Stimulation of inorganic-phosphate incorporation into phosphatidylinositol in rat thoracic aorta mediated through V_1 -vasopressin receptors

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Vasopressin stimulates the incorporation of [32 P]P_i into phosphatidylinositol but not into other phospholipids in rat thoracic aorta strips. The relative abilities of three vasopressin analogues to stimulate phosphatidylinositol labelling in rat aorta are similar to their relative pressor potencies *in vivo* and to their relative potencies in stimulating the metabolism of rat hepatocytes, but very different from their relative antidiuretic potencies. The vasopressor antagonist [1-(β -mercapto- β , β -cyclopentamethylenepropionic acid),8-arginine]vasopressin competitively inhibits [Arg⁸]vasopressin-stimulated phosphatidylinositol labelling in rat aorta with a pA₂ of 8.1. It is concluded that the Ca²⁺-mobilizing vasopressin receptors (V_1 -receptors) of the rat aorta stimulate phosphatidylinositol metabolism, probably by enhancing phosphatidylinositol breakdown.

Vasopressin, like some other hormones and neurotransmitters (e.g. catecholamines and histamine), can influence target tissues through more than one route of receptor–response coupling. It increases the permeability of the collecting ducts of mammalian kidney by activating adenylate cyclase (Nakahara *et al.*, 1978), but it activates glycogen phosphorylase and gluconeogenesis in liver without increasing the hepatic concentration of cyclic AMP (Hems & Whitton, 1973; Hems *et al.*, 1976; Kirk & Hems, 1974). There is considerable evidence that the hepatic effects of vasopressin are mediated through an increase in the concentration of Ca²⁺ in the cytosol (Stubbs *et al.*, 1976; Keppens *et al.*, 1977; Blackmore *et al.*, 1978; Chen *et al.*, 1978; Garrison *et al.*, 1979). In addition, vasopressin is a potent stimulus for the contraction of vascular smooth muscle. This response, like hepatic glycogenolysis, is abolished if the muscle is deprived of extracellular Ca²⁺ (Altura & Altura, 1976, 1977), suggesting that a rise in cytosolic Ca²⁺ concentration is also the initiator of vasopressin-induced vascular contraction.

More recently there have been two further advances in our knowledge of hepatic vasopressin responses. First, it was realized that hepatic vasopressin receptors, like many other cell-surface receptors that use Ca²⁺ as their ‘second messenger’ (Jones *et al.*, 1979; Michell, 1979) stimulate the

breakdown of phosphatidylinositol, a quantitatively minor membrane phospholipid. We have suggested that this reaction might be essential for the mobilization of Ca²⁺ in the cytosol by hepatic vasopressin receptors (Kirk *et al.*, 1977, 1978, 1979, 1980, 1981; Billah & Michell, 1978, 1979; Michell *et al.*, 1979). Secondly, studies with a variety of vasopressin analogues showed that the ligand selectivity of hepatic vasopressin receptors is very similar to the ligand selectivity of the vascular vasopressin receptors that mediate the pressor response to vasopressin *in vivo* (Kirk *et al.*, 1979, 1981; Michell *et al.*, 1979; Keppens & De Wulf, 1979). These studies have led us to suggest that the Ca²⁺-mobilizing vasopressin receptors of the liver and vasculature (V_1 -receptors; Michell *et al.*, 1979) might share a mechanism of action in which the breakdown of phosphatidylinositol is an essential step. The experiments described in the present paper were undertaken to test the essential prediction (Michell *et al.*, 1977, 1979) that vasopressin should stimulate phosphatidylinositol metabolism in those smooth muscles in which it evokes contraction, and to examine the nature of the receptors that mediate any such response.

Materials and methods

Preparation and incubation of aortic strips

Male Wistar rats (250–350 g) were killed by exsanguination from the abdominal aorta under

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diethyl ether anaesthesia. Their thoracic aortae were collected into an incubation medium consisting of bicarbonate-buffered physiological saline (Krebs & Henseleit, 1932), gassed with O_2/CO_2 (19:1) and containing 10 mM-glucose. The aortae were dissected free of connective tissue and fat, and the aortic arch was routinely discarded since preliminary experiments indicated that it exhibited rather variable sensitivity to vasopressin as compared with the thoracic aorta itself. The thoracic aortae were bisected first transversely and then longitudinally such that a single aorta yielded four strips, each of which weighed 5–10 mg.

