J. Physiol. (1965), **179**, *pp.* 185–191
With 2 text-figures
Printed in Great Britain

THE STIMULANT EFFECT OF COLD ON VASOPRESSIN RELEASE FROM THE NEUROHYPOPHYSIS *IN VITRO*

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(*Received* 7 *December* 1964)

From experiments carried out on the rat's neurohypophysis *in vitro*, it has been concluded that the physiological mechanism for the release of the posterior pituitary hormones involves the following steps: arrival of impulses in the neurosecretory terminals; depolarization of these terminals; and, finally, the activation of some calcium-dependent process (Douglas, 1963; Douglas & Poisner, 1964*a*, *b*). To learn more of 'stimulus–secretion coupling' in this preparation we have recently studied how it is influenced by temperature. During the course of this work, which will be described in another communication, we observed that cold was itself a strong stimulus for vasopressin release from the neurohypophysis *in vitro*. This report describes the phenomenon and some experiments made to analyse it.

METHODS

Preparation and incubation of glands. The experiments were performed on neurohypophyses taken from 165 male rats weighing about 250 g each. The animals were decapitated and the infundibular lobes removed, cut in half along the sagittal plane and suspended in groups of five (or occasionally ten) in 1 ml. of incubation medium kept in a water-bath at the appropriate temperature. The fluid was drawn off and replaced at 10 min intervals during the course of each experiment, and its content of vasopressin was assayed by its pressor effect on the rat anaesthetized with urethane and given large doses of the ganglion-blocking agent pentolinium. The procedure has already been described in detail (Douglas & Poisner, 1964*a*).

Incubation fluids. The principal incubation medium 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 glucose, 10. *Ca-free Locke's solution* was the same but with $CaCl_2$ omitted. In some experiments 1 mM ethylenediamine tetra-acetate (as the disodium salt) was added to this medium. In *Na-free Locke's solution*, NaCl, $NaHCO_3$ and KCl were omitted and replaced by 6 mM-$KHCO_3$ and sucrose sufficient to restore tonicity to that of Locke's solution. In *Low-Na Locke's solution*, all NaCl (154 mM) was replaced with sucrose but 6 mM-Na remained in the form of bicarbonate. *Ca-free K_2SO_4 Locke's solution* contained (mM): K_2SO_4, 79; $MgSO_4$, 1; $KHCO_3$, 6; sucrose, 94; glucose, 10. The various solutions were equilibrated with 5 % CO_2 in O_2. Some other solutions were employed containing excess K in different

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amounts; These will be described in the text. All the solutions used were isotonic with the *Locke's solution* described above; and, unless otherwise mentioned, isotonicity was achieved by adjusting the NaCl contents of the solutions.

Rate of temperature change. In most of the experiments the temperature changes were induced abruptly in the following way: first the test-tube containing the glands and incubation medium was removed from the constant temperature bath, and the medium drawn off; then, immediately, fresh medium at the new temperature was tipped in and the tube transferred to a second bath at this new temperature. A more gradual change of temperature was achieved in several experiments by replacing the fluid drawn off with fresh fluid at the same temperature and then transferring the incubation tube to a water-bath at the new temperature. In such experiments, however, although the rate of temperature change was less abrupt it was still rapid: for example, a thermocouple in the incubation medium showed that it took less than 90 sec for the temperature to fall from 37 to about 1° C or less when the tube was transferred from a bath at 37° C to one containing ice water. There were no obvious differences between the results obtained with the two techniques. In the remaining few experiments the temperature of the glands was varied much more slowly by progressively lowering or raising the temperature of the water-bath in which the incubation tube was suspended. This was done by allowing ice-water or hot-water to flow into the bath at a controlled rate. In such experiments the temperature of the incubation medium was continuously monitored by a thermocouple.

RESULTS

The stimulant effect of cooling on vasopressin output. When five hemisected rats' neurohypophyses are incubated together in 1 ml. of Locke's solution at 37° C the spontaneous output of vasopressin proceeds, after an initial equilibrium period of 20–30 min, at a level of about 0·5–2·5 m-u./5 glands per 10 min, and remains in this range during the course of experiments lasting 2–3 hr (Douglas, 1963; Douglas & Poisner, 1964*a*).

