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THE EFFECT OF METABOLIC INHIBITORS ON THE RELEASE OF VASOPRESSIN FROM THE ISOLATED NEUROHYPOPHYSIS

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Little is known of the intimate mechanism of release of the posterior pituitary hormones from the terminals of the hypothalamo-hypophyseal tract save that it involves some calcium-dependent process which appears to be set in motion by depolarization of the terminals on the arrival of impulses down the tract (Douglas & Poisner, 1964*a*, *b*). To learn more about the mechanism of release of posterior pituitary hormones we have now studied the effects of various metabolic inhibitors on the isolated neurohypophysis.

METHODS

The experiments were carried out on rats' neurohypophyses incubated *in vitro* at 37° C in Locke's solution or some other solution. In each experiment five hemisected neurohypophyses were incubated together in 1 ml. of incubation medium. The medium was replaced at 10 min intervals and its vasopressin content assayed by its pressor effect on the rat's blood pressure. Details of the procedure have already been published (Douglas & Poisner, 1964*a*).

Solutions. The principal solution used was a modified *Locke's solution* of the following composition (mM): NaCl, 154; KCl, 5·6; $CaCl_2$, 2·2; $MgCl_2$, 1·0; $NaHCO_3$, 6·0; dextrose, 10·0. *K_2SO_4 Locke's solution* contained (mM): Na_2SO_4, 39; K_2SO_4, 79; $MgSO_4$, 1·0; dextrose, 10·0; $NaHCO_3$, 6·0; $CaSO_4$, 8·0. In the Ca-free versions of these solutions, the Ca salt was omitted. The media were bubbled with 5% CO_2 in O_2.

Incubation procedure with metabolic inhibitors. Effect on spontaneous release: the glands were first incubated for 30–40 min in modified Locke's solution to allow the rate of vasopressin release to reach a low steady rate (Douglas & Poisner, 1964*a*). Then the metabolic inhibitor was added and incubation continued for a further 30–60 min. *Effect on K-evoked release:* each group of five glands was incubated for 30–60 min in Locke's solution containing the inhibitor. During the last 10 min of this period the potassium concentration of the medium was 56 mM (instead of the usual 5·6 mM). In untreated glands this K excess elicits a brisk release of vasopressin (Douglas & Poisner, 1964*a*); 50 mM sodium was omitted from this solution to maintain tonicity. *Effect on release evoked by introducing Ca during incubation with excess K:* glands were first incubated for 60 min in Ca-free Locke's solution and thereafter in Ca-free K_2SO_4 Locke's solution. At 40 min intervals the medium was changed to K_2SO_4 Locke's solution for 10 min (i.e. 8 mM–$CaSO_4$ was added), so that three successive responses to Ca introduction were obtained.

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Bioassay. All bioassays were conducted with Pitressin dissolved in the appropriate incubation solution as a standard.

Drugs. The following drugs were used: 2,4-dinitrophenol (DNP), 0·5 mM; antimycin A, 7·2 mM; NaCN, 3·0 and 10·0 mM; Na amytal, 2·0 mM; iodoacetate, 3·0 mM; *p*-chloromercuribenzoate (PCMB), 0·5 mM; salyrganic acid, 0·5 mM; N-ethylmaleimide (NEM), 10 and 20 mM; glutathione, 20 mM.

RESULTS

Effect of metabolic inhibitors on spontaneous release of vasopressin. In twenty-three experiments, each carried out on a different group of glands, there was little, if any, change in the rate of spontaneous release of vasopressin when the following substances were added during incubation with Locke's solution: NaCN, DNP, iodoacetate, Na amytal, and antimycin A. Observations were usually made for about 1 hr after adding the inhibitor. A typical result is shown in Fig. 1.

