J. Physiol. (1964), **175**, *pp.* 231–241
With 5 text-figures
Printed in Great Britain

THE EFFECTS OF ALKALINE EARTHS AND OTHER DIVALENT CATIONS ON ADRENAL MEDULLARY SECRETION

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(*Received* 11 *May* 1964)

Acetylcholine, released by sympathetic nerve endings in the adrenal gland, is believed to be the physiological stimulus which induces the medullary chromaffin cells to secrete their hormones, adrenaline and noradrenaline. Studies of the mode of action of ACh have established that some Ca-dependent process is involved, and have led to the suggestion that ACh stimulates the chromaffin cells by promoting an influx of Ca ions into them (Douglas & Rubin, 1961*a*, 1963; Douglas & Poisner, 1962). Among the main pieces of evidence are: first, that the stimulant action of ACh is lost when Ca is omitted from the extracellular environment; secondly, that ACh increases ^{45}Ca uptake in the medulla; and thirdly, that Ca itself stimulates the chromaffin cells to secrete in various conditions known to increase membrane permeability and where it may be assumed to penetrate readily the cell membrane.

In an attempt to gain some insight into the nature of the critical role Ca is playing in the secretory process at the adrenal medulla, we have now studied the effects of various other ions more or less closely related to Ca. Our approach has been to try the effect of adding one or other of these ions during adrenal perfusion with Ca-free Locke's solution, which is one of the conditions believed to increase the permeability of the chromaffin-cell membrane (Douglas & Rubin, 1963), and to see, first, whether it would evoke secretion, as does Ca in these circumstances, or secondly, restore responses to ACh or excess K as restoration of Ca would do. Our purpose has been to obtain information on the ion requirements of the unknown 'Ca-receptive process' involved in adrenal medullary secretion, information which might be of help in identifying this process.

METHODS

Adrenal glands of cats were used. They were acutely denervated and perfused *in vivo* by the method described in an earlier paper (Douglas & Rubin, 1961*a*). The basic perfusion fluid used was a '*Locke's solution*' of the following composition (mM): NaCl, 154; KCl, 5·6;

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$CaCl_2$, 2·2; $NaHCO_3$, 6; glucose, 10. It was equilibrated with 5% CO_2 in O_2 and had a pH close to 7·0. *Ca-free 'Locke's solution'* was the same but with $CaCl_2$ omitted. The various divalent ions such as Ca, Sr, Mg, Ba, Ni, Co, Zn and Mn were added as the chloride salts to Ca-free 'Locke's solution'. Except for Mg and Ba, which were used in various concentrations as will be described, these ions were always added to give a concentration of 2 mM. Solutions free of Ca but containing one or other of the other divalent ions will be referred to as *Sr-substituted 'Locke's solution'*, *Ba-substituted 'Locke's solution'*, etc. In further experiments Sr and Ba were added in different amounts to Locke's solution. Acetylcholine was used as the chloride in a concentration of 10^{-5} g/ml. *High K 'Locke's solution'* contained 56 mM-KCl and its NaCl content was lowered to 104 mM to maintain isotonicity: this solution contained hexamethomium (5×10^{-4} g/ml.) in an amount sufficient to block indirect effects due to stimulant effects of K on the cholinergic nerve endings present in the medulla (Douglas & Rubin, 1961*a*). Catecholamines, adrenaline plus noradrenaline, were measured by bio-assay with the rabbit's aortic strip as previously described (Douglas & Rubin, 1961*a*) or, in a few experiments, by the photofluorometric technique of von Euler & Floding (1956) as modified by Anton & Sayre (1962).

