Activation of membrane phospholipase C by vasopressin

A requirement for guanyl nucleotides

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Vasopressin stimulates the liberation of labelled inositol phosphate in partially purified plasma membranes prepared from *myo*-[^{3}H]inositol prelabelled WRK1 cells. This stimulatory effect was very rapid (165% stimulation of inositol trisphosphate accumulation after a 10 s incubation period in the presence of 1 μM vasopressin), concentration dependent ($EC_{50} = 12$ nM) and was abolished by an antagonist of the vasopressor response to vasopressin. GTP, even at high concentrations (0.1 mM), did not increase inositol phosphate release: it was found to be absolutely necessary for hormonal stimulation of phospholipase C activity. Non-hydrolysable analogues of GTP may also stimulate this enzyme activity.

Inositol trisphosphate *Vasopressin* *GTP-binding protein* *(WRK1 cell)* *Phospholipase C* *Guanyl nucleotide*

1. INTRODUCTION

Many hormones and neurotransmitters exert their biological actions through the mobilization of intracellular calcium (review see [1]). It is now well established that calcium mobilization is the consequence of receptor-mediated phospholipase C activity which specifically hydrolyses inositol lipids (review see [2]). Inositol trisphosphate, a product of PIP_2 breakdown, induces the release of calcium from intracellular stores [2,3]. The molecular mechanisms involved in the hormonal activation of phospholipase C have not yet been elucidated. Yet, there is evidence suggesting a possible role for a GTP binding protein in these activation processes [4–6].

In an earlier report [7], we showed that vasopressin induces inositol phospholipid breakdown in a rat mammary tumor cell line (WRK1). The results presented here in a partially purified preparation of WRK1 plasma membrane, demonstrate that vasopressin stimulates phospholipase C activity in a GTP-dependent manner.

Abbreviations: IP1, inositol monophosphate; IP2, inositol bisphosphate; IP3, inositol trisphosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; LVP, lysine vasopressin; GTPγS, guanosine 5′-*O*-thiotriphosphate; Gpp(NH)p, guanyl-5′-yl imidodiphosphate; des-Gly^9-d$(CH_2)_5$AVP, desglycine^9-[1-β-mercapto-β,β-cyclopentamethylene propionic acid]-arginine vasopressin

2. MATERIALS AND METHODS

2.1. *Products*

GTP, GppNHP and GTPγS were purchased from Boehringer, lysine vasopressin from Bachem, *myo*-[^{3}H]inositol (16.5 Ci/mol) from New England Nuclear, Eagle's minimum essential medium (MEM) and calf fetal serum from Gibco; Dowex, 1 × 10 (100–200 mesh, chloride form) from Fluka. Des-Gly^9-d$(CH_2)_5$AVP, a potent vasopressor antagonist [8], was a generous gift from Dr M. Manning (Toledo, USA).

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2.2. *Membrane preparation*

WRK1 cells were established by Monaco et al. [9] and grown in monolayer cultures 4 days in MEM medium containing 5% fetal calf serum, 2% rat serum, glutamine (292 mg/l), penicillin (100 units/ml), streptomycin (100 mg/ml) and *myo*-[^{3}H]inositol (1.5 μCi/ml). The culture medium was changed 2 days after seeding. 1 h before the experiment, cells were placed in a culture medium deprived of *myo*-[^{3}H]inositol, rat and calf serum. Cells were washed 3 times at 0°C with phosphate-buffered saline (PBS) without calcium and magnesium, scraped at 0°C with a rubber policeman into the following medium: 25 mM LiCl, 0.5 mM EDTA, 10 mM Tris-HCl (pH 7.4) and 0.1 mM ATP and homogenized at 4°C in a Dounce Potter homogenizer equipped with a loose piston (15-fold). After a rapid centrifugation (5 min, 100 × *g*), the pellet was discarded and the supernatant subjected to a second centrifugation (15 min, 30000 × *g*, 0°C). The final pellet was resuspended in the same buffer and used immediately.