In contrast, the SH reagents, PCMB, salyrganic acid and NEM, greatly stimulated the rate of vasopressin release in each of twelve tests. In one experiment, for example, the addition of 0·5 mM-PCMB during incubation

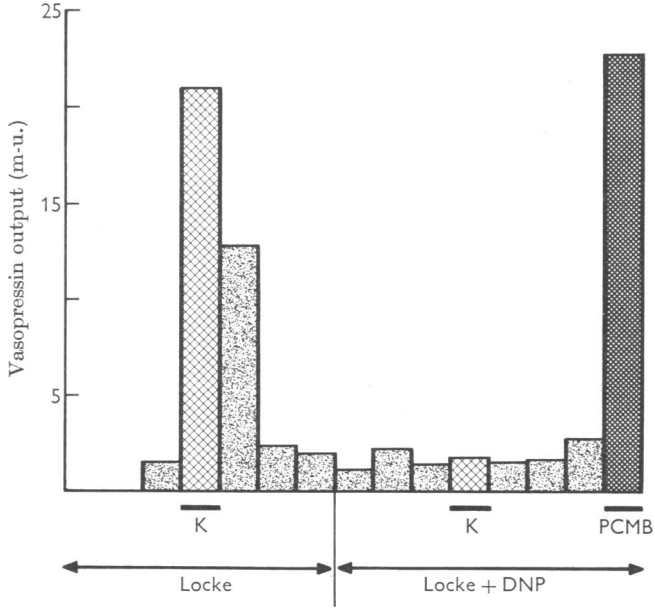

Fig. 1. An experiment showing that DNP inhibits the stimulant effect of excess K(56 mM) on vasopressin release from pituitary glands *in vitro*, but does not prevent the response to PCMB. The vertical columns represent the vasopressin output in successive 10 min periods from a group of five pituitary glands incubated first in Locke's solution and then in Locke's solution containing 0·5 mM-DNP. The first exposure to excess K (shown towards the left of the figure) was made 50 min after isolating the glands.

with Locke's solution, raised vasopressin output from 1·3 to 35·6 m-u./5 glands per 10 min, and the rate of release remained above 15 m-u./5 glands per 10 min during the following 50 min in the presence of PCMB. The contrasting effects of PCMB and DNP are illustrated in Fig. 1. The stimulant effect of these SH reagents, in contrast to that of excess K (Douglas & Poisner, 1964*a*), persisted when Ca was omitted from the incubation medium (Table 1). When, following a brief exposure to PCMB or NEM, incubation was continued with inhibitor-free media, vasopressin output returned slowly and incompletely toward control levels; but in each of three experiments (tests nos. 2, 8 and 9 in Table 1) a second exposure to the SH reagent raised vasopressin output further.

TABLE 1. Effect of sulphydryl reagents on vasopressin release

				Vasopressin output (m-u./5 glands. 10 min.)	
Test no.	Drug	Concentration	Incubation medium	Control	With SH reagent
1	PCMB	0·25 mM	Locke	2·9	9·8
2		0·50	Locke	4·2*	10·0
3		0·50	Locke	1·3	35·6
4		0·50	Locke + DNP 0·5 mM	2·8	22·8
5		0·50	Ca-free Locke	1·3	54·0
6		0·50	Ca-free Locke + EDTA 1 mM	2·0	72·4
7		0·50	Ca-free Locke + K 56 mM	1·2	35·8
8	NEM	10·0	Locke	5·0*	13·7
9		10·0	Locke	12·1*	72·0
10		20·0	Locke	1·3	14·7
11		10·0	Locke + EDTA 1 mM	1·7	7·2
12	Salyrganic acid	0·50	Ca-free Locke	2·0	40·0

* These responses were obtained from glands exposed for 10 min to the same SH reagent 50 min earlier. The elevated 'control' values are the consequence of this previous test.

Effect of metabolic inhibitors on release of vasopressin evoked by K and Ca. The increase in the rate of vasopressin output that normally accompanies a rise in the K concentration of the medium from 5·6 to 56 mM was absent or small when the medium contained any one of the following metabolic inhibitors: NaCN, DNP, Na amytal, antimycin A, and iodoacetate (Fig. 1).

