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Stimulation by prostaglandin E_2 of a high-affinity GTPase in the secretory granules of normal rat and human pancreatic islets

Anjaneyulu KOWLURU* and Stewart A. METZ

Department of Medicine and Section of Endocrinology, University of Wisconsin School of Medicine, Madison, WI 53792, and William S. Middleton Memorial VA Hospital, 2500 Overlook Terrace, Madison, WI 53705, U.S.A.

Recent reports of a pertussis-toxin (Ptx)-sensitive inhibition of glucose-induced insulin release by prostaglandin E_2 (PGE_2) in transformed β-cells prompted us to look for the presence of prostaglandin-regulatable GTP-binding proteins (G-proteins) on the secretory granules of normal pancreatic islets. PGE_2 (but not $PGF_{2\alpha}$, PGA_2, PGB_2 or PGD_2) stimulated in a concentration-dependent manner a high-affinity GTPase activity in the secretory-granule-enriched fractions of both normal rat and human islets. Similar results were found after sucrose-density-gradient-centrifugation-based isolation of secretory granules to those after a differential-centrifugation procedure. Half-maximal stimulation occurred at 800 nM PGE_2, a concentration known to inhibit both phases of glucose-induced insulin secretion from pure β-cell lines. The GTPase stimulatory effect of PGE_2 was blocked virtually totally by Ptx pretreatment; it was not due to an effect on substrate binding since no measurable effect of PGE_2 on binding of guanosine 5′-[γ-[^{35}S]thio]triphosphate was observed in cognate fractions. Other Ptx-sensitive inhibitors of insulin secretion (such as adrenaline or clonidine) also stimulated GTPase activity, suggesting that one (or more) inhibitory exocytotic G-proteins (i.e. a putative G_{Ei}) is located on the secretory granules. These studies demonstrate, for the first time in an endocrine gland, the presence of a regulatable G-protein, strategically located on the secretory granules where it might regulate the exocytotic cascade distal to both plasma-membrane events and the generation of soluble mediators of insulin secretion.

INTRODUCTION

Involvement of guanine nucleotide binding proteins (G-proteins), especially the heterotrimeric G-proteins, in the process of insulin secretion was first proposed by Katada and Ui [1], who demonstrated the reversal by pertussis toxin (Ptx) of adrenaline inhibition of glucose-induced insulin secretion. Several other laboratories have confirmed these findings in intact pancreas [2] as well as in isolated islets [3] and insulin-secreting cell lines [4], using adrenaline, clonidine, galanin or somatostatin as the inhibitory agent. Recent investigations have clearly demonstrated that prostaglandin E_2 (PGE_2), a major endogenous metabolite of arachidonic acid (AA) in islets [5], also inhibits glucose-induced insulin secretion *in vivo* and *in vitro* [6]; furthermore, these effects of PGE_2 are largely reversible by Ptx, suggesting a possible involvement of G_i- or G_o-like proteins, presumably at the plasma membrane [7]. More recent studies, employing specific antisera, have identified Ptx-sensitive trimeric G-proteins in plasma membranes of islets and insulin-secreting RINm5F and HIT cells [8–10]. Interestingly, the reversal by Ptx of either adrenergic- or PGE_2-induced inhibition of glucose-induced insulin secretion was only partial, suggesting that more than one mechanism might underlie such an inhibition, i.e. one presumably at the plasma membrane and another presumably distal to the plasma membrane at a subcellular locus [7].

Recent studies from our laboratory have identified the presence of a high-affinity GTPase activity on the secretory granules of normal rat and human islets [11–13]. Additional studies provided evidence of its heterotrimeric nature, since this G-protein is ribosylated by Ptx, and its ribosylation decreased dramatically in the presence of guanosine 5′-[γ-thio]triphosphate (GTP[S]), which dissociates the α and $\beta\gamma$ subunits of heterotrimeric G-proteins. In addition, the high-affinity GTPase activity intrinsic to this protein was decreased by Ptx treatment [11–13]. The present study describes, for the first time, regulation (by both α_2-adrenergic agents as well as by PGE_2) of a high-affinity GTPase activity in the secretory-granule fraction of a peptide-secreting gland. Similar findings were obtained with both rodent and human islets. These data suggest the presence of regulatable Ptx-sensitive substrates at a step in the exocytotic cascade distal to plasma-membrane events; the presence of such protein(s) on the granule itself may represent (one of) the group of inhibitory G-proteins ('G_{Ei}') that have been postulated to play a regulatory role(s) in exocytosis [14,15].

EXPERIMENTAL

Materials

[γ-^{32}P]GTP (30 Ci/mmol), [γ-^{32}P]ATP (600 Ci/mmol), [^{14}C]-UDP-galactose (298 mCi/mmol) and GTP[γ-^{35}S] (1200 Ci/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.). ATP, GTP[S] and adenosine 5′-[$\beta\gamma$-imido]triphosphate (p[NH]ppA) were purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Prostaglandins were from Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.). Pertussis toxin was purchased from List Biologicals (Campbell, CA, U.S.A.). Mastoparan and its inactive analogue mastoparan-17 were obtained from Peninsula Laboratories (Belmont, CA, U.S.A.). All other chemicals were of the highest available reagent grade and were purchased from Sigma (St. Louis, MO, U.S.A.).