RESULTS

Strontium. In each of five glands, Sr (2 mM) evoked an intense secretion of catecholamines when introduced after 12 min or more of perfusion with Ca-free Locke's solution. And on each occasion when the test was made, ACh (four glands) and excess K (one gland) proved effective stimuli for secretion in the Sr-substituted 'Locke's solution' although ineffective in Ca-free Locke's solution (Fig. 1). In both these respects Sr resembles Ca. Further analysis revealed more similarities. For example, there was little, if any, change in catecholamine secretion when adrenal perfusion was switched from Locke's solution to Sr-substituted Locke's solution (i.e. Ca-free Locke's solution with the addition of 2 mM-Sr). Furthermore, even after prolonged perfusion with this solution, the re-introduction of the normal amount of Ca (2·2 mM) failed to evoke secretion. In other words, the presence of 2 mM-Sr in the Ca-free Locke's solution prevented the chromaffin cells from developing the responsiveness to Ca which otherwise occurs during perfusion with Ca-free media. It was next noted that Sr (4–8 mM) had little effect on secretion when given during perfusion with Locke's solution or Sr-substituted Locke's solution (see left-hand part of Fig. 2), but did provoke secretion when the adrenal was perfused with high-K Locke's solution (right-hand part of Fig. 2) and, moreover, that this effect increased with increasing concentrations of Sr. This phenomenon too, is just like that previously reported for Ca (compare Douglas & Rubin, 1963, fig. 4). Finally, it was found that although the addition of excess Sr to Locke's solution did not itself evoke secretion, it augmented responses to ACh or excess K again as had already been found for Ca (Douglas & Rubin, 1961*a*). Two such experiments are shown in Fig. 3: a third experiment of the same type with ACh gave a similar result; three responses to ACh in the presence of 10 mM-Sr were 70, 64 and 82%

higher than the mean of the corresponding control responses immediately before and after.

Thus in all these experiments the effect of Sr was much like that of Ca. The question arose whether Sr acted directly on the secretory mechanism or whether it owed its effect to its liberating endogenous Ca from some binding site. This possibility seemed worth considering since a mechanism

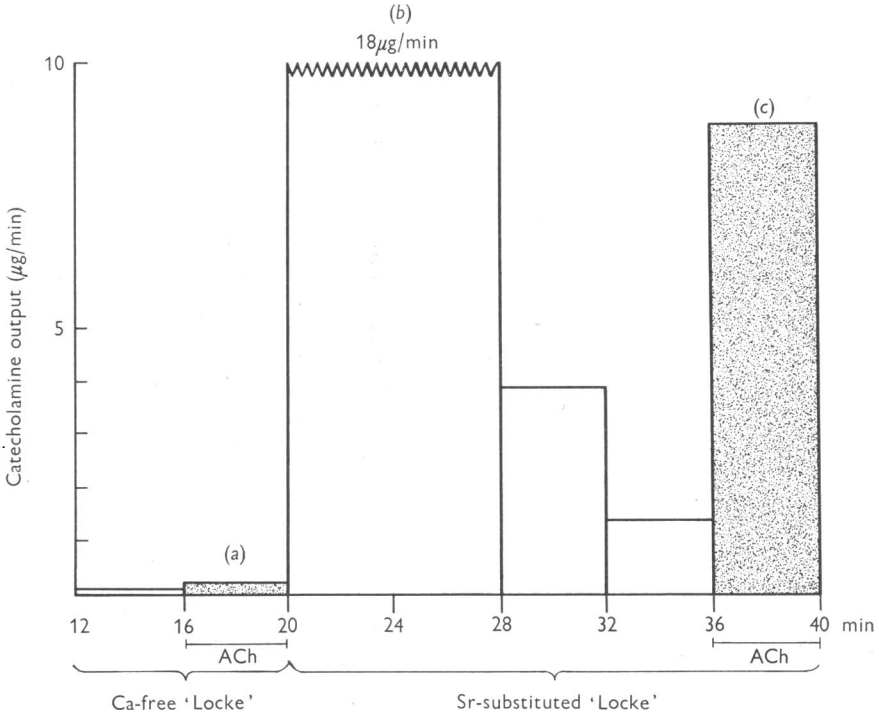

Fig. 1. The effect of adding Sr during adrenal perfusion with Ca-free Locke's solution. Sr (2M) stimulated secretion (*b*) and restored the response to ACh (10^{-5} g/ml.) (compare *c* with *a*). In this and all subsequent figures, the graphs show the rate of secretion of catecholamines, adrenaline plus noradrenaline, from an acutely denervated cat's adrenal gland perfused *in vivo*.

