Stimulation of specific GTPase activity by vasopressin in isolated membranes from cultured rat hepatocytes

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Membranes were isolated by isotonic homogenization and differential centrifugation from rat hepatocytes cultured overnight. The specific GTPase activity of the membranes was 1–1.3 pmol γ-labelled GTP hydrolysed/mg protein per min in the presence of 1.2 mM Na^+, 2 mM EGTA, 1 mM ATP and 0.2 mM 5-adenylyl imidodiphosphate. Under these conditions there was a stimulation of specific GTPase activity of no more than 20% by 11–115 nM vasopressin. No effect of vasopressin was seen in the presence of 1.7 μM free Ca^{2+} or 100 mM Na^+. The findings indicate that vasopressin is able to influence GTPase activity as well as accelerate phosphoinositide breakdown in rat hepatocytes.

GTPase *Guanine nucleotide binding protein* *Liver* *Phosphoinositide* *Vasopressin*

1. INTRODUCTION

The role of guanine nucleotides in the mode of action of hormones which do not affect adenylate cyclase is still unclear. However, recent findings show that guanine nucleotides are required for activation of phosphoinositide breakdown in a cell-free system from blowfly salivary glands by serotonin [1]. This suggested that there might be a function for a new guanine nucleotide binding protein. Hinkle and Phillips [2] found that thyrotropin-releasing hormone activated a specific GTPase activity in membranes from GH_4C_1 rat pituitary tumor cells. In many but not all systems, hormones which required the presence of guanine nucleotides for either stimulation or inhibition of adenylate cyclase also increase specific GTPase activity [3–5]. The guanine nucleotide binding protein involved in activation of adenylate cyclase is known as N_s or G_s and is ADP-ribosylated by cholera toxin [6]. The N_i or G_i protein thought to be involved in inhibition of adenylate cyclase is ADP-ribosylated by pertussis toxin [6]. In GH_4C_1 cells thyrotropin-releasing hormone has little effect on adenylate cyclase but activates phosphoinositide breakdown and elevates intracellular Ca^{2+} [7].

Vasopressin increases phosphoinositide breakdown as well as intracellular Ca^{2+} in rat hepatocytes but has little effect on cyclic AMP [8,9]. Guanine nucleotides will activate phosphoinositide breakdown in isolated membranes from rat hepatocytes [10] and several years ago it was demonstrated by Cantau et al. [11] that the binding of labelled vasopressin to rat hepatocytes was regulated by guanine nucleotides. The present studies were designed to investigate the possibility that vasopressin might affect the specific GTPase activity of rat hepatocyte membranes.

2. MATERIALS AND METHODS

Hepatocytes were prepared from male Wistar rats (200 g) as described [12,13]. The hepatocytes

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were attached to positively charged tissue-culture dishes (Primaria from Falcon) in L-15 medium containing 10% newborn calf serum and then incubated for 1–2 h at 37°C in humidified air. The medium was changed and the viable cells which remained were incubated for 7–8 h in L-15 medium. At the end of this period the L-15 medium was replaced with modified Leibovitz medium containing 0.2% fatty-acid-poor bovine albumin and the cells were incubated for 12–15 h [12,13]. The culture medium was removed and the cells washed with ice-cold homogenization buffer containing 0.25 M sucrose, 1 mM EDTA, 10 mM sodium azide, 10 mM sodium pyrophosphate and 10 mM Tricine at pH 7.5. The firmly attached cells from 4–6 culture dishes were removed by scraping using 5–7 ml buffer and 2.5-ml portions of cells plus medium were homogenized using a motor-driven homogenizer with Teflon pestle and glass homogeniser tube with 20 rapid strokes up and down. The homogenate was centrifuged at 5000 × g for 5 min at 3°C and the supernatant was then centrifuged at 26000 × g for 20 min to prepare the membrane preparation used for the studies. The membranes (10 μg of protein per assay tube) were incubated for 20 min in the presence of 65 nM GTP under Ca^{2+}-free conditions unless otherwise noted.