The aortic strips were carefully blotted to remove excess medium, weighed and placed in individual flat-bottomed plastic vials (1 cm diameter) containing 0.25 ml of incubation medium. The vials were gassed with O_2/CO_2 (19:1), capped and incubated at 37°C for 60 min in a gently shaking water bath. At the end of this period, hormones were added to the vials together with a further 0.25 ml of incubation medium containing 20 μ Ci of [32 P] P_i /ml. The vials were re-gassed, capped and further incubated with gentle shaking for the times indicated in the legends to the Figures. All vials were re-gassed at 30 min intervals throughout the incubations. It has been reported that the sensitivity of rabbit thoracic aorta to vasopressin diminishes towards the posterior end of the vessel (Altura & Altura, 1970). Our initial experiments suggested that this was not the case with rat aorta, and aortic strips from different regions of the vessel were distributed randomly amongst the incubation vials in the present study.

Lipid extraction and isolation

Incubations were terminated with the additions of 1.9 ml of chloroform/methanol (1:2, v/v) and the aortae were washed into plastic scintillation vials with a further 1.9 ml of chloroform/methanol (1:2, v/v). The tissue was homogenized with a top-drive rotary-blade homogenizer at full speed (14000 rev./min) for 2 min. The lipid extracts were transferred to glass test-tubes containing 1.25 ml of chloroform and 1.75 ml of 2 M-KCl and were thoroughly mixed. After gentle centrifugation, the lower chloroform phase was removed by aspiration and the upper phase was washed with a further small portion of chloroform. The chloroform phases were combined, evaporated to dryness under vacuum and the extracted lipids were separated on formaldehyde-treated papers (Kirk *et al.*, 1979).

Spots containing phosphatidylinositol and the other major phospholipids were identified with the aid of pure phospholipid standards, excised and wet-ashed in $HClO_4$ (Jones & Michell, 1974). In the time-course experiment (Fig. 1), the incorporation of P_i into phosphatidylinositol was expressed as ng-atoms incorporated/g of fresh tissue. However,

when results from separate experiments were expressed in this way, considerable variability was encountered due to the problem of weighing small quantities of tissue that may carry various amounts of surface water. In subsequent experiments, the incorporation of P_i into phosphatidylinositol has been expressed in terms of the ratio: radioactivity in phosphatidylinositol/radioactivity in other major

Fig. 1. Influence of vasopressin on the time course of P_i incorporation into phospholipids in rat thoracic aorta. Aortic segments were incubated with [32 P] P_i in the presence (closed symbols) or absence (open symbols) of 0.5 μ M-vasopressin for the times indicated. Lipids were extracted and chromatographed as described in the text to yield phosphatidylinositol (Fig. 1a) and a spot containing the other major phospholipids (phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, sphingomyelin; Fig. 1b). Results are means $\pm$ S.E.M. from aortic segments taken from three separate rats for each point. * P (versus hormone-free controls) < 0.01 .

phospholipids. This provides a sensitive measure of phosphatidylinositol labelling in conditions where it has been shown that the incorporation of P_i into other phospholipids remains unchanged (see Fig. 1 and Jones & Michell, 1974).

Materials

[Arg⁸]Vasopressin (grade V1) and phospholipid standards were from Sigma Chemical Co. [1-(β -Mercapto- β,β -cyclopentamethylenepropionic acid),8-arginine]vasopressin ([1-{ α -(1-mercapto-cyclohexyl)acetic acid},8-arginine]vasopressin) and [Val⁴,Arg⁸]vasopressin were gifts from Professor M. Manning, Medical College of Ohio, Toledo, OH, U.S.A. [D-Arg⁸]Vasopressin was a gift from Dr. H. Vilhardt, Ferring Pharmaceuticals, Malmo, Sweden.

Results

The incorporation of P_i into phosphatidylinositol followed a rising time course over the 60 min incubation period. At all times tested [Arg⁸]vasopressin (0.5 μ M) enhanced the incorporation of P_i into phosphatidylinositol, but not into other phospholipids (Fig. 1).

The dose-response curves for stimulation of phosphatidylinositol labelling by [Arg⁸]vasopressin, [Val⁴,Arg⁸]vasopressin and [D-Arg⁸]vasopressin are shown in Fig. 2. The latter two analogues exhibited only partial agonist activity with respect to the stimulation of phosphatidylinositol labelling. The relative abilities of these three peptides to promote phosphatidylinositol labelling in aortic strips are compared with their relative pressor, antidiuretic and hepatic potencies in Table 1.