of this sort seems to be responsible for the activity of a variety of cations which can substitute for Ca in maintaining muscular contraction. Thus Frank (1962, 1963) has found that in Ca-free media the addition of various divalent ions will restore contractions to excess K, but that responses to K rapidly dwindle during successive tests and are restored only by interpolating in this series of tests a brief exposure to Ca. He argued that the restoration of the response to K is due to a recharging of the sites of Ca binding in the muscle fibres. We carried out experiments to test this point by perfusing glands for long periods with Ca-free Locke's solution and then

introducing Sr for 4-min periods at regular intervals. When this was done, the successive secretory responses to Sr did wane—but the decline was not more rapid than we had previously found in similar experiments with Ca (compare, e.g. Douglas & Rubin, 1963, Fig. 8) and was presumably due to

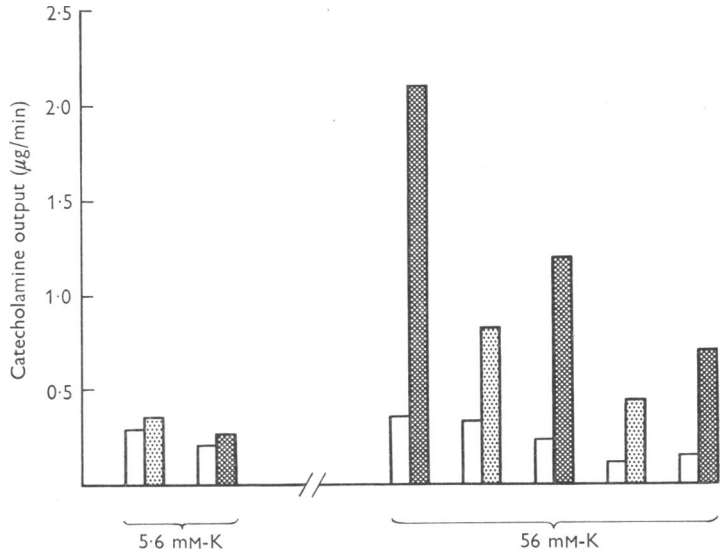

Fig. 2. The effect of different concentrations of Sr on catecholamine secretion in the presence of conventional and excess amounts of K. An adrenal gland was perfused first (left-hand part of Fig.) with Sr-substituted Locke's solution (Ca-free Locke's solution plus 2 mM-Sr) containing 5·6 mM-K; and then (right-hand part of Fig.) with Sr-substituted Locke's solution with 10 times this amount of K (Na was lowered to maintain tonicity). During each tenth minute the concentration of Sr was raised to 4 mM (light-stippled columns) or 8 mM (dark-stippled columns). The open columns show the 'control' outputs in the presence of 2 mM-Sr in the minute preceding each test. The first recorded response in the right-hand part of the figure was obtained 40 min after introducing excess K: the period of intense secretion immediately following its introduction is not shown.

the normal tendency of the perfused adrenal gland to give successively smaller responses (this phenomenon we have regularly seen during perfusion of more than 170 glands). Moreover, Ca re-introduced briefly to the perfusion medium did not reverse the trend (Fig. 4). Further evidence that Sr did not act by releasing Ca came from experiments in which glands were perfused with 3 mM-EDTA (disodium salt) in Ca-free Locke's solution. After 25–35 min of perfusion with this medium the introduction of 2 mM-Sr still provoked intense secretion in each of two glands. In one the output of catecholamines rose to 15·8 μg/min and in the other to 4·8 μg/min.