The assay for GTPase activity was based on the specific hydrolysis of [γ-^{32}P]GTP (65 nM) using a modification of the procedure of Koski and Klee [5] in the presence of 1×10^6 nM ATP and 0.2×10^6 nM 5-adenylyl imidodiphosphate (AMP-PNP) to correct for non-specific breakdown by nucleotidases that can utilize either GTP or ATP. These conditions are required in order to demonstrate specific GTPase stimulation by agents which affect N_i or N_s guanine nucleotide binding proteins. The final incubation buffer contained the following: 65 nM [γ-^{32}P]GTP, 200 μM Na-AMP-PNP, 1 mM Na-ATP, 1 mM ouabain, 1 mM dithiothreitol, 12.5 mM Tris with a pH of 7.5 (at 37°C) and 2 mM EGTA in a volume of 0.1 ml. The values for each experiment were based on the means of triplicate tubes for each point and the data in fig.1 and table 1 were based on 4 or more replications using separate cell preparations. Protein content of the membranes was measured by the dye-binding procedure of Bradford [14].

3. RESULTS AND DISCUSSION

The basal GTPase activity of hepatocyte membranes sedimented at 520000 × g·min was about 28 pmol phosphate released/mg protein per min in the absence of added Ca^{2+} and 240 in the presence of 1.7 μM free Ca^{2+} at 100 nM GTP. If the specific high-affinity GTPase activity was measured by including in the assay with the labelled GTP a large excess of ATP and AMP-PNP, then the basal activity at 65 nM GTP was around 1 pmol/mg per min in the absence of Ca^{2+} and increased to almost 3 pmol/mg per min in the presence of Ca^{2+} (fig.1).

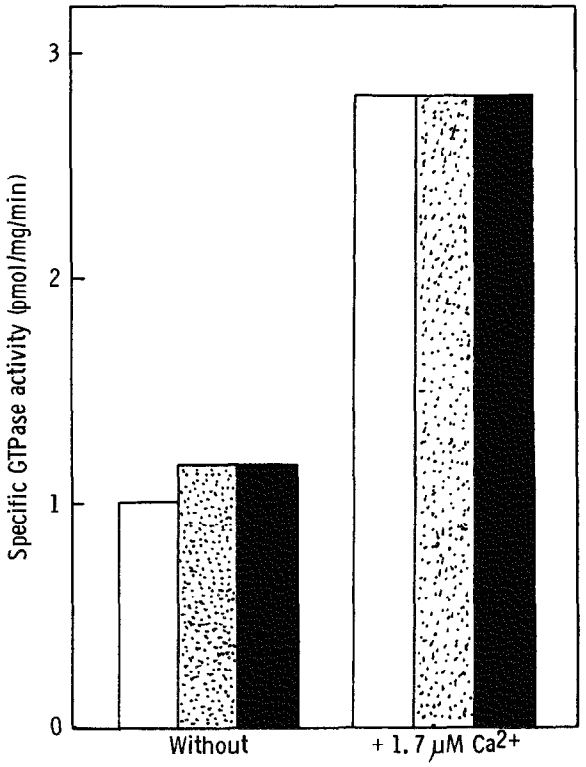

Fig.1. Stimulation by vasopressin of specific GTPase activity in membranes isolated from cultured rat hepatocytes. The crude membrane fraction obtained after centrifugation at 26000 × g for 20 min of a hepatocyte homogenate was incubated for 20 min. The values are the means of 4 paired replications incubated without (left) or with 1.7 μM free Ca^{2+} (right). This calculated free Ca^{2+} was obtained by adding 2 mM Ca^{2+} to the buffer containing 2 mM EGTA. Control values are shown by the open bars while those with 380 and 1150 nM vasopressin are shown, respectively, with stippled and solid bars.

This great reduction in activity is due to the fact that most nucleotidases are low affinity and will hydrolyse GTP or ATP. Their effects can be circumvented by including ATP plus AMP-PNP in the assay mixture [2–5]. Presumably the majority of the specific, high-affinity GTPase activity represents activity of guanine nucleotide binding proteins such as N_s, N_i and others.

The results in fig.1 indicate that in buffer free of added divalent cations, low concentrations of Na^+, 2 mM EGTA and no added Ca^{2+}, a stimulation of specific GTPase activity by vasopressin (380 or 1150 nM) can be seen. Presumably there is so much contribution of other GTPases in the presence of Ca^{2+} that no further effect of vasopressin can be seen in this condition (fig.1).

The activity of the specific GTPase was also examined at 10 min and the rate of GTP breakdown was 75% between 10 and 20 min of what it was during the first 10 min of incubation (not shown). The same stimulation of GTPase by vasopressin was seen during the first 10 min as during the second 10 min (13% during the first 10 min and 10% during the second 10 min as the effect of 11 nM vasopressin in one experiment).

The dose-response curve for activation of specific GTPase by vasopressin is shown in fig.2. The effect was significant at 11 nM vasopressin with near maximal effects seen at 115 nM vasopressin (fig.2).