In the present experiments we found that when the incubation medium at 37° C was drawn off and immediately replaced with fresh medium at 0° C there was a sharp increase in vasopressin output. The rate commonly rose 10- to 20-fold in the first 10 min of cooling to reach a value comparable with that caused by vigorous electrical stimulation or excess potassium in optimal amount (Douglas & Poisner, 1964*a*). This stimulant effect of cooling was seen in all of four experiments each carried out on a different batch of five glands. The rate of vasopressin output during the four 10 min incubations at 0° C was $29{\cdot}4 \pm 3{\cdot}3$ m-u./5 glands per 10 min (mean $\pm$ S.E.) compared with $1{\cdot}3 \pm 0{\cdot}5$ m-u./5 glands per 10 min in the corresponding control periods at 37° C immediately before cooling.

Since the neurosecretory terminals in the neurohypophysis in which the posterior pituitary hormones are stored are modified nerve endings, and since there is strong evidence (summarized by Douglas & Poisner, 1964*a*) that the arrival of action potentials in the terminals is the normal stimulus for hormone release, it was possible that cold stimulated vasopressin output by setting up impulses. Very rapid cooling has been reported to have

such an effect in a variety of nerve endings and axons (Hensel & Zotterman, 1951; Dodt, 1953). In each of three experiments, however, cooling to 0° C was still effective when glands were cooled at a rate not exceeding 2° C/min (Fig. 1), which made such an explanation improbable. An alternative possibility was that cooling depolarized the neurosecretory terminals by depressing metabolism: the powerful stimulant effect of excess K or electrical stimulation of the isolated neurohypophysis is probably mediated by depolarization (Douglas & Poisner, 1964*a*). But

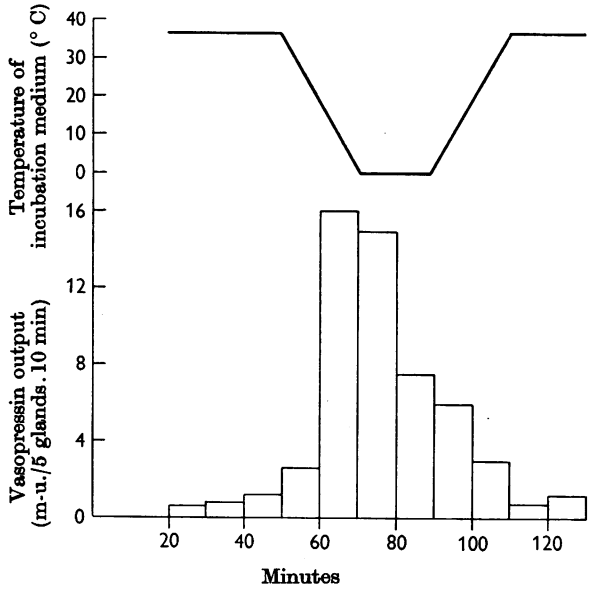

Fig. 1. The stimulant effect of cold on vasopressin output from the isolated neurohypophysis of the rat. Five neurohypophyses were incubated in 1 ml. Locke's solution which was replaced at 10 min intervals. The vasopressin content of the successive incubates is plotted in the lower part of the Fig. The upper curve shows the course of temperature change constructed from measurements made with a thermocouple placed in the incubation medium. Slow cooling and warming was achieved by changing the temperature of the bath.

this explanation too seemed improbable, for the effect of cold on vasopressin output was rapid in onset and the membrane potential of the neurosecretory endings would not be expected to fall so quickly; certainly that of non-myelinated fibres from the peripheral system does not (J. M. Ritchie, personal communication).

To obtain more direct evidence concerning these possibilities, neurohypophyses were cooled during incubation with Ca-free Locke's solution or Locke's solution containing excess Mg (20 mM) both of which media prevent secretory responses to electrical stimulation (Douglas & Mikiten,

unpublished results; Douglas & Poisner, 1964*a*). As illustrated in Fig. 2 the response to cold persisted in these conditions. Furthermore, cooling was also effective in each of three experiments where 1 mM EDTA was added to the Ca-free Locke's solution used to incubate the glands during cooling and for 30 min beforehand. In these experiments vasopressin

