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The Influence of Vasopressin and Related Peptides on Glycogen Phosphorylase Activity and Phosphatidylinositol Metabolism in Hepatocytes

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The relative abilities of seven vasopressin-like peptides to activate hepatic glycogen phosphorylase and stimulate phosphate incorporation into phosphatidylinositol were compared. Although the individual peptides differed in their potencies, the concentrations required to stimulate phosphatidylinositol metabolism were always greater (about 10 times) than those needed to activate phosphorylase. The molecular specificity of the hepatic vasopressin receptor and the role of vasopressin-stimulated phosphatidylinositol turnover are discussed.

Vasopressin is best known for its antidiuretic and vasopressor actions, but the hormone also exerts potent hepatic effects. These include stimulation of gluconeogenesis and glycogenolysis, and inhibition of fatty acid synthesis (Hems, 1977). Cyclic nucleotides are not implicated in the mechanism whereby vasopressin exerts these effects (Kirk & Hems, 1974; Hems *et al.*, 1978a), but a role for Ca^{2+} mobilization in the cytosol has been suggested (Keppens *et al.*, 1977; Blackmore *et al.*, 1978; Chen *et al.*, 1978).

We have previously shown that the Ca^{2+} -dependent glycogen breakdown induced by vasopressin in hepatocytes is associated with a marked stimulation of P_i incorporation into phosphatidylinositol, which is largely Ca^{2+} -independent (Kirk *et al.*, 1977, 1978). Billah & Michell (1978) have confirmed this observation and shown that vasopressin also stimulates the Ca^{2+} -independent breakdown of phosphatidylinositol in hepatocytes. Michell *et al.* (1977) have suggested that ligand-stimulated phosphatidylinositol breakdown in a variety of tissues is involved in the coupling between receptor–agonist interactions and enhanced Ca^{2+} -mobilization in the cytosol.

The present results described the relationship between the concentration-dependence of glycogen phosphorylase activation and that of stimulated phosphatidylinositol metabolism in hepatocytes exposed to various neurohypophyseal hormones and related analogues.

Materials and Methods

Preparation of hepatocytes

Isolated hepatocytes were prepared from fed male Wistar rats (200–240 g) by perfusion of the liver with Ca^{2+} -free bicarbonate-buffered saline (Krebs

& Henseleit, 1932) containing 0.04% collagenase (Kirk *et al.*, 1977). The separated cells were washed with bicarbonate-buffered saline containing 2.5 mM- Ca^{2+} and 2% (w/v) bovine serum albumin (fraction V) and suspended in this medium to a concentration of about 12 mg dry wt./ml. For each cell preparation, the dry weight of cells per ml of incubation medium was determined in duplicate. Cell viability was judged to be satisfactory by the criteria described previously (Hems *et al.*, 1978b).

Assay of glycogen phosphorylase activity

Glycogen phosphorylase activity was assayed in one portion of each hepatocyte preparation after preincubation for 30 min at 37°C in the presence of 20 mM-glucose. During this preincubation period, the activity of phosphorylase *a* fell from 70.7 ± 7.3 to 26.0 ± 4.0 μmol of glucose 1-phosphate/min per g dry wt. Hormones were then added to the cells, and the gas phase quickly flushed with O_2/CO_2 (19:1, v/v) before the vials were replaced in a shaking water bath at 37°C. The incubations were stopped 2 min after the addition of hormones by rapidly pipetting 0.5 ml of the cell suspensions into tubes pre-cooled in liquid N_2 . The hepatocytes were homogenized after the addition of 1 vol. of an ice-cold medium containing 100 mM-glycylglycine and 200 mM-NaF, pH 7.4. Glycogen phosphorylase *a* activity was assayed by the incorporation of ^{14}C from [^{14}C]-glucose 1-phosphate into glycogen at 30°C and pH 6.5 (Hems *et al.*, 1976).

Measurement of P_i incorporation into phosphatidylinositol

Another portion of the cells from each hepatocyte suspension was gassed with O_2/CO_2 (19:1, v/v) and preincubated for 20 min at 37°C with shaking in a large plastic vessel containing 15 μCi of [^{32}P] P_i /ml.

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After this preincubation period, 2ml portions of the cell suspension were rapidly transferred to plastic vials containing various hormones or 0.2ml of trichloroacetic acid (80%, w/v; 'initial control'). The vials were rapidly gassed, capped and transferred to a shaking water bath at 37°C. Incubation was for a further 20 min and was terminated with the addition of 0.2ml of trichloroacetic acid (80%, w/v) to each vial.