Abbreviations used: p[NH]ppA, adenosine 5′-[$\beta\gamma$-imido]triphosphate; AA, arachidonic acid; GTP[S], guanosine 5′-[γ-thio]triphosphate; PG, prostaglandin; Ptx, pertussis toxin; INT, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-tetrazolium chloride.

* To whom correspondence should be directed, at: Division of Endocrinology, H4/568 Clinical Sciences Center, 600 Highland Avenue, Madison, WI 53792, U.S.A.

Pancreatic-islet isolation and subcellular fractionation

Pancreatic islets were obtained from male Sprague–Dawley rats (300–400 g body wt.) by collagenase digestion followed by centrifugation on discontinuous Ficoll gradients as described previously [16]. Islets were manually picked twice under stereoscopic control to avoid any contamination by exocrine acinar tissue. Human islets were generously provided by Dr. David Scharp (Washington University Medical School, St. Louis, MO, U.S.A.) and were also picked again under stereomicroscopic control.

Subcellular fractions of rat islets were isolated as described previously [17,18], by using an Optima TL-100 ultracentrifuge (Beckman). All the procedures described below were carried out at 4 °C, unless stated otherwise. Briefly, 1000–1500 islets were washed once with 230 mM mannitol/70 mM sucrose/5 mM Hepes buffer, pH 7.4, containing 1 mM EGTA, and twice more with the same buffer containing no EGTA, and were homogenized manually in 1 ml of the same buffer (containing 1 mM dithiothreitol and 2.5 μg each of leupeptin and pepstatin/ml) in a 2 ml glass homogenizer. The homogenate was spun at 600 ***g*** for 5 min to remove the nuclear and cell-debris pellet. The resulting supernatant fraction was centrifuged at 5500 ***g*** for 10 min to yield a pellet enriched in mitrochondria. The resulting supernatant fraction was centrifuged at 25000 ***g*** for 20 min to yield a pellet enriched in secretory granules. Each of these pellets was washed twice with the homogenization medium and resuspended in a suitable volume of the buffer.

In some experiments, the secretory-granule fraction obtained after the differential-centrifugation procedure was reconstituted in 750 μl of 0.3 M sucrose/5 mM sodium phosphate buffer, pH 6.8, and subjected to a further density-gradient-centrifugation procedure as describe by Noe et al. [19]. In brief, the secretory-granule suspension was layered over 800 μl gradients of 1.5 M and 2.0 M sucrose in polyallomer (11 mm × 34 mm) tubes and centrifuged at 105000 ***g*** for 2 h in a TL-55 rotor. After the centrifugation, interfaces between the 0.3 M and 1.5 M sucrose layers, and between the 1.5 M and 2.0 M sucrose layers, as well as the pellets at the bottom of the tubes, were carefully removed by aspiration for the determination of insulin content [16] as well as high-affinity GTPase activity.

Purity of subcellular fractions

Previous work by others [20] and ourselves [17,18] has quantified the degree of purity of rat islet subcellular fractions, as judged by marker-enzyme assays. In the present studies, in addition to evaluating the possible contamination of the secretory-granule fraction by the nuclear, mitochondrial, microsomal and cytosolic fractions [17,18], we also assessed the possible contamination of the secretory-granule fraction with plasma membrane (ouabain-sensitive Na^+/K^+-ATPase as a marker), lysosomes (acid phosphatase as a marker), Golgi complex (UDP-galactosyltransferase and thiamin pyrophosphatase as markers), mitochondria (succinate–INT reductase as a marker), secretory granules (insulin as a marker) and cytosol (lactate dehydrogenase as a marker). The enzyme activities were quantified in homogenates as well as in the secretory-granule fraction in order to assess the degree of enrichment of the latter.

Enzyme assays

Ouabain-sensitive Na^+/K^+-ATPase was measured by monitoring the rates of hydrolysis of [γ-^{32}P]ATP in the absence or presence of ouabain (500 μM) as described in [21], and the activity was expressed as pmol of [γ-^{32}P]ATP hydrolysed/min per mg of protein. Lysosomal acid phosphatase was assayed colorimetrically (at 420 nm) with *p*-nitrophenyl phosphate as substrate as described in [22]. UDP-galactosyltransferase was measured by a method described by Fleischer [23], with [^{14}C]UDP-galactose and *N*-acetylgalactosamine. This activity was expressed as nmol of galactose transferred to *N*-acetylgalactosamine/min per mg of protein at pH 6.5. Lactate dehydrogenase was assayed spectrophotometrically (at 340 nm) by measuring the formation of NADH in the presence of lactate and NAD^+ by using a kit purchased from Sigma (procedure no. 228-UV). Succinate–INT reductase was assayed spectrophometrically (at 490 nm) with *p*-iodonitrotetrazolium, and the activity was calculated by using an absorption coefficient of 20.1×10^3 litre·mol^{-1}·cm^{-1} at 490 nm, as described in [24]. Thiamin pyrophosphatase was assayed by monitoring the hydrolysis of thiamin pyrophosphate at pH 8.0 as described by Morre [24], and insulin was assayed by a radioimmunoassay as described previously [16].