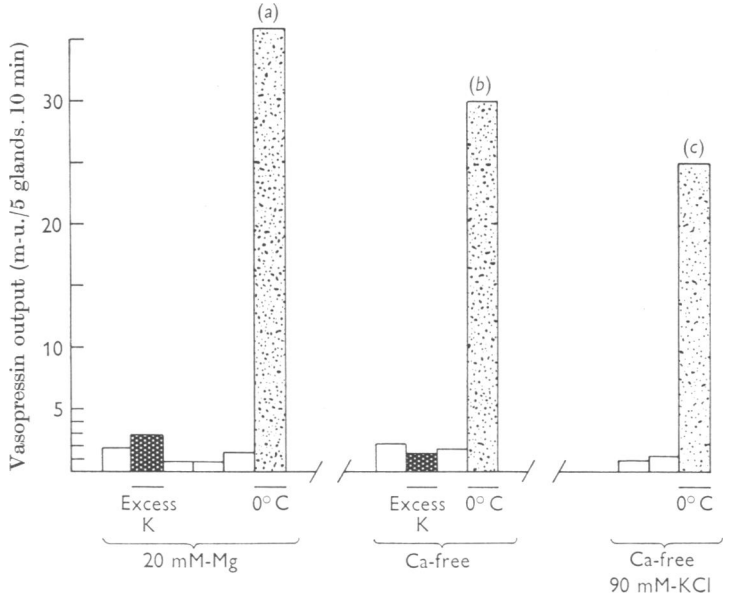

Fig. 2. Experiments showing that the stimulant effect of cold on vasopressin output from isolated neurohypophyses persists during incubation with media which suppress the response to excess K. (*a*) during incubation with Locke's solution containing 20 mM-Mg. (*b*) during incubation with Ca-free Locke's solution. (*c*) during incubation with Ca-free Locke's solution containing 90 mM-KCl. The three experiments were performed on three separate groups of neurohypophyses. The vertical columns represent vasopressin outputs in successive 10 min periods. During the periods marked excess K the K concentration of the media was raised to 56 mM (tonicity being maintained by a corresponding reduction in Na concentration). Incubation was at 37° C except for the periods at 0° C indicated.

outputs during 10 min incubations at 0° C were 84, 42 and 36 m-u./5 glands per 10 min compared with the corresponding control values in the preceding 10-min incubations at 37° C of 2·6, 2·8 and 2·2 m-u./5 glands per 10 min.

The fact that the effect of cold persisted in the absence of calcium offered a further means of testing its relation to membrane potential and electrical activity, for it was possible to examine the effect of cooling when the neurosecretory terminals were already depolarized by excess K and when secretion was held in check by omitting calcium from the medium. Cooling

to 0° C was effective in each of three groups of glands incubated in the presence of 90 mM-KCl, and with NaCl lowered to 70 mM to maintain isotonicity (*c.* Fig. 2). In these experiments a rough indication of the threshold for the effect of cooling was sought. Lowering the temperature to 15° C had no obvious effect in each of three tests; but at lower temperatures (10° in two experiments, 7° in the third) vasopressin output was clearly stimulated. The control values before cooling were 1·3, 1·6 and 1·3 m-u./5 glands per 10 min, and during cooling, 10, 15 and 13 m-u./5 glands per 10 min. Cooling was still effective in an incubation medium, Ca-free K_2SO_4 Locke's solution, containing a still higher concentration (158 mM) of K although the responses were smaller. In the four groups of glands so tested, the control outputs were 1·9, 1·5, 1·9 and 1·3 m-u./5 glands per 10 min.

In the last-mentioned group of experiments, the incubation medium (Ca-free K_2SO_4 Locke's solution) was free of sodium. Under somewhat different conditions, however, it was found that Na-poor solutions prevented the stimulant effect of cold. Thus cooling to 0° C caused little or no change in vasopressin output in glands incubated in Na-free Locke's solution (three experiments) or Low-Na Locke's solution (two experiments). In two of these experiments incubation was subsequently continued with Locke's solution, and in this medium the glands which had previously failed to respond to cooling were stimulated in the usual way, vasopressin output rising from control values of 0·9 and 1·8 to 24 and 35 m-u./5 glands per 10 min. In each of two final experiments the addition of 90 mM-KCl to Na-free Locke's solution (with removal of an osmotically equivalent amount of sucrose) restored responsiveness to cooling: lowering the temperature to 0° C stimulated vasopressin output to 9·5 and 11·6 m-u./5 glands per 10 min from control values of 1·5 and 1·8 m-u./5 glands per 10 min, respectively.

