HORMONE-STIMULATED GTPase ACTIVITY IN RAT PANCREATIC PLASMA MEMBRANES

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1. Introduction

Pancreatic plasma membranes have an adenylate cyclase system responsive to pancreozymin and secretin [1–3]. Both hormones activate a common catalytic subunit via distinct receptors [1,2,4] which are not permanently coupled with the catalytic subunit [5]. Guanyl nucleotides act synergistically with these hormones [6].

In numerous eukaryotic systems, Gpp(NH)p and GTPγS are better activators than GTP and allow a quasi-irreversible activation of adenylate cyclase following binding to regulatory guanyl nucleotide site(s) [6–9]; a finding which raises the question of the role of GTP hydrolysis in the adenylate cyclase system. The catecholamine-stimulated adenylate cyclase of turkey erythrocyte membranes has been shown to be associated with a catecholamine-stimulated GTPase (EC 3.6.1.-) [10,11]. This GTPase is inhibited when adenylate cyclase is activated by isoproterenol and GTPγS [12] or by cholera toxin [13,14]. Based upon these observations a model has been proposed [15,16] in which adenylate cyclase oscillates between the active and inactive state and is regulated by the hydrolysis of GTP. GTP binds to the inactive adenylate cyclase system, this complex is activated by the hormone and is subsequently deactivated by hydrolysis of GTP by the GTPase. After the detachment of GDP + P_i the system can again bind GTP.

Indirect evidence for the existence of a GTPase as a regulatory component of the adenylate cyclase system has also been presented for hepatocytes [17] and neuroblastoma cells [18]. The present work on rat pancreatic plasma membranes illustrates a dose-related activation of a specific GTPase by the pancreozymin family of peptides and by secretin. The possible relevance of this GTPase activity to the regulation of adenylate cyclase is discussed.

Abbreviations: Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; GTPγS, guanosine 5′-*O*(3-thiotriphosphate); App(NH)p, adenosine 5′-(β,γ-imido)triphosphate; cyclic GMP, guanosine 3′:5′-monophosphate; P_i, inorganic phosphate; NTPase, non-specific nucleoside triphosphatase; EGTA, ethylene glycol-bis-(2-aminoethylether) *N,N,N′,N′*-tetraacetic acid; OC-PZ, C-terminal octapeptide of pancreozymin; Boc, *t*-butyl-oxycarbonyl; VIP, vasoactive intestinal peptide; D_{50}, concentration required to exert half-maximal response (hormone binding, activation)

2. Materials and methods

2.1. *Peptides and reagents*

The peptides of the pancreozymin family were obtained from sources detailed in [19]. Synthetic secretin was a gift from Dr W. Koenig (Hoechst Pharma, Frankfurt). Trasylol was a gift from Bayer-Pharma (Brussels). Bacitracin, creatine phosphate, creatine phosphokinase, App(NH)p and ATP were purchased from Sigma (St Louis, MO) and carbamylcholine from Federa (Brussels). GTP was obtained from Boehringer (Mannheim). [γ-^{32}P]GTP (15–20 Ci/mmol) and [α-^{32}P]ATP (0.5–3.0 Ci/mmol) were purchased from the Radiochemical Centre (Amersham). All other reagents were of analytical grade.

2.2. *Rat pancreatic plasma membranes*

These were prepared as in [2] and stored in liquid nitrogen until use.

2.3. *GTPase assay*

The method in [11] was used with slight modification. Membrane proteins (5–10 μg) were incubated for 6 min at 37°C in 0.1 ml medium containing 50 mM imidazole–HCl (pH 6.7 or 7.4), 0.3 mg/ml bacitracin, 500 UIK/ml trasylol, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM ATP, 2 mM creatine phosphate, 3 U creatine phosphokinase, 2 mM β-mercaptoethanol, 0.5 mM App(NH)p, and 0.25 μM or 30 μM [γ-^{32}P]GTP (1–2 × 10^5 cpm/assay). The reaction was initiated by the addition of membranes and stopped by adding 0.1 ml of 2.5% sodium dodecylsulfate. The P_i liberated was isolated by chromatography on Celite-Norite [11] and the radioactivity was determined by liquid scintillation counting. For blank evaluation, the membranes were added after the incubation was stopped with sodium dodecylsulfate. All assays were performed in triplicate.

2.4. *Adenylate cyclase assay*

The hormone responsiveness of adenylate cyclase was tested as in [6] or in the medium detailed for GTPase assay (see above) except that 0.25 μM GTP and 1 mM cyclic AMP were added, and that [α-^{32}P]ATP (3 × 10^6 cpm/assay) was used in place of [γ-^{32}P]GTP. The membranes were incubated for 6 min at 37°C (pH 7.4) and radioactive cyclic AMP was separated and determined according to [20].

2.5. *Protein determination*

This was by the Lowry method of [21].

3. Results

Orientation experiments following the method in [11] allowed the detection of a specific GTPase in rat pancreatic plasma membranes. The hydrolysis of GTP at 0.25 μM (fig.1, curve A) was observed in the presence of 0.5 mM App(NH)p (to inhibit nonspecific NTPase(s)) and of an ATP-regenerating system (to suppress the transfer of γ-^{32}P from GTP to ADP).