Magnesium. In previous experiments we found that the addition of magnesium to Ca-free Locke's solution protects the chromaffin cells from the changes which otherwise occur in them on Ca deprivation and which cause them to secrete when Ca is re-introduced. In this respect magnesium was clearly able to substitute for Ca. But in other tests it behaved quite differently from Ca. When added during perfusion with a Ca-free medium, it neither evoked secretion nor restored responses to ACh or K. And when added to Locke's solution containing the normal amount of Ca, it inhibited responses to ACh or K (Douglas & Rubin, 1963). Since many antagonists are known to act as weak agonists when given in sufficient concentration,

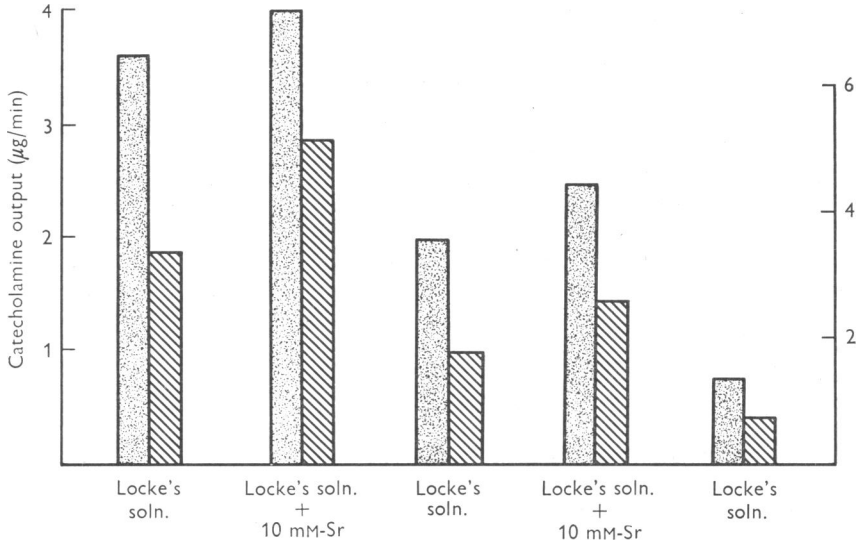

Fig. 3. The potentiating effect of Sr (10 mM) on catecholamine output evoked by ACh (stippled columns) and excess K (cross-hatched columns). Each series of responses was obtained sequentially from a different adrenal gland perfused for alternate periods of 15 min with Locke's solution or Locke's solution with the addition of 10 mM-Sr. The stimulating agents were introduced during the final few minutes of perfusion with one or the other solution, ACh (10^{-5} g/ml.) for 2 min and excess K (56 mM) for 4 min. The left and right-hand scales apply to the ACh experiment and K experiment respectively.

and since Schümann & Philippu (1963) have observed stimulant effects of magnesium on catecholamine release, further experiments were done to see whether still higher doses of magnesium might stimulate secretion. In each of two experiments glands were perfused with Ca-free Locke's solution for 12 min or more and then perfusion was switched to a similar solution containing 80 mM-Mg. In this latter solution the Na concentration was lowered by an appropriate amount to maintain tonicity: lowering

Na does not prevent the effect of introducing Ca (Douglas & Rubin, 1961*a*). In these two experiments the introduction of magnesium had little or no effect on the rate of release of catecholamines which remained in the control range (less than 70 ng/min). Nor did it restore responses to ACh (10^{-5} g/ml.).

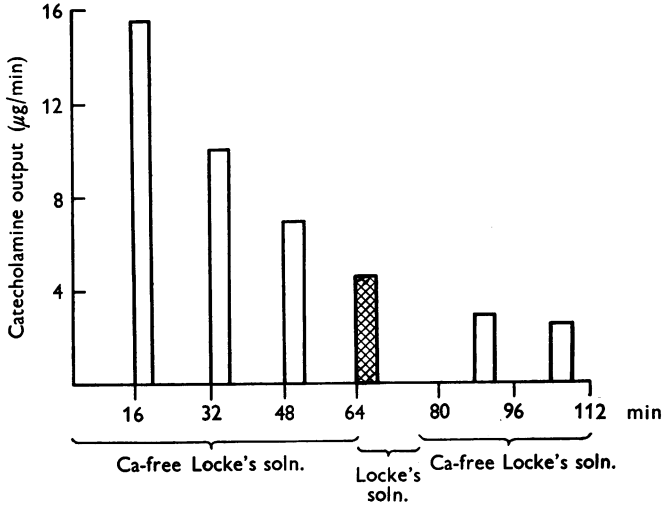

Fig. 4. An experiment showing repeated secretory responses to Sr during adrenal perfusion with Ca-free Locke's solution; and that although these responses wane, the trend is not reversed by exposing the gland to Ca. The output of catecholamines during 4-min periods of exposure to Sr (2 mM) is represented by the open columns. The cross-hatched column shows the corresponding effect of Ca (2 mM) in the first 4 min after it was introduced. The 'control' rate of catecholamine output in the 4 min preceding each test was less than 50 μg/min.