The failure of excess K to evoke vasopressin release in such concentrations might possibly be due to the inhibitors causing a fall in membrane potential of the neuro-secretory terminals such that the addition of excess K had relatively little depolarizing effect. This suggested that the effect of inhibitors on vasopressin release be tried under conditions in which the glands were already depolarized by incubation in Ca-free K_2SO_4 Locke's solution (containing 158 mM-K), and where vasopressin release was evoked by the addition of Ca. As previously reported (Douglas

& Poisner, 1964*a*), the rate of release of vasopressin in Ca-free K_2SO_4 Locke's solution was low and comparable with that occurring in Locke's solution but rose to a high level when $CaSO_4$ (8 mM) was added. These responses to Ca were much smaller in glands treated with various inhibitors (Table 2).

TABLE 2. Effect of metabolic inhibitors on Ca-evoked vasopressin release

			Vasopressin output (m-u./5 glands. 10 min)					
			1st stimulation		2nd stimulation		3rd stimulation	
Inhibitor	Concentration (mM)	No. of expts.	m-u.	inhibition %	m-u.	inhibition %	m-u.	inhibition %
None (control)	—	5	82·2 ± 6·6	—	36·5 ± 2·5	—	23·3 ± 5·7	—
DNP	0·5	4	6·6 ± 0·7	92·0	3·7 ± 1·3	89·8	1·8 ± 0·6	92·3
Iodoacetate	3·0	2	2·7 ± 0·6	96·7	1·2 ± 0	96·7	1·2 ± 0	94·8
Cyanide	3·0	2	14·7 ± 1·3	82·1	11·2 ± 1·0	69·3	9·7 ± 0·1	58·4
Cyanide	10·0	1	11·5	86·0	6·9	81·0	6·6	71·7
Amytal	2·0	1	5·8	92·9	1·2	96·7	1·2	94·8
Antimycin A	$7·2 \times 10^{-3}$	1	37·8	54·0	10·0	72·6	2·5	89·3

In each experiment 5 glands were incubated in Ca-free K_2SO_4 Locke's solution. At 40 min intervals the incubation medium was changed, for 10 min, to K_2SO_4 Locke's solution containing 8 mM-$CaSO_4$. The inhibitors were present in both the Ca-free and Ca-containing media. The vasopressin outputs (m-u.) during three successive exposures to Ca are shown, along with the percentage inhibition relative to the responses obtained from the control glands.

Lack of effect of DNP on cold-evoked vasopressin release. When the temperature of the incubation medium is lowered below about 15° C, the rate of vasopressin release from isolated neurohypophyses is increased sharply (Douglas & Ishida, 1965). This effect was not suppressed by DNP. Thus in an experiment where two groups of glands were cooled from 37 to 0° C the output in one group pretreated for 50 min with 0·5 mM-DNP reached 40 m-u./5 glands per 10 min during cooling, a value that exceeded the rate of release in the second, control, group: 37·6 m-u./5 glands per 10 min. And in a third set of glands incubated at 37° C for 80 min with Locke's solution containing 0·5 mM-DNP, cooling to 0° C raised the rate of vasopressin release to 120 m-u./5 glands per 10 min.

Effect of glutathione on response to PCMB and cold. In a single experiment, a group of glands was incubated in the presence of 20 mM glutathione. This blocked the stimulant effect of PCMB (0·5 mM) on vasopressin output (output during exposure to PCMB was 1·0 m-u./5 glands per 10 min compared with a control output in the preceding 10 min of 1·3 m-u./5 glands per 10 min). However, glutathione did not abolish the response to cooling: lowering the temperature of the incubation medium to 0° C caused vasopressin output in the same preparation to rise from 1·3 to 66 m-u./5 glands per 10 min.