An isotope dilution curve of GTP hydrolysis (fig.2) indicated the presence of two enzymatic activities: one with high affinity for GTP (app K_m 0.1–0.2 μM, data not shown); a second with a lower affinity (app K_m > 30 μM). Pancreatic plasma membrane preparations have a NTPase activity (app K_m 80 μM for

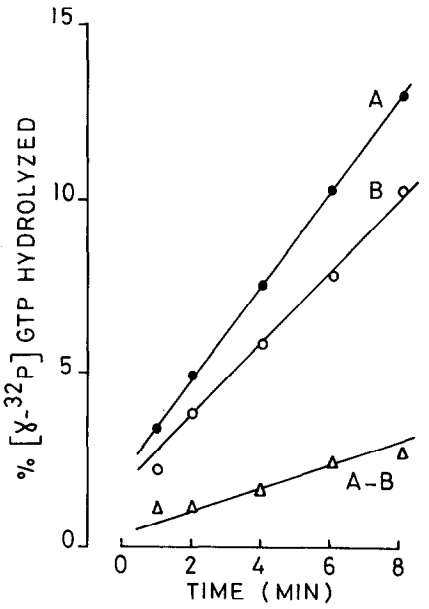

Fig.1. Time course of GTP hydrolysis at pH 6.7 in the presence of 0.25 μM GTP (curve A, •—•) or 30 μM GTP (curve B, ○—○); basal specific GTPase (△—△) was defined as A minus B. Results are representative of 4 experiments.

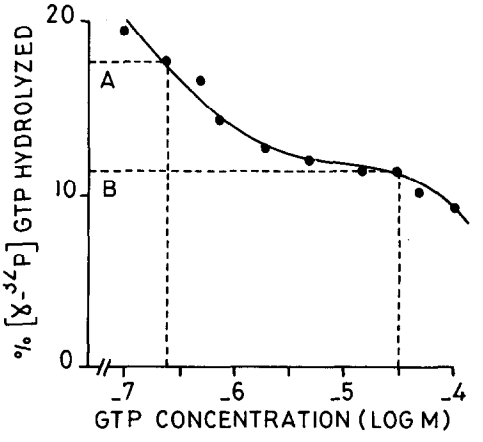

Fig.2. Isotope dilution curve of GTP hydrolysis. Unlabeled GTP was added to a fixed quantity of [γ-^{32}P]GTP to give the final concentration indicated in the abscissa. Pancreatic plasma membranes were incubated for 6 min at pH 6.7. The dotted lines indicate the % of GTP hydrolyzed in the presence of 0.25 μM GTP (A) and 30 μM GTP (B). Results are representative of 4 experiments.

Mg-GTP) which could account for the activity at 30 μM GTP [22]. The contribution of the residual NTPase activity to hydrolysis of GTP at 0.25 μM was estimated by subtracting the proportion of GTP hydrolyzed at 30 μM since the increase in GTP from 0.25–30 μM must cause a relatively proportional increase in NTPase activity, considering the app K_m value of this activity with Mg-GTP [22]. The mean activity of nonspecific NTPase was 110 pmol GTP/min.mg protein at pH 7.4 and 70 pmol GTP/min.mg protein at pH 6.7 whereas the basal specific GTPase was 25 pmol GTP/min.mg protein at both pH values. The basal specific GTPase represented 18–30% of the total activity measured with 0.25 μM GTP depending on the membrane batch utilized.

In routine assays, the reaction time was fixed at 6 min so that GTP hydrolysis was ⩽ 20% at either pH 7.4 or 6.7. Under these conditions, the reaction rate was proportional to the amount of membranes added in the 10–60 μg protein range. Addition of 1 μM OC-PZ caused a 2-fold increase in specific GTPase activity at pH 6.7 whereas nonspecific residual NTPase activity was unaffected (fig.3). Secretin did not, however, provoke a stimulation of specific GTPase activity at pH 6.7.

As adenylate cyclase activity is poorly stimulated by secretin at pH 6.7, hormone stimulations of GTPase

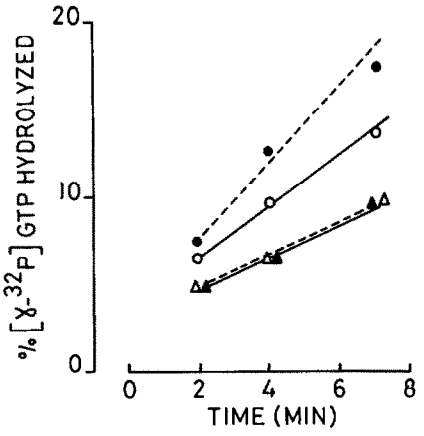

Fig.3. Effect of 1 μM OC-PZ on GTP hydrolysis. Pancreatic plasma membranes were incubated at pH 6.7 with 0.26 μM [γ-^{32}P]GTP in the presence (● and ▲) or absence (○ and △) of 1 μM OC-PZ. GTP was 0.25 μM (● and ○) or 30 μM (▲ and △). Mean of 2 experiments performed in triplicate.