DISCUSSION

The stimulant effect of cold on vasopressin release cannot readily be interpreted in terms of the events at present known or suspected to be involved in the physiological mechanism of release of vasopressin: electrical activity of the neurosecretory terminals followed by depolarization and calcium entry (see Douglas & Poisner, 1964*a*, *b*). It was obtained not only in Locke's solutions containing no calcium or an excess of magnesium, both of which suppress the secretory response to electrical stimulation or depolarization (Douglas & Poisner, 1964*a*; Douglas & Mikiten, unpublished observations), but also in Na-free media rich in K in which the neurosecretory terminals may be supposed to have been depolarized and unable to discharge impulses. It is conceivable that cold may act by liberating Ca

from some bound site on or within the neurosecretory terminals. On this view the inhibitory effect of removing all or nearly all Na as well as K from the environment (as in the Na-free (sucrose) Locke experiments) might be interpreted as due to a lack of monovalent ions to exchange with calcium at the site of binding. A similar idea has been explored by Daniel (1964) and Daniel, Sehdev & Robinson (1962) in connexion with cold excitation of smooth muscle. The calcium-liberation hypothesis is not altogether satisfactory, however, for the response to cold was not inhibited by magnesium which previous evidence suggests may oppose the stimulant action of calcium at some intracellular site (Douglas & Poisner, 1964*b*). Other possible explanations which we have considered for the effect of cold include the following: (1) that the storage mechanism that retains vasopressin in the neurosecretory terminals is metabolically dependent. This, however, does not seem likely in the light of some recent experiments we have carried out with metabolic inhibitors (Douglas, Ishida & Poisner, unpublished results). (2) That cooling may cause some structural change which facilitates hormone leakage such as dissociation of the pituitary hormones from neurophysin, or molecular rearrangement of the membrane of the hormone storage granules or plasma membrane of the secretory terminals. And, (3) that cooling activates or 'disinhibits' some enzyme whose activity then promotes loss of vasopressin: for example, a proteolytic enzyme activated at temperatures below about 15° C has been described by Diniz & Carvalho (1963). The true explanation may well have escaped us and whether or not it will have a bearing on the physiological mechanism of release of posterior pituitary hormones is quite uncertain but the phenomenon is of immediate practical importance, for it indicates that neurohypophyses which are chilled for biochemical or histological purposes may well be actively secreting and not 'resting' as might otherwise be supposed.

SUMMARY

1. The rate of vasopressin release from rats' neurohypophyses incubated *in vitro* rose sharply when the glands were cooled to 10° C or below from 37° C.

2. The response to cooling persisted in the absence of Ca and in the presence of excess Mg in contrast to the response to excess potassium. Cold, moreover, was still an effective stimulus in the presence of depolarizing concentrations of K.

3. The stimulant effect of cold does not, therefore, seem to depend on action potentials, membrane depolarization and entry of calcium from the external medium, which are steps believed to be involved in the physiological process of 'stimulus-secretion coupling'. Among the explanations considered is that cooling may free 'bound Ca'.

We wish to thank Miss Agatha Palazzolo for valuable assistance during the course of these experiments. The work was supported by grants B-4006 and NB 01093 from the National Institute of Neurological Diseases and Blindness of the National Institute of Health, United States Public Health Service.

REFERENCES

DANIEL, E. E. (1964). Effect of drugs on contractions of vertebrate smooth muscle. *Annu. Rev. Pharmacol.* **4**, 189–222.

DANIEL, E. E., SEHDEV, H. & ROBINSON, K. (1962). Mechanisms for activation of smooth muscle. *Physiol. Rev.* **42**, suppl. **5**, 228–260.

DINIZ, C. R. & CARVALHO, I. F. (1963). A micromethod for determination of bradykininogen under several conditions. *Ann. N.Y. Acad. Sci.* **104**, 77–89.

DODT, E. (1953). Differential thermosensitivity of mammalian A-fibres. *Acta physiol. scand.* **29**, 91–108.

DOUGLAS, W. W. (1963). A possible mechanism of neurosecretion: release of vasopressin by depolarization and its dependence on calcium. *Nature, Lond.*, **197**, 81–82.

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.

HENSEL, H. & ZOTTERMAN, Y. (1951). The response of mechanoreceptors to thermal stimulation. *J. Physiol.* **115**, 16–24.