Fig. 2. Concentration dependence of stimulated P_i incorporation into phosphatidylinositol in the presence of [Arg⁸]vasopressin, [Val⁴,Arg⁸]vasopressin and [D-Arg⁸]vasopressin in rat thoracic aorta
Aortic segments were incubated for 30 min with [³²P] P_i , and [Arg⁸]vasopressin (O), [Val⁴,Arg⁸]vasopressin (Δ) or [D-Arg⁸]vasopressin ($\bullet$) at the concentrations indicated. The incorporation of P_i into phosphatidylinositol is expressed as the ratio: radioactivity (d.p.m.) in phosphatidylinositol/radioactivity (d.p.m.) in other phospholipids as described in the text. Results are means $\pm$ S.E.M. from aortic segments taken from three to five rats for each point.

Table 1. Influence of vasopressin and related peptides on cellular responses

Results were obtained from Fig. 2 and the references listed. The relative molar activities are compared with [Arg⁸]vasopressin = 1. References: *Sawyer *et al.* (1974); †Kirk *et al.* (1978); ‡Keppens & De Wulf (1979).

	Vascular activity		Hepatic activity		Renal antidiuretic activity (in vivo)*
	Pressor (in vivo)*	Phosphatidylinositol labelling (aortic strips)	Phosphorylase activation	Phosphatidylinositol labelling†	
[Arg ⁸]vasopressin	1.0	1.0	1.0†	1.0	1.0
[Val ⁴ ,Arg ⁸]vasopressin	0.09	0.06	0.07‡	—	2.2
[D-Arg ⁸]vasopressin	0.003	0.07	0.005†	0.004	0.8

Fig. 3. Inhibition by [1-(β -mercapto- β , β -cyclopentamethylenepropionic acid),8-arginine]vasopressin of [Arg^8]vasopressin-induced phosphatidylinositol labelling in rat thoracic aorta

[1-(β -Mercapto- β , β -cyclopentamethylene propionic acid),8-arginine]vasopressin was added to vials containing aortic segments 5 min before the addition of [^{32}P]P_i and [Arg^8]vasopressin to give the following final concentrations: 50 nM (Δ), 0.5 μM ($\square$) and 1.5 μM (∇). Results in the absence of antagonist (O) are taken from Fig. 2. Results are means $\pm$ S.E.M. from aortic segments taken from three to four rats for each point. When replotted by the method of Schild (1947) these results yield a pA_2 value for the inhibition of phosphatidylinositol labelling of 8.1 (inset).

The antipressor antagonist [1-(β -mercapto- β , β -cyclopentamethylenepropionic acid),8-arginine]vasopressin competitively inhibited the stimulation of phosphatidylinositol labelling provoked by [Arg^8]vasopressin (Fig. 3). The pA_2 [$-\log(\text{antagonist concentration that doubles the concentration of agonist required to evoke a half-maximal response})$] for this inhibition was calculated by the method of Schild (1947) and found to be 8.1 (Fig. 3, inset).

Discussion

Rat aorta is a tissue that supports a rapid rate of P_i incorporation into phosphatidylinositol even when unstimulated, with this lipid incorporating about half of the [^{32}P]P_i that is found in total aortic lipids. The only other aorta to have been investigated is that from cat, where about one-quarter of the incorporated [^{32}P]P_i is found in phosphatidylinositol (Lapetina *et al.*, 1976). By contrast, in unstimulated hepatocytes much less of the [^{32}P]P_i incorporated into lipids (about 12%) is found in phosphatidylinositol (Kirk *et al.*, 1977). Despite this difference between the unstimulated tissues, the present results show that in aorta, as in hepatocytes, vasopressin stimulates the incorporation of [^{32}P]P_i into phosphatidylinositol,

but not into other phospholipids. Lapetina *et al.* (1976) have previously reported that α -adrenergic stimuli selectively enhance phosphatidylinositol labelling in isolated cat aorta. The selective stimulation of [^{32}P]P_i incorporation into phosphatidylinositol observed in the present study is similar to that provoked by many other ligands whose influence on their target tissues is mediated by a rise in intracellular Ca^{2+} concentration (Michell, 1975, 1979). All of the available evidence is consistent with the view that in each of these situations, including the vasopressin-stimulated hepatocyte (Billah & Michell, 1979; Kirk *et al.*, 1981), the initial event that is stimulated by receptor occupation is phosphatidylinositol breakdown (Michell, 1975; Jones *et al.*, 1979). We would expect that this is also the initiating event in the stimulation of phosphatidylinositol metabolism in rat aorta by vasopressin, but we have thus far been unable to make appropriate measurements with the small amounts of available tissue.