The relative specific activity of each of the marker proteins was expressed as the ratio of its initial specific activity in the homogenate to the final specific activity in the secretory-granule fraction. We observed the following relative specific activities of each marker in the secretory-granule fraction obtained by differential centrifugation: total ATPase, 0.09; acid phosphatase, 0.39; succinate–INT reductase, 0.39; UDP-galactosyltransferase, 1.50; lactate dehydrogenase, 0.26; insulin, 4.18. We failed to detect any Na^+/K^+-ATPase activity in the secretory-granule fraction. Although UDP-galactosyl transferase was slightly enriched in the secretory-granule fraction, a second Golgi marker, thiamin pyrophosphatase, was not detectable there, although significant activity was found in homogenate. As described above, the secretory-granule fraction obtained by differential centrifugation was further purified by sucrose-density-gradient centrifugation. Owing to limitations of protein, we measured only the relative specific activities of insulin in these fractions; the data are provided in the Results section.

Evaluation of the purity of the secretory-granule fraction by electron microscopy

The pellets of the secretory-granule fractions obtained by differential centrifugation as well as by sucrose-density-gradient centrifugation were washed twice with 0.3 M sucrose/5 mM sodium phosphate, pH 6.8, and fixed in 2.5 % glutaraldehyde in 100 mM Sorensen's phosphate buffer, pH 7.4, at 22 °C for 2 h, and was washed (four times) with the above buffer at 4 °C for a total of 60 min. This was followed by a post-fixation step in 2 % OsO_4 in phosphate buffer at 25 °C for 2 h. The material was then dehydrated in a graded series of alcohol, and was subjected to three washes with propylene oxide, and embedded in epoxy resin for 48 h at 65 °C. Thin sections were cut, stained with uranyl acetate and lead citrate and viewed under a Philips 310 electron microscope.

Measurement of GTP binding and GTPase activities

GTPase activity was assayed by a modification of a method originally described by Cassel and Selinger [25]. We initially observed high activities of non-specific nucleoside triphosphatases in islet homogenates [11–13]. In order to saturate these activities, millimolar concentrations of ATP and p[NH]ppA, a non-hydrolysable analogue of ATP, were included along with an ATP-regenerating system comprising phosphocreatine and creatine kinase. In brief, the GTPase assay system, in a total volume of 100 μl, consisted of 110 mM NaCl, 20 mM Tris/HCl,

pH 7.4, 2 mM $MgCl_2$, 120 μM EGTA, 1 mM dithiothreitol, 1 mM ATP, 1 mM p[NH]ppA, 0.4 mg of BSA, 5 mM phosphocreatine, 50 units/ml creatine kinase (type 1; rabbit muscle) and 5–10 μg of secretory-granule protein. The reaction was started by adding 14.3 nM [γ-^{32}P]GTP (250000 c.p.m./pmol) and was carried out at 37 °C for 30 min, since this activity was found to be linear up to 30 min in the secretory-granule fraction. It was terminated by adding 300 μl of an ice-cold solution of 6% charcoal in 10% trichloroacetic acid and 5 mM NaH_2PO_4. The tubes were centrifuged at 2500 *g* for 10 min, and 100 μl of the supernatant was counted for radioactivity. Specific activity was expressed as pmol of [^{32}P]P_i released/min per mg of protein. In some experiments, results were confirmed by separating the [^{32}P]P_i (when [γ-^{32}P]GTP was used as substrate) or [^{32}P]GDP (when [α-^{32}P]GTP was used as substrate) on polyethyleneimine (PEI)-cellulose thin-layer plates (E. M. Separations, Gibbstown, NJ, U.S.A.) by using 0.75 M KH_2PO_4, pH 3.4, followed by autoradiography and/or scintillation spectrometry.

Ptx-mediated ADP-ribosylation

ADP-ribosylation was carried out as previously described [26]. Ptx was first activated with 30 mM dithiothreitol, to release its A-protomer, the peptide responsible for its ribosyltransferase activity. The ribosylation mixture (in a total volume of 100 μl) consisted of 50 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM ATP, 0.1 mM GTP, 20 mM thymidine, 10 μM NAD^+ and islet secretory-granule protein (30–50 μg of protein), and incubated at 37 °C for 60 min. As a control, secretory-granule proteins were incubated in the absence of Ptx, but in the presence of appropriate diluents, under the above conditions. GTPase activity was measured in these preparations in the absence and presence of PGE_2 as described above.

GTP[γ-^{35}S] binding assay

GTP[γ-^{35}S] binding was measured by the rapid filtration assay described recently [26]. Briefly, the reaction mixture (100 μl) consisted of 50 mM Tris/HCl, pH 7.4, 10 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl and 5–10 μg of secretory-granule protein. The reaction was started by adding 1 μM GTP[γ-^{35}S] (10000 c.p.m./pmol), and was carried out at 37 °C for 30 min. Then 80 μl of the reaction mixture was applied on to a Millipore filter (0.45 μm pore; HA filter; Millipore, Boston, MA, U.S.A.) and washed with 4 × 4 ml of wash buffer, consisting of 10 mM Tris/HCl, pH 7.4, 1 mM $MgCl_2$ and 1 mM EGTA. The radioactivity bound to the filter was determined by scintillation spectrometry. Non-specific binding was determined under similar conditions in the presence of 100 μM unlabelled GTP[S] and was subtracted from each value to calculate specific binding, which was expressed as pmol of GTP[S] specifically bound/mg of protein.

