omitted from the extra-cellular environment (Douglas *et al.*, 1967b) just as do many other cells (Brink, 1954 ; Shanes, 1958 ; Cerf, 1963).

In some preparations (see Ritchie & Greengard, 1966) local anaesthetics prevent the depolarization that occurs when calcium is withdrawn from the environment, but amethocaine did not have such an effect on chromaffin cells. In each of two experiments on cells exposed to amethocaine (0.33 mM) the mean potential fell from about 30 mV to less than 17 mV on withdrawing calcium. Amethocaine (0.33 mM) also failed to prevent the depolarization of chromaffin cells that occurs when the potassium concentration of the medium is raised. Thus, increasing the potassium concentration from 5.6 to 40 mM in the presence of amethocaine (0.33 mM) lowered membrane potential in a population of cells from 30.7 ± 2.0 to 12.3 ± 0.7 mV. This is similar to the effect of potassium observed in the absence of amethocaine (Douglas *et al.*, 1967b).

DISCUSSION

There has been evidence for some time now that local anaesthetics have a depressant effect on secretory activity of the adrenal medulla evoked by various substances (Eichholtz & Roesch, 1949 ; Lecomte, 1953 ; Philippu & Schümann, 1962). The inhibitory effect of amethocaine on the secretory response of perfused gerbil adrenal glands to acetylcholine conforms to this general pattern. What is of especial interest is that electrophysiological analysis of this effect of amethocaine appears to provide a valuable insight into the mode of action of acetylcholine in stimulating catecholamine secretion, or what has been termed " stimulus-secretion coupling " (Douglas & Rubin, 1961). The analysis indicates, first, that amethocaine, in a concentration that strongly inhibits catecholamine secretion in response to acetylcholine, has little or no effect on the inward currents of sodium resulting from exposure to acetylcholine. This is fresh evidence that neither sodium entry nor the attendant depolarization are tightly coupled to the secretory response. Previously this view has rested on evidence of quite a different sort based partly on the observation that acetylcholine retains its stimulant activity in sodium-free media (Douglas & Rubin, 1963) when its depolarizing effect is profoundly reduced (Douglas *et al.*, 1967b) and partly on the observation that excess potassium, which depolarizes chromaffin cells (Douglas *et al.*, 1967b) does not evoke secretion provided calcium is absent from the extracellular environment (Douglas & Rubin, 1961). The second, and contrasting, finding that amethocaine does block the depolarization that is attributable to inward movement of calcium ions in response to acetylcholine, clearly supports the view that calcium entry is intimately related to the secretory response. The various pieces of evidence favouring this view have been reviewed elsewhere and need not be recapitulated (Douglas, 1966a, b).

Although we were led to suspect that local anaesthetics might inhibit the secretory response to acetylcholine by interfering with inward calcium current on learning that amethocaine blocks the stimulant effect of calcium on medullary secretion observable in certain circumstances (Rubin, Jaanus, Feinstein & Paimre, 1965), it was surprising to

find that the effect of the local anaesthetic was so selective as to involve calcium but not sodium. This differential effect renders amethocaine a singularly useful tool with which to analyse the mechanism of action of acetylcholine on the chromaffin cell, but it is unusual and deserves additional comment.

The most familiar effect of local anaesthetics such as amethocaine is to suppress membrane excitability by interfering with the process fundamental to the generation of the action potential, namely the large transient rise in the permeability of the membrane to sodium ions that occurs on partial depolarization (Ritchie & Greengard, 1966). However, this effect is one exerted on electrically excitable membranes and it seems likely that chromaffin cells do not fall into this category. Although older, indirect, experiments have offered conflicting testimony on the electrical excitability of medullary tissue (Rosenblueth & Cannon, 1934 ; Sgrosso, 1935 ; Hermann, Jourdan, Morin & Vial, 1936 ; Cannon & Rosenblueth, 1937), we have never detected any spike generation in isolated chromaffin cells when we have passed depolarizing current across the membrane (unpublished observations). The fact that chromaffin cells yield vigorous secretory responses to acetylcholine in sodium-free environments (Douglas & Rubin, 1963) certainly demonstrates that no sodium-dependent mechanism is necessary for the response to acetylcholine. Evidence that local anaesthetics interfere with processes other than sodium-dependent spike generating mechanisms, and with calcium movement in particular, can be found in a number of reports (see Shanes, 1958, and Ritchie & Greengard, 1966). It is particularly relevant that local anaesthetics decrease calcium fluxes in depolarized skeletal and uterine muscles (Feinstein, 1963, 1964a). And, moreover, that even the classical effect of local anaesthetics on the sodium-dependent spike generating mechanism of excitable tissues may be due to an interaction between the local anaesthetics and membrane calcium, the interference with calcium movement in the membrane leading to the failure of gNa to increase on depolarization (Feinstein, 1964b ; Blaustein & Goldman, 1966 ; see also Ritchie & Greengard, 1966). We are unaware, however, of any instance of selective block of calcium movement of the type observed in the chromaffin cell.

The present evidence may shed some light on the inhibitory effect of local anaesthetics on the release of substances from other cells, particularly nerves and neuroendocrine systems developmentally related to chromaffin cells where calcium is also critically involved (Birks & MacIntosh, 1957 ; Douglas & Poisner, 1964a,b ; Katz & Miledi, 1965 ; Mikiten & Douglas, 1965). Local anaesthetics are known to depress acetylcholine output from cholinergic nerves in relatively low concentrations (Harvey, 1939 ; Furukawa, 1957 ; Straughan, 1961 ; Matthews & Quilliam, 1964 ; Paton & Thomson, 1964) and also inhibit the output of vasopressin from neurohypophyses stimulated electrically (Haller, Sachs, Sperelakis & Share, 1965). The only explanation that has been offered for these effects is that the local anaesthetics may block impulse propagation in the terminals. The results obtained on the chromaffin cell suggest an alternative hypothesis: that inhibition of release of neurohumours or neurohormones may be attributable to block of calcium entry.

Finally, we should like to point out that local anaesthetics inhibit contractile responses in both skeletal and smooth muscle and that this effect also is associated with block of calcium movement (Feinstein, 1963 ; 1964a). Thus the present findings further extend the remarkable parallelism between the processes of stimulus-secretion coupling in the

chromaffin cell and excitation-contraction coupling in muscles to which attention was drawn earlier (Douglas & Rubin, 1961, 1963 ; Douglas, 1966a, b).

SUMMARY

1. Amethocaine inhibited secretion of catecholamines from perfused adrenal glands of gerbils stimulated by acetylcholine.

2. Intracellular records from isolated chromaffin cells showed that amethocaine only partially depressed the depolarizing effect of acetylcholine when the extracellular environment contained both sodium and calcium, but blocked completely the depolarizing effect of acetylcholine in sodium-free environments where depolarization is dependent on extracellular calcium.

3. The results indicate that amethocaine, in the concentration studied, has a preferential blocking action on inward calcium current.

4. The findings support the view that inward movement of calcium is critically involved in evoking secretion from the chromaffin cell, and that neither sodium entry nor the attendant depolarization is tightly coupled to the secretory response.

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