Barium. Like Ca and Sr, Ba evoked an intense secretory response when introduced during perfusion with Ca-free Locke's solution. The effect could be obtained repeatedly on a single preparation, and once again did not seem to be indirectly mediated. An experiment carried out in the same way as that illustrated in Fig. 4 but using 2 mM-Ba instead of 2mM-Sr gave a very similar result: Ba still raised secretion more than one hundred-fold (from 51 ng to 5·8 μg/min) on the fourth occasion it was introduced and the interpolated response to Ca (2 mM) which followed was no greater —secretion rose from 46 ng to 4·9 μg/min. Moreover, this brief exposure to Ca did not prevent the responses to Ba from continuing to wane. And finally, as with Sr, the stimulating effect of Ba persisted in glands perfused with EDTA: for example, in a gland perfused for 70 min with Ca-free Locke's solution containing 3 mM-EDTA, barium (2 mM) introduced for 4 min at the 20th, 40th, and 60th min caused secretion to rise to 13, 7·7 and 6·7 μg/min in the three tests.

However, Ba had certain properties distinct from those of Ca and Sr. For example, an intense stimulant effect on secretion was seen in each of three glands when perfusion was switched from Locke's solution to Ba-substituted Locke's solution without any preceding period of Ca deprivation. And indeed the addition of Ba during perfusion with Locke's solution was sufficient to evoke secretion. These stimulant actions of Ba are described in detail elsewhere (Douglas & Rubin, 1964). The effect was of significance for the present experiments inasmuch as it complicated the experiments made to test whether or not Ba would substitute for calcium in restoring responsiveness of a gland to ACh or K. For example, in each of six experiments the stimulant effect of Ba-substituted Locke's solution (containing 2 mM-Ba) was persistent and when ACh or K was given the rate of secretion, already very high, was not further increased. An answer to this question was finally obtained by changing the experimental conditions. It was found that the stimulant effects of Ba could be avoided by using it in low concentration (0·2 mM) and having 2 mM-Mg present during Ca-free perfusion (magnesium, as mentioned above, probably prevents the chromaffin cells from becoming excessively permeable in the Ca-free medium). In each of three glands where this procedure was adopted Ba proved capable of restoring responses to ACh and K (Fig. 5).

Cations other than alkaline earths. In experiments on five adrenal glands a variety of divalent ions other than alkaline earths were added during perfusion with Ca-free Locke's solution. The cations tested were nickel, cobalt, zinc and manganese. Each was introduced in a concentration of 2 mM/l. about 30 min after beginning perfusion with Ca-free Locke's solution.

None of these cations had any significant stimulant effect on catecholamine secretion, and only Mn showed any tendency to restore responsiveness to ACh; and this was feeble (Table 1).

DISCUSSION

The Ca-dependent process which has been found to be involved in the secretion of catecholamines from the adrenal medulla (Douglas & Rubin, 1961*a*, 1963; Douglas & Poisner, 1962) can clearly be activated by the related alkaline earths, Sr and Ba. Like Ca, these evoked secretion when introduced during adrenal perfusion with Ca-free Locke's solution and restored the secretory responses to ACh or K which are otherwise absent in this medium. From our analysis of their effects, they seem to act in their own right and not merely by liberating Ca from some site in the cell. Barium differed from the other alkaline earths in that it stimulated catecholamine secretion without the presence of ACh, excess K or Ca-depri-