Protein assay

Protein concentration was determined by a dye-binding assay described by Bradford [27], with BSA as standard.

RESULTS

Purity of the rat islet secretory-granule fraction isolated by the differential-centrifugation method

In earlier publications [17,18] we described the purity of the secretory-granule fraction obtained by the differential-centrifugation method, using individual marker enzymes for nuclear, mitochondrial, cytosolic and microsomal fractions. In addition to these markers, in the present studies we also analysed quantitatively the secretory-granule fraction for contaminating plasma membrane, lysosomes and Golgi complex (see the Experimental section). The total ATPase activity in the secretory-granule fraction represented only 9% of the specific activity measured in homogenates, and is presumably attributed to the presence of Ca^{2+}-ATPase [28] and H^+-ATPase activity [29] on islet secretory granules. Specific ouabain-sensitive Na^+/K^+-ATPase activity was unmeasurable in the granule fraction, suggesting that this fraction is devoid of detectable plasma-membrane contamination. The specific activity of acid phosphatase activity (enriched in lysosomal fraction) was at least 2.5 times higher in the homogenates than in the secretory-granule fraction, demonstrating that it is not enriched in the secretory-granule fraction. The detectable levels of this activity may be attributable to some acid phosphatase activity known to be associated with the secretory-granule fraction [30]. Detectable activity of succinate–INT reductase activity in the secretory-granule fraction (although not enriched) may be due to modest (< 10%) contamination of heavy mitochondria with the secretory-granule fraction, as indicated by the electron-microscopic data (see below). The data of relative specific activities of Golgi markers also indicate no enrichment of the Golgi complex and/or fragments in the secretory-granule fraction.

In addition, the purity of the secretory-granule fraction was evaluated by electron microscopy (see the Experimental section). A sectional view of the secretory-granule pellet (obtained by the differential-centrifiguation method) was found to be highly enriched in β-cell secretory granules, most of them having an intact limiting membrane. No visual evidence of plasmalemma, Golgi or lysosomes was seen contaminating this preparation. However, this fraction was slightly contaminated with heterogeneous structures, denser than secretory vesicles; these appeared to be heavy mitochondria trapped between secretory vesicles. Howell et al. [31] have also reported a modest contamination with mitochondria of the secretory vesicles isolated by the differential-centrifugation method. Visual analyses of three separate sections revealed that this preparation contained > 90% β-cell secretory granules and < 10% contaminating mitochondria (additional results not shown). These results, in combination with marker-enzyme analyses, demonstrate a high

Table 1 Stimulation of high-affinity GTPase activity in normal rat islet secretory granules by PGE_2, α_2-adrenergic agents and mastoparan

High-affinity GTPase activity was measured in the secretory-granule fraction obtained by differential centrifugation as described in the text. This activity in the control secretory-granule preparations (expressed as 100%) represented 4 pmol/min per mg of protein. PGE_2 effects were also studied in the Ptx-treated secretory-granule fraction (see the Experimental section). Data are means ± S.E.M. of the numbers of determinations given in parentheses. Experimental values were significantly different ($P < 0.001$) from controls (values in the absence of agonists), whereas PGE_2 no longer had a significant effect in the Ptx-pretreated secretory-granule fraction.

Condition(s)	High-affinity GTPase activity (% of control)
Control	100 ± 3 (9)
PGE_2 (10 μM)	175 ± 7 (9)
PGE_2 (10 μM) (Ptx-pretreated secretory granules)	110 ± 7 (6)
Clonidine (10 μM)	140 ± 13 (6)
Adrenaline + propranolol (10 μM)	150 ± 10 (6)
Mastoparan (50 μM)	200 ± 25 (6)

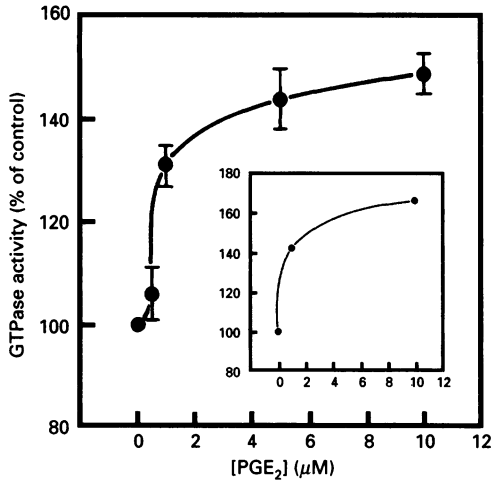

Figure 1 Concentration-dependent stimulation of secretory-granule high-affinity GTPase activity by PGE_2

Effects of PGE_2 (0–10 μM) on high-affinity GTPase activity in normal rat (main panel) and human (inset) islet secretory granules were studied. Data are means ± S.E.M. of 3–4 determinations at each concentration.

Table 2 Specificity of prostaglandin effects on high-affinity GTPase activity in rat islet secretory granules

Effects of prostaglandins (1 μM) on high-affinity GTPase activity in the secretory-granule fraction obtained by differential centrifugation of normal rat islets were determined. Data are means ± S.E.M. of the numbers of individual determinations given in parentheses: *only PGE_2 significantly ($P = 0.004$) activated GTPase activity compared with control.