2.3. *Electron microscopic controls*

WRK1 membrane pellet was fixed in a 5% glutaraldehyde solution buffered with 0.1 N cacodylate (pH 7.0), post-fixed in the same buffer with 1% osmium tetroxide, dehydrated in alcohol and embedded in Spurr resin. Ultrathin sections stained with uranyl acetate and lead citrate were examined in a Jeol 2000 EX electron microscope. As seen in fig.1, the preparation lacked intact or partially lysed cells. It was constituted essentially of plasma membranes with few cytoplasmic contaminants (Golgi elements, mitochondria and endoplasmic reticulum).

2.4. *Assay of phospholipase C activity*

Membranes prepared from prelabelled WRK1 cells (30–60 μg protein per assay) were incubated at 37°C in 300 μl in a medium similar to that described by Litosch et al. [5]. It was composed of 25 mM LiCl, 0.1 mM ATP, 0.25 mM EDTA, 5 mM Tris-HCl (pH 7.4), 50 mM Na-phosphate buffer (pH 7.0), 1 μM $CaCl_2$, 5 mM $MgCl_2$. When specified vasopressin and/or guanyl nucleotide were added. The reaction was stopped by the addition of 300 μl of 10% $HClO_4$, 100 μl of 20 mg/ml BSA and the cooling of the tube to 0°C. The pH

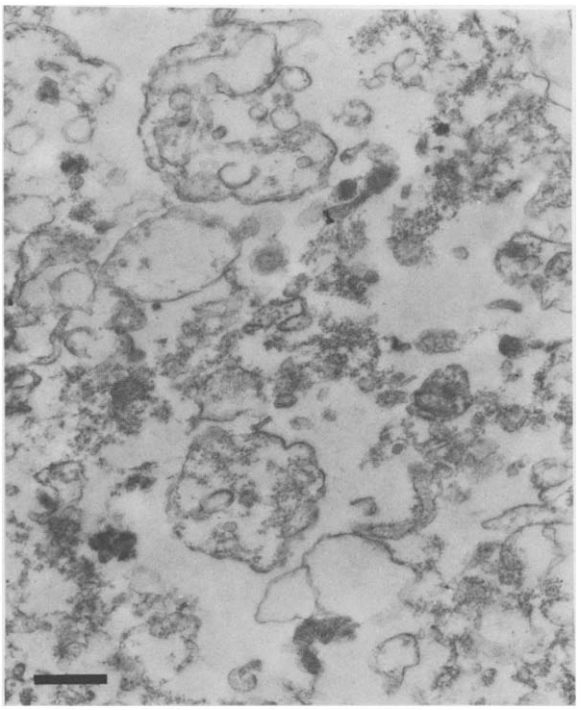

Fig.1. An electron micrograph of the WRK1 membrane prepration. Bar, 500 nm.

of the extract was adjusted to 7.0 by adding 0.1 M KOH. After a rapid centrifugation of the $HClO_4$ extracts, IP1, IP2 and IP3 present in the supernatant were separated by chromatography on Dowex, 1 × 10 (100–200 mesh, formate form) columns (4.0 × 0.6 cm) by sequential elutions as described by Berridge et al. [10] with minor modifications [11]. This protocol was established using purified labelled IP1, IP2 and IP3 obtained from Dr C. Kirk (Birmingham, England). Labelled inositol lipids were determined after chloroform/methanol/HCl (100:100:1) extraction and deacylation in the presence of NaOH as described earlier by Creba et al. [12]. Radioactivity found in the IP1, IP2 and IP3 fractions was measured by liquid scintillation spectrometry. All results were corrected for quenching and expressed in dpm. They represent the mean ± SE of 3 distinct determinations.

3. RESULTS AND DISCUSSION

Fig.2 shows the kinetic evolution of inositol lipids present in the WRK1 membrane preparation as well as inositol phosphate accumulation in the incubation medium. Under control conditions (in the absence of vasopressin) the membrane amount of PI and PIP remained stable. A slight but significant decrease in PIP2 was observed. The relative proportions of PI, PIP and PIP2 in membranes prepared from WRK1 cells are not markedly different from those found in intact cells (not shown), suggesting that no preferential breakdown of one of these 3 inositol lipids occurs during the course of membrane preparation. The reduction in PIP2 content was accompanied by a progressive and almost linear accumulation of IP2 and IP3. There was a fairly good correlation between the amount of PIP2 which disappeared during the 30 min incubation period and the amount of IP2 + IP3 which accumulated. All together these observations suggest the presence in this membrane