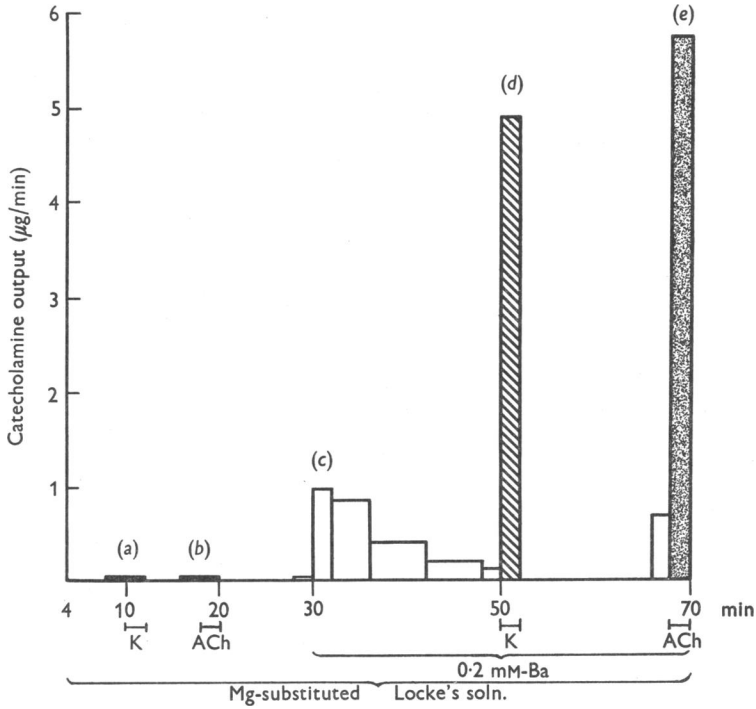

Fig. 5. The effect of adding Ba during adrenal perfusion with Mg-substituted Locke's solution (Ca-free Locke's solution plus 2 mM-Mg). Ba (0·2 mM) stimulated secretion slightly (*c*), and restored responses to excess (56 mM) K and ACh (10^{-5} g/ml.)—compare *d* with *a*, and *e* with *b*.

TABLE 1. The effect of divalent cations on adrenal medullary secretion

		Catecholamine output (ng/min)			
		In Ca-free Locke's soln.		In Ca-free Locke's soln. containing test cation	
		(*a*)	(*b*)	(*c*)	(*d*)
Gland	Test cation	Control	On adding test cation	Control	On adding ACh
A	Ni	35	40	34	44
A	Co	30	27	26	20
B	Mn	100	150	135	425
C	Mn	33	40	103	431
D	Zn	28	37	47	77
E	Zn	38	141	261	220

The columns *a*, *b*, *c* and *d* show catecholamine output in 4-min periods: *a* and *b* immediately before and after the introduction of the test cation, and *c* and *d* 20 min later immediately before and after addition of ACh (10^{-5} g/ml.).

vation. It thus seems to possess a unique ability to gain access to the secretory apparatus. This additional aspect of its action is analysed elsewhere (Douglas & Rubin, 1964). Strontium, on the other hand, resembled Ca very closely. Like Ca, and in contrast to Ba, it did not evoke secretion when added during perfusion with Locke's solution but did so after a period of Ca-deprivation or during exposure to ACh or excess K, all of which procedures probably increased the permeability of the chromaffin cell membrane (Douglas & Rubin, 1963) and allowed Sr to penetrate the cells. Furthermore, Sr could substitute for Ca in preventing this change from occurring in the chromaffin cells perfused with Ca-free media. And, finally, although it was without stimulant effect when added during perfusion with Locke's solution, it then potentiated responses to ACh or K just as does excess Ca (Douglas & Rubin, 1961*a*).

The ability of Sr and Ba to substitute for Ca in the secretory process is obviously not simply due to the fact that they are divalent ions. The other divalent ions tested were ineffective and amongst these was the closely related alkaline earth magnesium. It is, of course, possible that all these ions failed to substitute for Ca because they could not gain access to the site which is critical for initiating secretion. This explanation, however, is unsatisfactory, for according to Mullins (1961), magnesium and the other cations we have found to be ineffective have hydrated-ion radii smaller than those of the alkaline earths which stimulate and would thus be expected to penetrate no less readily. It thus seems preferable at present to interpret the results as meaning that the ineffective cations do not have the properties required to activate the secretory apparatus even when presented to it. Indirect support for this view has come from recent experiments on the neurohypophysis where secretion is also a Ca-dependent process (Douglas, 1963; Douglas & Poisner, 1964*a*) and where magnesium appears able to inhibit secretion by some intracellular action (Douglas & Poisner, 1964*b*).