Condition(s)	High-affinity GTPase activity (% of control)
Control	100 ± 4 (4)
PGA_2	110 ± 5 (4)
PGB_2	96 ± 3 (4)
PGD	91 ± 2 (4)
PGE_2	120 ± 5 (10)*
PGF_2	93 ± 5 (4)

degree of purity and enrichment of the β-cell secretory-granule fraction isolated by this procedure. Nonetheless, critical results were verified after a further purification by sucrose-density-gradient centrifugation (see below).

Effects of prostaglandins on GTPase activity in the secretory granules isolated by differential centrifugation

We have recently reported the presence of a Ptx-sensitive high-affinity GTPase activity, with an apparent K_m for GTP of 370 ± 52 nM ($n = 5$ determinations) in the crude membrane and secretory-granule fraction of normal rat islets and in homogenates of pure insulin-secreting β-cells (HIT cells) ([11–13]; A. Kowluru and S. A. Metz, unpublished work). Data in Table 1 show that well-studied receptor-mediated inhibitors of glucose-induced insulin secretion, such as α_2-adrenergic-receptor agonists (clonidine and adrenaline) or PGE_2, stimulated this high-affinity GTPase activity in the secretory-granule fraction by 40–75% above control values. This magnitude of stimulation by PGE_2

Table 3 Relative distribution of insulin in islet subcellular fractions

Subcellular fractions were isolated from normal rat islets as described in the Experimental section. Insulin content was measured in each fraction by radioimmunossay [16]. Relative specific activity is defined as the ratio of final to initial specific activity (homogenates) of insulin in each fraction. Data are means ± S.E.M. of four individual preparations.

Fraction(s)	Insulin (m-units/mg of protein)	Relative sp. act.
Differential centrifugation		
(i) Homogenate	1544 ± 127 (4)	1
(ii) Nuclear and cell debris	1918 ± 121 (4)	1.24
(iii) Mitochondria	4116 ± 408 (4)	2.66
(iv) Secretory granules	6460 ± 510 (4)	4.18
(v) Post-secretory-granule supernatant	178 ± 32 (4)	0.12
Sucrose gradients of secretory granules (fraction (iv) from above)		
(i) 0.3/1.5 M interface	1255 ± 487 (2)*	0.81
(ii) 1.5/2.0 M interface	10338 ± 1401 (4)	6.69
(iii) 2.0 M pellet	Undetectable	—

* We failed to detect any insulin in this fraction in two other preparations.

(Table 1) was comparable with the stimulatory effects observed in the presence of mastoparan, an amphiphilic peptide which has been shown to stimulate directly the functions of G-proteins (GDP/GTP exchange and GTPase activity [32,33]). Mastoparan-17, an inactive analogue of mastoparan [32], failed to stimulate GTPase activity in this fraction (additional results not shown). Prior treatment of the secretory-granule fraction with Ptx (see the Experimental section) completely abolished the stimulatory effects of PGE_2, suggesting that the latter stimulated a GTPase activity largely intrinsic to one (or more) Ptx-sensitive G-protein(s).

Data in Figure 1 demonstrate that the stimulation by PGE_2 of the secretory-granule high-affinity GTPase activity of normal rat (Figure 1) and human (Figure 1, inset) islets is concentration-dependent and saturable. A similar degree of stimulation was observed in both rat and human islets. Half-maximal stimulation was observed at 800 nM PGE_2 (Figure 1).

Additional studies were carried out to evaluate the structural specificity for PG stimulation of this GTPase activity in the rat islet secretory-granule fraction (Table 2). In these experiments, PGs were tested at 1 μM, a concentration which has been shown to inhibit both phases of glucose-induced insulin secretion from HIT cells [7]. Of the five PGs studied, only PGE_2 stimulated the GTPase activity significantly ($P = 0.004$), whereas others were essentially inactive, suggesting a marked structural selectivity for PGE_2 stimulation of high-affinity GTPase activity.

PGE_2 effects on GTPase activity in the secretory granules isolated by sucrose-density-gradient centrifugation

All of the above studies were carried out on the secretory-granule fraction isolated by the differential-centrifugation method. As indicated in the Experimental section, electron-microscopic examination of this fraction indicated a modest (< 10%) contamination by heavy-mitochondrial fraction. Therefore we further purified the secretory-granule fraction, using a sucrose-density-gradient-centrifugation method (see the Experimental section). In order to determine the insulin enrichment of these fractions, relative specific activity of insulin was measured in each fraction (Table 3). Sucrose-density-gradient centrifugation yielded a significantly greater degree of enrichment (nearly

Table 4 Effect of PGE_2 on the high-affinity GTPase activity in islet mitochondrial and purified secretory-granule fractions isolated by differential and density-gradient centrifugation

High-affinity GTPase activity was measured in islet fractions as described in the text in the absence (control) or presence of PGE_2 (1 μM). Fractions 1 and 2 represent interfaces between 0.3/1.5 M and 1.5/2.0 M sucrose, respectively, whereas fraction 3 represents the pellet from 2.0 M sucrose (see the Experimental section). *$P = 0.004$ versus control.