DISCUSSION

It is clear that a variety of metabolic inhibitors can prevent potassium from releasing vasopressin from the isolated neurohypophysis. Since it has been proposed that excess potassium evokes vasopressin release by depolarizing the neurosecretory terminals and thus promoting the influx of calcium (Douglas & Poisner, 1964*a*, *b*), one possible explanation of the observed results would be that the effect of the metabolic inhibitors is to cause a progressive fall in membrane potential such that the depolarizing action of K is lost. However, the experiments carried out on glands incubated in excess K (158 mM), in which the metabolic inhibitors were also effective, require another explanation, for the terminals may be supposed to have been already depolarized by this high concentration of potassium. If Ca entry across the cell membrane in such conditions is passive, and hence uninfluenced by the metabolic inhibitors, these experiments would indicate that it is the link between Ca entry and vasopressin extrusion that the metabolic inhibitors disrupt. A possible clue to the inhibitors' action may reside in the recent evidence that Ca may regulate oxidative phosphorylation (Rossi & Lehninger, 1965): conceivably the calcium-induced secretion might be accompanied by, and tightly coupled to, energy metabolism. Alternatively, the inhibitors might act by blocking production of some intermediate required to 'prime' the hormone extrusion mechanism that calcium activates. Such an intermediate might be ATP. There are now many examples of interactions of Ca with ATP: Ca can activate ATPases in various tissues (Martonosi & Feretos, 1964*b*; Woodin & Wieneke, 1964); Ca can bind free and protein-bound ATP (Lowenstein, 1960; Martonosi, Molino & Gergely, 1964); influx and efflux of Ca is accompanied by similar movements of ATP in various tissues or cell fractions (Carafoli & Lehninger, 1964; Abood, Koketsu & Miyamoto, 1962); and—perhaps particularly relevant here—extrusion of hormones from the developmentally related chromaffin cells of the adrenal medulla, a process that is also calcium-dependent (Douglas & Rubin, 1961, 1963), is accompanied by the appearance of large amounts of AMP and related metabolites of ATP in the venous effluent from the gland (Douglas, Poisner & Rubin, 1965). However, it remains to be seen whether adenine nucleotide metabolism is intimately involved in vasopressin extrusion.

The present results have a bearing on the curious phenomenon of release of vasopressin as a result of lowering the temperature of the incubation medium below 15° C (Douglas & Ishida, 1965). Since a fall in temperature slows metabolic processes, Douglas & Ishida suggested that one possible explanation of this stimulant effect of cold on vasopressin release might

be an inhibition of a metabolically dependent hormone storage process. Some precedent for this view can be found in the metabolically dependent uptake of catecholamines by adrenal medullary storage granules (Carlsson, Hillarp & Waldeck, 1963). However, the present evidence that various metabolic inhibitors fail to elicit any comparable increase in spontaneous release excludes this explanation. Moreover, the stimulant effect of cold was also unaffected by DNP or glutathione, drugs which block the stimulant effect of excess K and PCMB. The cause of the response to cold thus remains obscure.

The release of vasopressin by sulphydryl reagents calls to mind the finding of D'Iorio (1957) that PCMB increases catecholamine release from isolated chromaffin granules. D'Iorio suggested that PCMB may act by disrupting the membrane of the chromaffin granules and a similar explanation could conceivably account for vasopressin release from the neurohypophysis where the hormone is likewise stored in membrane-limited vesicles. Another possibility is that the sulphydryl reagents may release bound calcium inside the cell, the calcium, in turn, evoking hormone release. Thus Martonosi & Feretos (1964*a*) have shown that salyrganic acid causes the release of calcium bound to microsomes, and in some unpublished experiments we have found that ^{45}Ca is released from neurohypophyses *in vitro* when they are cooled or exposed to PCMB. The fact that metabolic inhibitors interfere with the link between stimulation and vasopressin release from the neurohypophysis is reminiscent of evidence that the inhibitors act to 'uncouple' excitation-contraction coupling in muscles (Timms, 1964). The present observations thus provide yet another illustration of the parallelism between stimulus-secretion coupling and excitation-contraction coupling to which attention has been drawn in previous reports (Douglas & Rubin, 1961, 1963; Douglas & Poisner, 1964*a*, *b*).