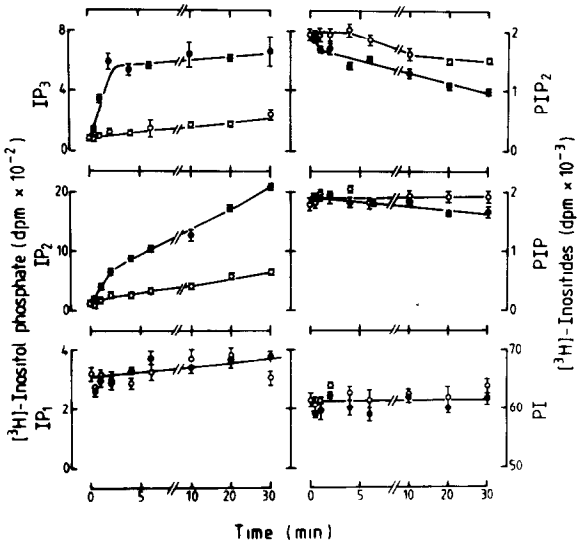

Fig.2. The time course of the accumulation of inositol phosphates and the breakdown of phosphoinositol lipids in WRK1 membranes. Membranes prepared from WRK1 cells were grown 4 days in the presence of *myo*-[^{3}H]inositol and were incubated in the presence of 0.1 mM GTP for various times at 37°C with (●) or without (○) 0.2 μM LVP (see section 2). Inositol phosphates (left panel) and phosphoinositol lipids (right panel) were determined as indicated in section 2.

preparation of a phospholipase C activity using PIP2 as a substrate. They are compatible with the hypothesis that IP2 originates from IP3 hydrolysis rather than from an enzyme attack on PIP. IP1 content increased at a very slow rate. The origin of IP1 found at zero time is not clear. It might have resulted from a slow inositol lipid breakdown during the process of membrane preparation.

In the presence of vasopressin, a marked but transient increase in IP3 accumulation and a more sustained increase in IP2 accumulation were observed (fig.2). The amounts of IP2 and IP3 which accumulated were about twice the observed reduction in PIP2 and PIP content, suggesting that PIP and PIP2 are produced by PI phosphorylation during the 30 min incubation period. The rapid fade of the vasopressin response may be a consequence of a rapid reduction in the amount of the phospholipase C specific substrate. Indeed, studies of Monaco et al. [13] on WRK1 cells indicate that only a fraction of the PI pool is sensitive to vasopressin-induced breakdown.

Nevertheless, the experiment illustrated in fig.2 clearly shows that vasopressin-induced inositol lipid breakdown can be demonstrated using acellular preparations derived from prelabelled WRK1 cells. Based on the results shown in fig.2, a 6 min incubation period was chosen for all further experiments.

As shown in table 1, the vasopressin-sensitive phospholipase C in WRK1 cell membranes is activated by guanyl nucleotides. The non-hydrolysable GTP analogues, Gpp(NH)p and GTPγS produced a marked stimulation of IP_2 and IP_3 formation, while GTP was inactive. The most salient result shown in table 1 is that LVP, when added in the absence of GTP, was almost inactive. In the presence of GTP (0.1 mM), vasopressin induced a 3–4-fold increase in IP2 and IP3 formation. The responses to vasopressin + GTP and GTPγS were of a similar magnitude.