The trichloroacetate-precipitated material was collected by centrifugation, and the lipids extracted as described previously (Kirk *et al.*, 1977), and washed with 0.2 vol. of 0.1M-tripotassium citrate (Webster & Folch, 1961). Phosphatidylinositol was separated by chromatography on formaldehyde-treated papers (Letters, 1964) with water/n-butanol/acetic acid (10:8:1, by vol., upper phase). The separated phospholipids were visualized by immersion in 0.1% Nile Blue for 30 min. Spots containing phosphatidylinositol were excised and placed in scintillation vials containing 10ml of scintillant [8g of 5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole/l of toluene]. The radioactivity of these samples was determined in a Packard Tri-Carb liquid-scintillation counter. The efficiency of counting was calculated by means of a computerized channels-ratio quench-correction technique.

Incubations with each peptide were performed in duplicate. The incorporation of P_i into phosphatidylinositol during the experimental period was calculated by subtraction of the amount of P_i incorporation observed in the 'initial control' samples extracted after the preincubation period.

Chemicals

[Arg⁸]Vasopressin and phospholipid standards were from Sigma Chemical Co. (Kingston upon Thames, Surrey, U.K.). [1-deaminocysteine, 2-phenylalanine, 7-(3,4-didehydroproline), 8-arginine]-Vasopressin was a gift from Dr. R. Walter (University of Illinois Medical Centre, Chicago, IL, U.S.A.). [Lys⁸]Vasopressin, [1-deaminocysteine, 8-arginine]-vasopressin, [D-Arg⁸]vasopressin, [Arg⁸]vasotocin ([Ile³, Arg⁸]vasopressin) and oxytocin ([Ile³, Leu⁸] vasopressin) were kindly provided by Dr. H. Vilhardt (Ferring Pharmaceuticals, Malmo, Sweden). Collagenase (grade II) was from C.F. Boehringer Corp., Lewes, East Sussex BN7 1LG, U.K. and bovine serum albumin (fraction V) was supplied by Miles Laboratories, Stoke Poges, Bucks., U.K. [³²P] P_i was from The Radiochemical Centre, Amersham. All other chemicals were of the highest grade commercially available.

Results and Discussion

All the peptides tested in the present study caused a maximal stimulation of hepatic glycogen phos-

phorylase *a* activity to about 250% of control values (Fig. 1). However, the sensitivity of glycogen phosphorylase activity to the seven peptides varied considerably. Native [Arg⁸]vasopressin was the most potent agonist and [D-Arg⁸]vasopressin was least potent (concentrations that induce half-maximal phosphorylase activation are indicated in Fig. 1). A similar pattern of relative potencies was observed

Fig. 1. Influence of vasopressin-related peptides on glycogen phosphorylase activity and phosphatidylinositol metabolism in isolated hepatocytes

Glycogen phosphorylase activity and the incorporation of phosphate into phosphatidylinositol were measured as described in the text. The control values for these parameters were $26.0 \pm 4.0 \mu\text{mol/min per g dry wt.}$ and $27.6 \pm 2.0 \text{ ng-atom/20 min per g dry wt.}$ respectively. The results for each peptide are derived from three to four separate hepatocyte preparations, and each point represents the mean for duplicate determinations from one of these preparations. Maximally effective concentrations of [Arg⁸]vasopressin were included in each experiment. For each peptide, the concentration effective in producing half-maximal stimulation of the two processes is indicated. The seven vasopressin-like peptides were as follows: (a), [Arg⁸]vasopressin; (b), [Lys⁸]vasopressin; (c), [Arg⁸]vasotocin, (d), [1-deaminocysteine]vasopressin, (e), [1-deaminocysteine, 2-phenylalanine, 7-(3,4-didehydroproline), 8-arginine]-vasopressin; (f), oxytocin; (g), [D-Arg⁸]vasopressin. Symbols: ■, phosphorylase *a* activity; □, incorporation of P_i into phosphatidylinositol.

with respect to the stimulation of P_i incorporation into phosphatidylinositol (Fig. 1). However, of the three least-potent agonists, at least two (oxytocin and [1-deaminocysteine, 2-phenylalanine, 7-(3,4-didehydroproline), 8-arginine]vasopressin) induced a maximal stimulation of phosphatidylinositol metabolism that was considerably smaller than that provoked by the native hormone. Hence these analogues may be regarded as partial agonists with respect to the stimulation of phosphatidylinositol metabolism.

These differing effects of the four naturally occurring hormones suggest that the hepatic potency of these peptides is critically dependent on the nature of the amino acid in position 8. Those molecules with basic amino acids in this position all exerted half-maximal effects on phosphorylase activity and phosphatidylinositol labelling at about 10^{-10} and 10^{-9} M respectively. In contrast, oxytocin (similar to [Arg⁸]vasotocin, but with a hydrophobic leucine residue in position 8) was considerably less potent. This observation is in keeping with the results of a previous study that demonstrated that oxytocin is considerably less potent than vasopressin in stimulating glucose mobilization and activating phosphorylase glycogen in hepatocytes (Hems *et al.*, 1978b).