The four vasopressin-like peptides used in the present study were chosen for their qualities as three agonists of widely differing pressor potencies and as a selective inhibitor of the pressor effects of vasopressin. The relative abilities of the three

agonists to stimulate aortic phosphatidylinositol metabolism were very similar to their relative pressor potencies *in vivo* (see Fig. 2 and Table 1). Similarly, the potency of [1-(β -mercapto- β , β -cyclopentamethylenepropionic acid),8-arginine]-vasopressin as a competitive inhibitor of vasopressin-stimulated phosphatidylinositol metabolism ($pA_2 = 8.1$; Fig. 3) closely matched its potency as a competitive inhibitor of the pressor effect of vasopressin *in vivo* ($pA_2 = 8.35$; Kruszynski *et al.*, 1980). In the absence of published information on the relative abilities of these four ligands to affect contractility in isolated aorta, these results suggest that the same type of vasopressin receptor is responsible both for stimulating aortic phosphatidylinositol labelling, presumably by stimulation of phosphatidylinositol breakdown, and for initiating the contraction of arterial smooth muscle that contributes to a vasopressin-induced increase in blood pressure *in vivo*.

The concentrations of [Arg⁸]vasopressin and [D-Arg⁸]vasopressin that provoked half-maximal phosphatidylinositol labelling in the present study of aorta (5.5 nM and 790 nM respectively) were very similar to the concentrations that provoked half-maximal labelling in hepatocytes (2.7 nM and 710 nM respectively; Kirk *et al.*, 1979). Furthermore, [D-Arg⁸]vasopressin was a partial agonist that showed about the same efficacy in both systems, and the vasopressin antagonist [1-(β -mercapto- β , β -cyclopentamethylenepropionic acid),8-arginine]-vasopressin showed approximately similar affinities in competitively inhibiting the phosphatidylinositol labelling responses of the two tissues ($pA_2 = 8.1$ in aorta and 9.0 in hepatocytes; see Kirk *et al.*, 1981). The relative potencies of the chosen agonists as antidiuretic agents are quite different from their relative pressor potencies or their relative abilities to stimulate phosphatidylinositol turnover (Table 1). These observations are therefore in keeping with the suggestion that the hepatic and arterial effects of vasopressin are mediated through receptors that show a variety of marked similarities; they both exhibit similar ligand selectivities, they both stimulate the metabolism of phosphatidylinositol and they both use Ca^{2+} as their intracellular signal. Mechanistically, these receptors resemble α_1 -adrenergic receptors and H_1 -histamine receptors, and we therefore suggested (Michell *et al.*, 1979) that they might appropriately be termed V_1 -receptors, so as to distinguish them from adenylyl cyclase-coupled renal receptors that are responsible for inhibiting diuresis (V_2 -receptors).

The present results are also consistent with our previous suggestion (Kirk *et al.*, 1979) that stimulated phosphatidylinositol degradation is a direct consequence of interaction between vasopressin and the V_1 -receptors, and that it may be

intrinsic to the mechanism whereby the hormone mobilizes Ca^{2+} in the cytosol. It is striking that the concentrations of [Arg⁸]vasopressin necessary to provoke half-maximal contraction of rat thoracic aortic strips (3.5 nM; Altura, 1974), half-maximal phosphatidylinositol labelling in aorta (5.5 nM) and half-maximal phosphatidylinositol breakdown and labelling in liver (4.7 nM and 2.7 nM respectively; Kirk *et al.*, 1981) are all very similar. These concentrations are also very similar to the concentration of [Lys⁸]vasopressin that gives half-maximal occupation of hepatic receptors (4.9 nM; Cantau *et al.*, 1980). If the stimulation of phosphatidylinositol metabolism in aorta is closely coupled to receptor occupation (as seems to be the case in the liver) then these results suggest that, at least in relation to vasopressin-stimulated contraction, there is no 'receptor reserve' of aortic V_1 -receptors. This is in contrast with the substantial receptor reserve observed in the activation of hepatic phosphorylase by vasopressin (Kirk *et al.*, 1979). Altura (1974, 1975) has reported that arterioles and muscular venules are much more sensitive to vasopressin than is aorta, and he has suggested that their contraction contributes more to the pressor effect of the hormone than does contraction of the major blood vessels. We would predict that these smaller vessels, which should possess a greater concentration of vasopressin receptors, would exhibit a receptor reserve with respect to vasopressin-induced contraction but not with respect to phosphatidylinositol labelling. Effective concentrations of the hormone should also provoke a greater stimulation of phosphatidylinositol metabolism in these vessels than is observed in aorta.

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