Fraction(s)	GTPase activity (% of control)
(A) Fractions obtained by differential centrifugation	
(i) Mitochondria	100 ± 4 (12)
(ii) Secretory granules	120 ± 5 (10)*
(B) Secretory granules from (ii) (above), after density-gradient centrifugation	
Fraction 1	103 ± 11 (5)
Fraction 2	125 ± 3 (13)*
Fraction 3	Not detectable

6.7-fold) compared with that obtained by the differential-centrifugation method (4.2-fold). Moreover, the low specific activity of insulin in the post-secretory-granule supernatant (comprising mainly cytosol and microsomes) suggests that the homogenization and/or centrifugation procedures caused very little disruption of the secretory-granule fraction (Table 3); loss of integrity of the limiting membrane would be expected to allow much of the insulin contained therein to become soluble and remain in the cytosol. These data are compatible with the electron-microscopic data (see above) that the limiting membrane of the β-cell granules was largely intact. These fractions were used in the following studies to demonstrate the association of the PGE_2-regulatable high-affinity GTPase activity specifically with the secretory granules.

The effects of PGE_2 on GTPase activity in individual fractions were then studied (Table 4). PGE_2 (1 μM) specifically stimulated the GTPase activity in fraction 2 of the sucrose gradients, which also contained the highest relative specific activity of insulin. The degree of stimulation was comparable with that seen for the secretory-granule fraction obtained by the differential-centrifugation method from the same batch of islets (Table 4). Additional studies using the mitochondrial fraction obtained by differential centrifugation (see the Experimental section) demonstrated that the GTPase specific activity was several-fold higher in the secretory-granule fraction (4 pmol/min per mg) than in the mitochondrial fraction (1.7 pmol/min per mg). Moreover, PGE_2 (1 μM) failed to stimulate the (low) GTPase activity intrinsic to the mitochondrial fraction, additionally suggesting that the stimulatory effects of PGE_2 on GTPase activity are specific to the secretory-granule fraction, and cannot be explained by the small contamination with heavy mitochondria.

Structural specificity of PGE_2 effects

To examine further the structural specificity of these effects of PGE_2 effects, the effects of AA and its major metabolites on GTPase activity were studied. We recently reported a marked concentration-dependent inhibition of this GTPase by unmetabolized AA in the membrane and cytosolic fractions of normal rat and human islets as well as in HIT cells [11–13]; A. Kowluru and S. A. Metz, unpublished work). In the present study, we examined the dose-dependent effects of AA on the high-affinity GTPase activity in the secretory-granule fraction (Figure 2). For this purpose, we utilized t.l.c. to confirm the identification of reaction products, namely either [^{32}P]P_i (when [γ-^{32}P]GTP was used as the substrate) or [^{32}P]GDP (when [α-^{32}P]GTP was used as the substrate). Data in Figure 2 demonstrate virtually identical rates of hydrolysis of either form of labelled GTP at all the protein concentrations (up to 5 μg) of the secretory-granule fraction. The effects of AA (0–330 μM) on this activity were then evaluated with [γ-^{32}P]GTP as substrate. Significant inhibition of the secretory-granule GTPase activity by AA was demonstrable starting at AA concentrations $\leqslant 82.5$ μM (Figure 2b). These data demonstrate parallelism between the effects of these lipids and their effective concentrations on insulin secretion; AA, which is a stimulator of insulin secretion at these concentrations [5], inhibited the GTPase, whereas PGE_2, an inhibitor of insulin secretion at the concentrations used [7], stimulated this enzyme. These data are thus compatible with regulatory role(s) for this G-protein in insulin secretion. 12-Hydroxyeicosatetraenoic acid, a

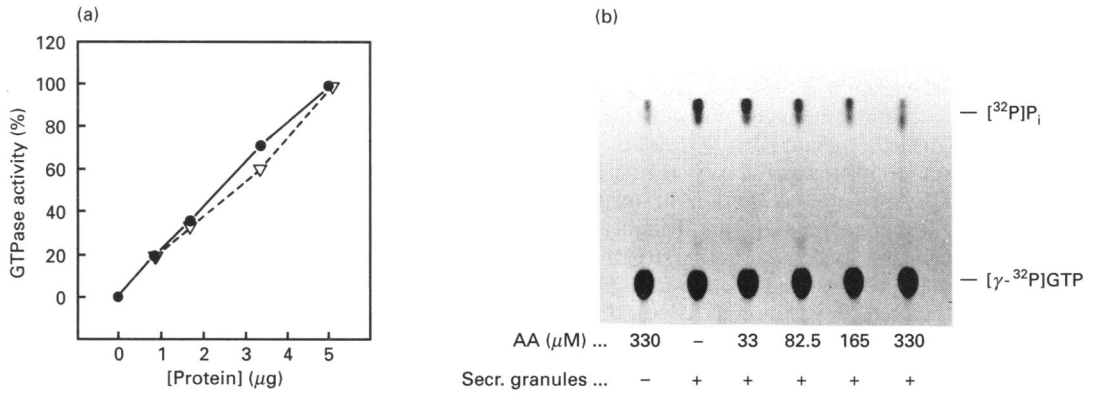

Figure 2 Concentration-dependent inhibition of GTPase activity in the secretory-granule fraction by AA

GTPase activity was assayed in the secretory-granule fraction (0–5 μg of protein; (**a**), with either [γ-^{32}P]GTP (●) or [α-^{32}P]GTP (▲) as substrate. Activity obtained at 5 μg of granule protein was used as 100% to calculate relative rates of hydrolysis at each protein concentration for each substrate. The reaction products were separated on a polyethyleneimine-cellulose t.l.c. plate as described in the text. (**b**) GTPase activity was assayed in the secretory-granule fraction in the presence of AA (0–330 μM) with [γ-^{32}P]GTP as substrate. Data are representative of two experiments with similar results.

predominant lipoxygenase-derived metabolite of AA in islets [34,35], had no effect on GTPase activity at 30 μM (additional results not shown).