Some synthetic analogues of vasopressin were also tested. Removal of the amino group from residue 1 yields [1-deaminocysteine]vasopressin, which has 4 times the antidiuretic potency of [Arg⁸]vasopressin, but relatively unchanged vasopressor activity (Sawyer *et al.*, 1974). In contrast, [D-Arg⁸]vasopressin retains most of the antidiuretic potency of the native hormone, but exhibits only about 1% of its pressor activity (Sawyer *et al.*, 1974). The increased antidiuretic potency of [1-deaminocysteine]vasopressin may derive from its increased resistance to enzymic degradation in the kidney (Le Bourhis & Sawyer, 1974). However, the susceptibility of the various peptides to hepatic degradation would not be likely to influence the present results in view of the large medium/tissue ratio employed. Recently Smith & Walter (1978) have synthesized the peptide [1-deaminocysteine, 2-phenylalanine, 7-(3,4-didehydroproline), 8-arginine]vasopressin in an attempt to devise a molecule with characteristics ideal for interaction with the renal antidiuretic receptor coupled to adenylate cyclase. They report that this molecule has 250 times the antidiuretic potency of [Arg⁸]vasopressin, but no detectable pressor activity.

In the present study, [1-deaminocysteine]vasopressin was nearly as potent as [Arg⁸]vasopressin in stimulating both glycogen phosphorylase *a* activity and P_i incorporation into phosphatidylinositol. This observation is consistent with the results of Cash & Caplan (1964) who showed that the removal of the amino group at residue 1 of [Lys⁸]vasopressin

did not influence the glucose-mobilizing effect of the hormone *in vivo*. In contrast, the other two synthetic analogues ([D-Arg⁸]vasopressin and [1-deaminocysteine, 2-phenylalanine, 7-(3,4-didehydroproline), 8-arginine]vasopressin) exhibited markedly diminished hepatic potency compared with the native hormone. In fact, the relative potencies of the peptides tested in the present paper are very similar to those observed for pressor activity in intact rats (Altura, 1974; Sawyer *et al.*, 1974; Smith & Walter, 1978). Hence vasopressin receptors in the liver may have characteristics in common with those responsible for pressor activity *in vivo*, whereas the renal receptor coupled to adenylate cyclase has quite different characteristics.

The mechanism whereby vasopressin exerts these metabolic effects in the liver is unknown, but it has been suggested that the hormone activates hepatic phosphorylase activity by increasing cytosol Ca^{2+} concentrations (Keppens *et al.*, 1977; Blackmore *et al.*, 1978; Chen *et al.*, 1978). We have previously suggested that enhanced phosphatidylinositol turnover in the liver might be involved in the coupling of receptor-agonist interaction with Ca^{2+} mobilization and subsequent glycogenolysis (Kirk *et al.*, 1977). Such a mechanism has been proposed for a variety of agonists that cause Ca^{2+} -dependent effects in their target tissues (Michell *et al.*, 1977).

The parallelism between the dose-response curves for phosphorylase activation and for enhanced phosphatidylinositol metabolism caused by the four potent analogues suggests that the same receptor population is responsible for triggering both responses. The relationship between the peptide concentrations that produced a half-maximal stimulation of the two hepatic processes was fairly constant for these four analogues. Thus: (concentration of agonist that causes half-maximal stimulation of P_i incorporation into phosphatidylinositol)/(concentration of agonist that causes half-maximal stimulation of phosphorylase *a* activity) = 6.9–12.6. Further, the concentrations of the seven peptides that gave half-maximal phosphorylase activation were always within the range of concentrations over which phosphatidylinositol labelling began to increase appreciably.

The probability of very low concentrations of hormones interacting with specific cellular receptors is proportional to the number of such receptors carried by the cell. Many cell types exhibit maximal physiological responses in the presence of very low concentrations of agonist that are only sufficient to stimulate a small proportion of the receptor population (Levtzki, 1976). This observation implies that the stimulation of this small fraction of total cellular receptors generates an intracellular signal (perhaps cyclic AMP accumulation or Ca^{2+} mobilization) that is sufficient to induce the maximal

physiological response. The stimulation of further receptors may activate additional coupling elements and hence increase the amount of intracellular messenger produced, but it cannot increase the physiological response; thus such cells are said to have a 'receptor reserve'. The presence of such a 'receptor reserve' implies that biochemical processes involved in stimulus-response coupling in cells will exhibit decreasing sensitivity to a given concentration of hormone as they are more intimately coupled with the hormone-receptor interaction itself (Michell *et al.*, 1976). Thus the occurrence of maximally stimulated phosphorylase activity at ligand concentrations that cause only a small increase in phosphatidylinositol labelling, taken together with the relatively constant relationship between the concentration-dependencies of these two processes, is consistent with the suggestion that enhanced phosphatidylinositol turnover might be involved in stimulus-response coupling in vasopressin-treated hepatocytes.

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