The majority of the particulate protein in homogenates from cultured hepatocytes sedimented during the initial 25000 × g·min centrifugation. The basal GTPase activity of the membranes sedimented at 25000 × g·min was 0.43 pmol phosphate released from GTP/mg protein per min which is about one-third the specific activity of membranes sedimented at 520000 × g·min. In the same experiments 1.25 pmol/min per mg protein were released by the 520000 × g·min membranes. If 100 mM Na^+ was present the basal activity of the membranes doubled to 2.37 pmol/mg per min.

The stimulation by vasopressin of GTPase activity was no greater in the presence of 101.5 mM Na^+ as contrasted to that with 1.5 mM Na^+ (not shown). However, high concentrations of Na^+ (around 100 mM) are required for stimulation of specific GTPase activity by hormones which inhibit adenylate cyclase [4].

These data indicate that the direct addition of

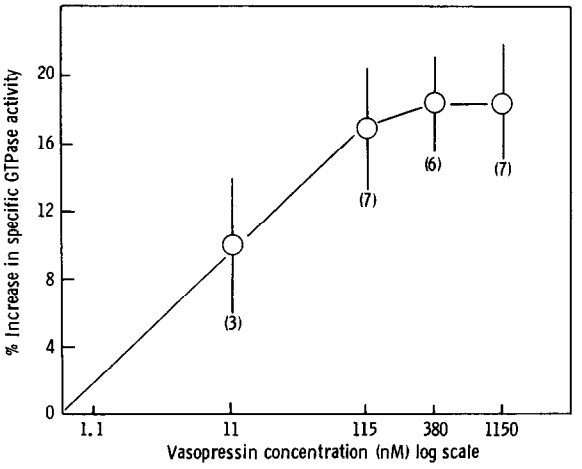

Fig.2. Dose-response relationship for vasopressin stimulation of specific GTPase activity in membranes from cultured rat hepatocytes. The membranes were incubated for 20 min and basal GTPase activity was around 1 pmol phosphate released/mg protein per min. The effects of the indicated concentrations of vasopressin are the mean ± SE of the % stimulation for the number of experiments indicated in brackets for each concentration of hormone.

vasopressin can activate, by about 20%, the specific GTPase activity of crude membranes prepared from cultured rat hepatocytes. Thyrotropin-releasing hormone has similarly been shown to stimulate GTPase activity of cultured GH_4C_1 cells [2]. Both hormones increase phosphoinositide breakdown with few other known effects in these cells and possibly hormonal activation of GTPase is linked to phosphoinositide breakdown.

While under certain conditions angiotensin II inhibited adenylate cyclase activity of rat liver membranes no effect of vasopressin was observed [15]. Crane et al. [16] did observe an inhibition of glucagon-stimulated cyclic AMP formation in isolated rat hepatocytes by 0.1 μM vasopressin but this may well be secondary to Ca^{2+} activation of cyclic AMP phosphodiesterase.

In chick heart an unusual inhibition of adenylate cyclase by muscarinic cholinergic activation has been noted which was relatively insensitive to pertussis toxin [17]. This toxin ADP-ribosylated the N_i protein and blocked the ability of guanine nucleotides to affect muscarinic cholinergic

agonist displacement of binding to membranes by a labelled antagonist [17]. Adenylate cyclase inhibition by GTP was reversed by pertussis toxin treatment in contrast to the lack of any effect on muscarinic cholinergic inhibition of adenylate cyclase. It would be of interest to examine pertussis toxin effects on vasopressin activation of specific GTPase as the effects of agents working through N_i protein are abolished by the toxin [18].

This report provides further evidence that guanine nucleotides are possibly involved in vasopressin stimulation of phosphoinositide breakdown in rat hepatocytes. This supports the findings of Cantau et al. [11] that guanine nucleotides affected vasopressin binding to rat liver membranes. Recently it has been found that guanine nucleotides increase polyphosphoinositide breakdown in rat liver membranes [10]. In blowfly salivary glands, Litosch et al. [1] found that guanine nucleotides were required in order to observe hormonal activation of phosphoinositide breakdown in isolated membranes. Cockcroft and Gomperts [19] have also found stimulatory effects of guanine nucleotides on phosphoinositide breakdown of membranes from mast cells. However, it remains to be established whether there is any relationship between vasopressin stimulation of GTPase activity of membranes from cultured rat hepatocytes and phosphoinositide breakdown in these cells.

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