PGE_2 effects on substrate (GTP) binding

Additional studies were carried out to exclude the possibility that PGE_2 stimulation of GTPase activity might be due to its ability to increase GTP binding to the putative G-protein, thereby increasing the apparent GTPase activity. Such experiments indicated that PGE_2 (1 μM), which stimulated GTPase activity significantly ($P = 0.004$), had no significant effect on GTP[S] binding in the secretory-granule fraction ($98 \pm 7\%$ of control; $n = 5$).

DISCUSSION

Evidence is emerging to suggest the presence of low-molecular-mass and heterotrimeric G-proteins on the secretory granules of several cell types, including parotid gland [36], neutrophils [37] and chromaffin cells [38]. However, the present studies are the first to describe the presence of both functional and regulatable G-proteins on the secretory-granule fraction of a peptide-secreting endocrine cell in general, and pancreatic islets in particular. Most previous studies investigating the putative role(s) for G-proteins in insulin secretion were carried out on plasma membranes [8] or crude membrane fractions [9] of insulin-secreting transformed cell lines (HIT cells and RIN5mF cells). The present studies were conducted with normal rat and human islets, for two reasons. First, the insulin-secretory dynamics of transformed β-cell lines are not normal [39]. Secondly, the metabolism of *ras*-like G-proteins has been shown to be altered in several transformed cell lines [40]. In fact, we [13] and others [41] have observed significant differences in the abundance and distribution (membrane versus cytosolic) of low-molecular-mass as well as heterotrimeric G-proteins in transformed β-cell lines compared with normal rat islets. Although it is tedious to isolate a purified secretory-granule fraction from normal islet tissue, it was considered necessary to carry out the present studies in this fraction in order to relate the functional regulation of G-proteins on the secretory-granule fraction to the physiological modulation of insulin secretion in a normally functioning preparation.

Earlier studies from our laboratory [42] and by others [35,43] have indicated that PGE_2 is a major endogenous product of AA metabolism in islets and that glucose amplifies the synthesis of PGE_2 in islets. It is well established that PGE_2 inhibits glucose-induced insulin secretion *in vivo* and *in vitro* [6]. Conversely, inhibitors of PGE_2 synthesis generally augment glucose-induced insulin secretion from rat islets [42], human islets [44] and intact humans [45]. All these data point toward a key regulatory mechanism(s) involving PGE_2-mediated inhibition of insulin secretion. Recent studies by Robertson et al. [4] and Seaquist et al. [46] have provided convincing evidence on the structure-specific inhibition of glucose-induced insulin secretion from HIT cells by PGE_2. Using Ptx-mediated ribosylation as a tool, these investigators have demonstrated the involvement of an inhibitory G-protein in PGE_2-mediated inhibition of insulin secretion. However, Ptx prevented the inhibition of PGE_2 of insulin release only partially; thus it was proposed that PGE_2 regulates islet metabolism at site(s) distal to the plasma membrane [7]. The present studies provide the first direct evidence for the presence of such a locus on the secretory-granule fraction.

Recent studies from our laboratory have described the presence of at least three specific GTPase activities in homogenates of normal rat islets and HIT cells, based on their K_m for GTP (A. Kowluru and S. A. Metz, unpublished work; and [11–13]). Further studies on the subcellular distribution of these specific GTPases revealed that the high-affinity (and Ptx-sensitive) enzyme is localized in the membrane as well as the secretory-granule fraction. This may represent a G_i- or G_o-like G-protein, since it has been shown recently that α_2-adrenergic agents such as adrenaline, clonidine [47] or galanin stimulated a high-affinity GTPase activity in the membrane fraction of insulin-secreting RIN5mF cells [48], and the present studies show a similar degree of stimulation of the high-affinity GTPase activity by these agents in the secretory granules of normal islets. Indeed, more recent serologic studies by Seaquist et al. [9], Sharp et al. [8], Berrow et al. [10] and Morgan and Berrow [41] have provided convincing evidence for the presence of G_i or G_o subspecies in insulin-secreting cell lines as well as normal rat islets. The present study thus identifies a high-affinity GTPase activity in the secretory-granule fraction from normal rat and human islets; thus this GTPase might be located in the exocytotic cascade at a site functionally downstream of proximal (metabolic and ionic) events (see below). Based on the findings reported herein (the stimulation of high-affinity GTPase activity by PGE_2 without significant effects on GTP binding), it seems likely that PGE_2 (or α_2-agonists) would increase the 'cycling' [49] of an inhibitory G-protein, culminating in PGE_2- (or α_2-receptor)-mediated inhibition of glucose-induced insulin secretion.