The tentative conclusion which emerges is that the unknown Ca-dependent process involved in the secretion of catecholamines from the medulla is one which can be activated by Sr and Ba but not by a variety of other divalent ions including the closely related alkaline earth magnesium. While this information is insufficient in itself to establish the nature of the process, it may possibly contribute towards that end. The information as it stands, however, would appear to be of value in testing existing and future hypotheses on the role of Ca in the secretory process. For example, since we reported on the importance of Ca for the actions of ACh, various nicotine-like drugs and excess K (Douglas & Rubin, 1961*a*, *b*), evidence has been provided by Philippu & Schümann (1962) that calcium's role may be to act directly on the chromaffin granules within the

cell to promote the release of their stored catecholamines. Although this is an attractive hypothesis, the experimental evidence in its support is not yet convincing. As we have previously pointed out, the concentration of Ca required in their experiments to release catecholamines from the granules *in vitro* was considerably higher than one might expect ever to find in the cell sap; and its stimulant effects on the granules were very small in comparison with those we have observed in the intact gland. The present results with magnesium give further reason to be cautious in accepting the catecholamine granules *in vitro* as a model of the Ca-sensitive mechanism involved in adrenal medullary secretion, for Schümann & Philippu (1963) found that magnesium also was effective in increasing the rate at which the granules released catecholamines. This is in striking contrast to the purely inhibitory effects of magnesium we have observed in the intact gland. The site as well as the mode of action of Ca within the cell seems still to be an open question. An earlier suggestion (Douglas & Rubin, 1961*a*) that Ca may be involved in some secretory process which involves the cell membrane and the granule, such as 'reverse pinocytosis', cannot be discounted.

Finally, as knowledge of the effect of ions on the adrenal medulla and posterior pituitary gland has accumulated, it has become increasingly clear that their influence on 'stimulus-secretion coupling' in these organs is similar to their effects in 'excitation-contraction coupling' in muscles (Douglas & Rubin, 1961*a*, 1963; Douglas & Poisner, 1964*a*, *b*). The present experiments extend this parallelism. The effects of Ca, Sr, Ba and Mg on secretion in the Ca-deprived gland (where we believe these ions gain ready access to the cell interior) are comparable to their effects when injected into the interior of muscle fibres. Thus Ca, Sr and Ba evoke contraction but magnesium does not (Heilbrunn & Wiercinski, 1947; Caldwell & Walster, 1963). It would seem to be worth while exploring the possibility that a common process is involved in excitation as it occurs in muscles and in at least some secreting cells.

SUMMARY

1. Strontium and Ba have been shown to be like calcium in that each stimulated catecholamine secretion when introduced during perfusion with Ca-free 'Locke's solution' and restored the responses to ACh and excess K otherwise absent in this medium. These ions can substitute for Ca in the secretory process.

2. Barium differed from Sr and Ca in that it stimulated catecholamine secretion when introduced during perfusion with Locke's solution (i.e. without prior Ca deprivation).

3. A variety of other divalent cations, including magnesium, were without stimulant effects on catecholamine secretion and failed to restore responses to ACh or K when added during perfusion with Ca-free Locke's solution.

4. The unknown Ca-dependent process involved in the release of the hormones from the adrenal medulla thus appears to be one which can be activated by Ca, Sr and Ba but not by other divalent cations, including the closely related alkaline earth Mg (which indeed has powerful inhibitory effects).

We would like to thank Miss Siret Jaanus for valuable assistance in these experiments. The work was supported by Grants NB 1093 and NB 4006 from the National Institute of Neurological Diseases and Blindness, and Grant 2M6418 from the National Institute of Mental Health, United States Public Health Service.

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