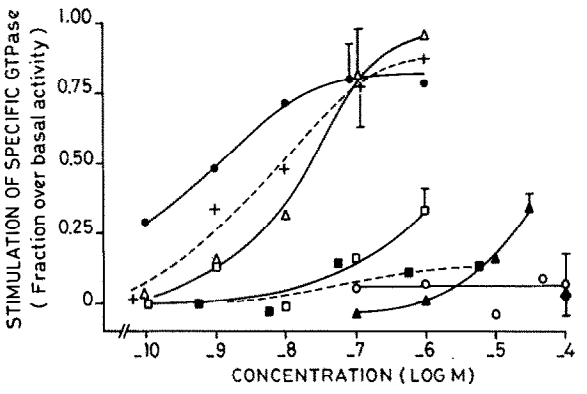

Fig.4. Dose–effect relationship of hormones and hormone analogs on the specific GTPase activity. Pancreatic plasma membranes were incubated for 6 min at pH 7.4 with 0.25 μM or 30 μM GTP. Each increment over specific basal value is the mean of 2–5 separate experiments (vertical bars indicate SEM values in the latter case). Legend (from left to right): (●—●) OC-PZ; (+ - - -+) Boc (4–10)-[Nle^8]-caerulein; (△—△) caerulein; (□—□) secretin; (■—■) VIP; (▲—▲) pentagastrin; (○—○) Tyr(SO_3H)-(7–10)-caerulein; (♦) carbamylcholine.

were reexamined at pH 7.4. pH 7.4 was indeed optimal for adenylate cyclase activation with GTP used in combination with either OC-PZ or secretin; adenylate cyclase activity measured at pH 7.4 in the medium used for GTPase assays, i.e., in the presence of 0.25 μM GTP, was activated 3.6-fold with 0.1 μM secretin and 5.8-fold with 0.1 μM OC-PZ (data not shown).

Dose–response curves for GTPase stimulation with hormones and hormone analogs at pH 7.4 are shown in fig.4. OC-PZ was the most potent activator: a stimulation occurred at 0.1 nM. Boc-(4–10)-(Nle^8)-caerulein and caerulein, two full agonists of OC-PZ vis-à-vis adenylate cyclase [19], produced the same maximal effect on GTPase activity but their potency was ~10-times lower. Pentagastrin, a weak and partial agonist of OC-PZ on pancreatic adenylate cyclase [19], produced a 30% increase in specific GTPase activity at the highest concentration tested (33 μM). Tyr (SO_3H)-(7–10)caerulein, which is even less efficient than pentagastrin on adenylate cyclase [19], did not produce any significant increase in GTPase activity at 0.1 mM.

Secretin was found to be a weak activator of GTPase: a 33% stimulation was observed at 1 μM.

VIP at $\leqslant 6$ μM did not significantly activate the specific GTPase. Carbamylcholine which does not activate pancreatic adenylate cyclase [23], was also without effect on GTPase activity even when used at 0.1 mM.

4. Discussion

The high activity of NTPase(s) has made it difficult to demonstrate a hormone-stimulated GTPase activity in pancreatic plasma membranes. Despite the presence of this high residual nonspecific activity, an 80% increase in specific GTPase activity could be observed with OC-PZ. Basal and specific GTPase activities, on a mg protein basis, were 5-fold higher in rat pancreatic plasma membranes than in turkey erythrocyte membranes [12] while maximal adenylate cyclase activity (800 pmol/min.mg protein) in the same pancreatic plasma membranes stimulated according to [6] with 1 μM OC-PZ plus 50 μM Gpp(NH)p was twice that reported for turkey erythrocyte membranes activated with isoproterenol and Gpp(NH)p [12].

Three indirect pieces of evidence suggest that the GTPase activity was coupled with adenylate cyclase activation and OC-PZ receptors.

(1) Dose–effect curves of adenylate cyclase activation and GTPase stimulation by OC-PZ and related peptides indicated similar relative affinities [19,23].
(2) The 5×10^{-10} M concentration of OC-PZ which activated half-maximally (D_{50}) the specific GTPase (fig.4) was lower than the D_{50} for adenylate cyclase activation in the presence of GTP and Gpp(NH)p (3×10^{-8} M and 1×10^{-8} M, respectively [6]).
(3) The app K_m for GTP of the specific GTPase, the D_{50} of GTP which activates adenylate cyclase in the presence of OC-PZ and the D_{50} of GTP and Gpp(NH)p inhibiting.[8-^{3}H]Gpp(NH)p binding to high affinity binding sites are similar, i.e., $1-2 \times 10^{-7}$ M (fig.2 and [6]).

With secretin, lesser stimulation of specific GTPase was observed than that with OC-PZ. The secretin activation of adenylate cyclase is, however, not maximal at low concentrations of GTP (see results and fig.3A in [6]): at 0.25 μM GTP, the concentration required for the assay of the specific GTPase, an optimal concentration of secretin produces only half the effect of OC-PZ.

Acknowledgements

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