SUMMARY

1. The effect of metabolic inhibitors on the release of vasopressin was studied on rats' neurohypophyses *in vitro*.

2. The following inhibitors had little or no effect on the spontaneous release of vasopressin, but all greatly reduced the effect of excess potassium in evoking vasopressin release: NaCN, DNP, iodoacetate, Na amytal and antimycin A.

3. These same inhibitors also blocked the release of vasopressin evoked by introducing Ca during incubation with a Ca-free high K medium.

4. Three sulphydryl reagents (PCMB, salyrganic acid and NEM) released vasopressin and, unlike the stimulating effect of excess K, this action persisted in the absence of calcium.

5. The vasopressin-releasing action of PCMB was blocked by glutathione but not by DNP. The vasopressin releasing action of cold was not blocked by either of these agents.

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REFERENCES

ABOOD, L. G., KOKETSU, K. & MIYAMOTO, S. (1962). Outflux of various phosphates during membrane depolarization of excitable tissues. *Amer. J. Physiol.* **202**, 469–474.

CARAFOLI, E. & LEHNINGER, A. (1964). Binding of adenine nucleotides by mitochondria during active uptake of calcium. *Biochem. biophys. Res. Commun.* **16**, 66–70.

CARLSSON, A., HILLARP, N. A. & WALDECK, S. (1963). Analysis of the Mg-ATP dependent storage mechanism in the amine granules of the adrenal medulla. *Acta physiol. scand.* **59**, suppl. 215, 1–38.

D'IORIO, A. (1957). The release of catecholamines from the isolated chromaffine granules of the adrenal medulla using sulfhydryl inhibitors. *Canad. J. Biochem. Physiol.* **35**, 395–400.

DOUGLAS, W. W. & ISHIDA, A. (1965). The stimulant effect of cold on vasopressin release from the neurohypophysis *in vitro*. *J. Physiol.* **179**, 185–191.

DOUGLAS, W. W. & POISNER, A. M. (1964*a*). Stimulus-secretion coupling in a neurosecretory organ: the role of calcium in the release of vasopressin from the neurohypophysis. *J. Physiol.* **172**, 1–18.

DOUGLAS, W. W. & POISNER, A. M. (1964*b*). Calcium movement in the neurohypophysis of the rat and its relation to the release of vasopressin. *J. Physiol.* **172**, 19–30.

DOUGLAS, W. W., POISNER, A. M. & RUBIN, R. P. (1965). Efflux of adenine nucleotides from perfused adrenal glands exposed to nicotine and other chromaffin cell stimulants. *J. Physiol.* **179**, 130–137.

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.

LOWENSTEIN, J. M. (1960). Synergism of bivalent metal ions in transphosphorylation. *Nature, Lond.*, **187**, 570–573.

MARTONOSI, A. & FERETOS, R. (1964*a*). Sarcoplasmic reticulum. I. The uptake of Ca^{++} by sarcoplasmic reticulum fragments. *J. biol. Chem.* **239**, 648–658.

MARTONOSI, A. & FERETOS, R. (1964*b*). Sarcoplasmic reticulum. II. Correlation between adenosine triphosphatase activity and Ca^{++} uptake. *J. biol. Chem.* **239**, 659–668.

MARTONOSI, A., MOLINO, C. M. & GERGELY, G. (1964). The binding of divalent cations to actin. *J. biol. Chem.* **239**, 1057–1064.

ROSSI, C. S. & LEHNINGER, A. (1965). Stoichiometry of respiratory stimulation, accumulation of Ca and phosphate, and oxidative phosphorylation in rat liver mitochondria. *J. biol. Chem.* **239**, 3971–3980.

TIMMS, A. R. (1964). The coupling of electrical and mechanical activities in intestinal smooth muscle in relation to metabolism. *Trans. N.Y. Acad. Sci.* Ser. II, **26**, 8, 902–913.

WOODIN, A. M. & WIENEKE, A. A. (1964). The participation of calcium, adenosine triphosphate, and adenosine triphosphatase in the extrusion of the granule proteins from the polymorphonuclear leucocyte. *Biochem. J.* **90**, 498–509.