Recently Han et al. [50] demonstrated that very high concentrations (160–180 μM) of $PGF_{2\alpha}$ and PGA_2 stimulated, and that PGI_2 inhibited, the GTPase-activating protein (GAP) associated with *ras*-like G-proteins. However, we believe that their findings differ mechanistically from those of the present studies. First, in contraindication to their findings, prostaglandin effects are structurally specific (i.e. only E-series PGs are effective in stimulating GTPase activity; Table 2). Second, our studies provide evidence for direct effects of PGE_2 (i.e. presumably apart from indirect effects on GAP or exchange protein) on the high-affinity GTPase activity. Most of the regulatory proteins (i.e. GDP/GTP-exchange protein and GAP) for G-proteins are predominantly cytosolic in their distribution [51,52], whereas the present studies were carried out in a secretory-granule-enriched fraction, with virtually no cytosolic contamination (as judged by $< 3\%$ lactate dehydrogenase, a cytosolic marker). This suggests that such intermediary proteins may not be present. However, we cannot totally exclude the presence of such regulatory proteins in islet secretory granules, since, as Ullrich et al. recently reported [53], a small fraction of such proteins may be associated with the membranes.

The degree of stimulation of the high-affinity GTPase activity by PGE_2 was comparable with the degree of stimulation induced by mastoparan (Table 1), which is a frequently used direct (receptor-independent) stimulator of GDP/GTP-exchange and GTPase activities [32,33]. Its inactive analogue mastoparan-17 [32] was without any effect (results not shown). The stimulation of GTPase activity (Table 1) both by mastoparan (which promotes insulin release) as well as by adrenaline, clonidine or PGE_2, which inhibit insulin secretion, might suggest the presence of both stimulatory (G_E) and inhibitory (G_{Ei}) G-proteins involved in exocytosis on the secretory granules. However, a more parsimonious alternative explanation can be found in our recent observations [54] that insulin secretion by mastoparan (but not its inactive analogue mastoparan-17) was resistant to inhibition of GTP-binding-protein function (by interference with their isoprenylation or carboxyl methylation). In fact, interference with GTP-binding-protein functions potentiated mastoparan-induced insulin secretion from normal rat islets [54]. This suggests

that (in addition to its stimulatory effects) mastoparan might activate the same inhibitory GTP-binding proteins stimulated by PGE_2 or α_2-agonists.

There is emerging evidence to suggest that heterotrimeric GTP-binding proteins may not be restricted only to the control of coupling of cell-surface receptors to their respective intracellular effectors. Several studies have demonstrated the presence of heterotrimeric GTP-binding proteins on the secretory-granule membranes, including parotid [36], chromaffin cells [38] and neutrophils [37]. More recent evidence indicates the presence of mastoparan-regulatable heterotrimeric GTP-binding proteins on secretory granule membranes on chromaffin cells [55]. These data support our present observations concerning the presence of such regulatable GTP-binding proteins in human and rat islet secretory-granule membranes. The present data do not address the mechanism whereby receptor agonists could gain access to an intracellular organelle (e.g. the secretory granule) in order to regulate its intrinsic GTP-binding-protein function. PGE_2 is synthesized in islets, and therefore might regulate intracellular GTPase activity directly within the cell. However, other agonists (e.g. adrenaline or clonidine) are restricted to the extracellular space. One possibility is that the secretory granules transfer their GTPases into the plasma membrane during the docking of the secretory granule at the plasma membrane, or when the two become interconnected by the fusion-pore as proposed by Monck et al. [56]. These concepts obviously require further study. However, studies by Draznin et al. [57] may be relevant in this regard; these investigators have identified the presence of biologically functional somatostatin receptors on the secretory granules of rat islets. These receptors are recruited to the plasma membrane during secretory-granule margination, rendering islets more sensitive to inhibition by somatostatin. Thus exocytosis might bring inhibitory sites of PGE_2 (or α_2-agonist) action to the secretory-granule/plasma-membrane interface, where they might inhibit insulin release.

Moreover, recent evidence also suggests that receptor activation results in the translocation of GTP-binding proteins associated with intracellular organelles (e.g. secretory granules) to the plasma membrane. Recently, Rotrosen et al. [37] have demonstrated that exposure of neutrophils to *N*-formyl-methionyl-leucyl-phenylalanine resulted in a marked translocation of Ptx-sensitive GTP-binding proteins from the secretory granules to the plasma membrane, suggesting yet another mechanism for cellular regulation of GTP-binding-protein activity.

The specific regulatory effects of PGs on the high-affinity GTPase activity may imply the presence of PG receptors localized on the granule membrane, although we cannot yet directly assess this possibility, owing to limitations of the amount of tissue available for study. However, the structural selectivity requirement (for the E series of PGs), as well as the low concentrations (nanomolar) required for stimulation, are compatible with observations by Robertson et al. [4], which indicated the presence of specific binding sites for PGs on HIT-cell and guinea-pig islet membranes. Additional studies are needed to substantiate directly the presence of PGE_2-binding sites in these subcellular fractions, especially the secretory-granule fraction.

The present studies also identify functional and PGE_2-regulatable G-proteins on the secretory granules of human islets. Interestingly, it has been shown that human islets also synthesize PGE_2 [44] and that inhibitors of PGE_2 synthesis, such as indomethacin [44,58] or sodium salicylate [58], augment glucose-induced insulin secretion in human subjects or isolated human islets. Therefore the findings of a negative modulatory role for PGE_2, acting in part via its regulatory effects on GTP-binding proteins on the secretory granules, which were originally deduced by using intact humans *in vivo* [45,58] or from rodent β-cells *in vitro*, probably does extend to human β-cells as well.

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