The data presented in fig.3 show that in the presence of 0.1 mM GTP, the stimulation of phospholipase C by vasopressin was concentration dependent. The vasopressin-induced IP3 accumulation (426 ± 116% of basal values) was more pronounced than the increase in IP2 and IP1 accumulations (271 ± 50 and 116 ± 16%, respectively). Yet, the concentration of vasopressin leading to the half-maximum response (EC_{50}) was identical

Table 1

Effect of guanyl nucleotides, vasopressin and vasopressin antagonist on the phospholipase C activity present in WRK1 cell membranes

Additions		Inositol phosphate accumulation (dpm per assay)		
Hormone	Nucleotide	IP1	IP2	IP3
–	–	515 ± 31	285 ± 15	146 ± 16
–	0.1 mM GTP	542 ± 7	305 ± 10	170 ± 8
–	0.1 mM Gpp(NH)p	576 ± 50	506 ± 38	209 ± 20
–	0.1 mM GTPγS	603 ± 76	869 ± 48	517 ± 12
0.1 μM LVP	–	484 ± 19	383 ± 8	175 ± 13
0.1 μM LVP	0.1 mM GTP	494 ± 4	966 ± 56	636 ± 43
0.6 μM des-Gly^9-d(CH$_2$)$_5$AVP	0.1 mM GTP	472 ± 25	292 ± 21	175 ± 14
0.1 mM des-Gly^9-d(CH$_2$)$_5$AVP + 0.1 μM LVP	–	482 ± 53	248 ± 20	140 ± 17

myo-[^{3}H]Inositol prelabelled WRK1 membranes (50 μg per assay) were incubated for 6 min at 37°C in the presence of the indicated effectors. Inositol phosphates (IP1, IP2 and IP3) liberated in the medium were determined as indicated in section 2

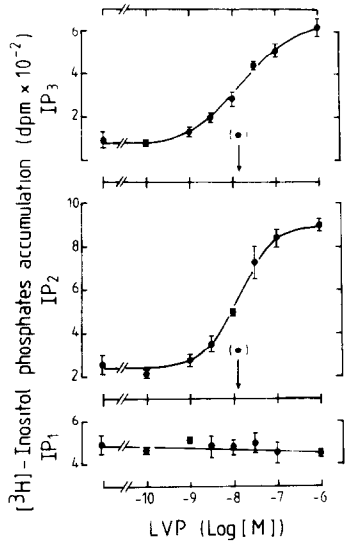

Fig.3. Dose-dependent activation of phospholipase C by vasopressin. *myo*-[^{3}H]Inositol prelabelled WRK1 membranes were incubated in the presence of 0.1 mM GTP for 6 min at 37°C with increasing amounts of vasopressin. Inositol phosphates liberated in the incubation medium were determined (see section 2).

regardless of the inositol phosphate considered. The mean EC_{50} values derived from 3 independent determinations were 13 ± 3 and 12 ± 4 nM for IP3 and IP2, respectively. These values are very close to the dissociation constant (K_d) determined for [^{3}H]lysine-vasopressin binding to WRK1 membranes derived from these cells ($K_d \simeq 10$ nM, Guillon et al., personal communication). Moreover, the vasopressin-induced IP2 and IP3 accumulation could be completely inhibited by the vasopressin antagonists des-Gly^9-d(CH$_2$)$_5$AVP (table 1). The observed vasopressin effect on phospholipase C thus appeared to be receptor mediated.

In conclusion, we demonstrate the presence of a guanyl nucleotide and hormone-sensitive phospholipase C in a plasma membrane fraction derived from WRK1 cells.

Our results confirm the previous studies of Wallace and Fain [14] in rat liver membranes showing a vasopressin-induced phosphatidylinositol breakdown. Moreover, they are in good agreement with those of Litosch and co-workers [5] who demonstrated, in a blowfly salivary gland membrane preparation, the presence of a GTP and serotonin-sensitive stimulation of inositol

phosphate production. Activation of phospholipase C by non-hydrolysable analogues of GTP was also described in other systems [6,15]. All together these data indicate that GTP binding protein(s) is (are) probably involved in mediating the effect of hormones that act via the breakdown of phosphoinositides. In line with this hypothesis, it is interesting to mention that vasopressin binding to WRK1 cells and liver membrane is sensitive to GTP ([16] and unpublished). The role and the nature of the putative GTP binding protein(s) involved in these mechanisms remain unclear. By analogy with hormonally regulated adenylate cyclase systems, this (these) protein(s) may exhibit a GTPase activity and may be responsible for the reversal of phospholipase C activation. Such a hypothesis would account for the marked activation induced by the non-hydrolysable analogues of GTP, Gpp(NH)p and GTPγS (see table 1).

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