Modulation of insulin secretion from normal rat islets by inhibitors of the post-translational modifications of GTP-binding proteins

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Many GTP-binding proteins (GBPs) are modified by mevalonic acid (MVA)-dependent isoprenylation, carboxyl methylation or palmitoylation. The effects of inhibitors of these processes on insulin release were studied. Intact pancreatic islets were shown to synthesize and metabolize MVA and to prenylate several candidate proteins. Culture with lovastatin (to inhibit synthesis of endogenous MVA) caused the accumulation in the cytosol of low-*M_r* GBPs (labelled by the [α -³²P]GTP overlay technique), suggesting a disturbance of membrane association. Concomitantly, lovastatin pretreatment reduced glucose-induced insulin release by about 50 %; co-provision of 100–200 μ M MVA totally prevented this effect. Perillic acid, a purported inhibitor of the prenylation of small GBPs, also markedly reduced glucose-induced insulin secretion. Furthermore, both *N*-acetyl-*S*-

trans,trans-farnesyl-L-cysteine (AFC), which inhibited the base-labile carboxyl methylation of GBPs in islets or in transformed β -cells, and cerulenin acid, an inhibitor of protein palmitoylation, also reduced nutrient-induced secretion; an inactive analogue of AFC (which did not inhibit carboxyl methylation in islets) had no effect on secretion. In contrast with nutrients, the effects of agonists that induce secretion by directly activating distal components in signal transduction (such as a phorbol ester or mastoparan) were either unaffected or enhanced by lovastatin or AFC. These data are compatible with the hypothesis that post-translational modifications are required for one or more stimulatory GBPs to promote proximal step(s) in fuel-induced insulin secretion, whereas one or more inhibitory GBPs might reduce secretion at a more distal locus.

INTRODUCTION

Regulated exocytotic secretion is one example of vectorial transport and cellular trafficking in which a vesicle buds off of a donor compartment (e.g. the *trans*-Golgi face) and moves in a directed fashion to an acceptor compartment (e.g. the plasma membrane), where the vesicle docks, fuses, transfers its contents (unless they are resident proteins) and then recycles (for reviews, see [1,2]). There is evidence from yeast [3] and eukaryotic cells that low-*M_r* monomeric GTP-binding proteins (GBPs) [1,4] and perhaps heterotrimeric GBPs [5,6] may participate in this exocytotic-endocytotic cycle. A GBP may be required for secretory vesicle biogenesis [7]. In yeast having mutations in the SEC4 gene, secretory vesicles are unable to fuse with the plasma membrane and therefore accumulate [1,3] rather than undergoing exocytosis. In mammalian secretory cells, such as pancreatic islets, monomeric [8–11] and heterotrimeric [12–14] GBPs have been identified; in neutrophils [15], parotid gland [16], neuronal cells [4], pancreatic exocrine cells [17], adrenomedullary chromaffin cells [18,19] and pancreatic islets [20,21], GBPs have been localized specifically on the secretory granules (or secretory vesicles) themselves. In neuronal cells, the monomeric GBP *rab3a*, which is associated with secretory vesicles, translocates to the cell surface during massive exocytosis [4], and a *rab3a* effector domain synthetic peptide stimulates amylase release from permeabilized pancreatic acini [22] or pancreatic islets [23]. Thus one or more such GBPs has been postulated [24] to be '*G_{ex}*', a putative GBP that directly modulates or mediates terminal steps in exocytotic hormone release. However, the evidence for the existence of such a protein is largely based on the stimulation of secretion by GTP analogues provided exogenously to permea-

bilized cells [25]. Additionally, recent evidence suggests that small proteins apparently involved in the exocytotic process can leak from such porated cells [6,26–28] and that some of these may be GBPs [6]. However, a direct link between GBPs and secretion has not yet been established.

Most GBPs are subject to post-translational modifications (PTMs), such as isoprenylation, α -carboxyl methylation and protein palmitoylation (for reviews, see [29–32]). Isoprenylation involves the addition of a 15- or 20-carbon polyisoprenoid group (farnesyl or geranylgeranyl respectively) in thioether linkage to a cysteine residue. These moieties are derived from mevalonic acid (MVA) [32,33]; MVA, in turn, is synthesized from acetate via hydroxymethylglutaryl-CoA (HMG-CoA) reductase. Isoprenylation is an early post-translational event which is irreversible and therefore probably not an acute regulatory mechanism [30]; isoprenylated GBPs are stable and have long half-lives [30,34–37]. After isoprenylation (at a cysteine residue upstream of the C-terminal amino acid), the terminal amino acids are removed; the isoprenylated truncated GBP may then be methylated on the newly exposed α -carboxy group [30,32]. In some cases, the GBP is also acylated, usually with palmitic acid [31], on upstream cysteine residues. Isoprenylation is a prerequisite for proteolytic cleavage, carboxyl methylation and acylation [35,38]. The latter two are late post-translational events which are both acutely regulated and reversible [37,39,40]. These sequential modifications render proteins progressively more hydrophobic [41] and thus able to associate more tightly with relevant membranes [31,37,42]. Although isoprenylation alone may be sufficient for GBPs to associate with membranes, methylation and acylation may be required for maximal membrane association [30,31,38,42–46] or for recognition of the GBP by target proteins.

Abbreviations used: PTM, post-translational modification; GBP, GTP-binding protein; MVA, mevalonic acid; AFC, *N*-acetyl-*S*-*trans,trans*-farnesyl-L-cysteine; AGC, *N*-acetyl-*S*-*trans*-geranyl-L-cysteine; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; HMG-CoA, hydroxymethylglutaryl-CoA; PTx, pertussis toxin; GTP[S], guanosine 5'-[α -thio]triphosphate.

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Interference with PTM may cause unmodified precursor GBPs to accumulate in the cytosol [11,30,34–37,43], thereby inhibiting their translocation on the vesicle/granule to sites of subsequent fusion events important for regulated secretion.

Despite the attractiveness of this widely held formulation, few data exist to demonstrate that the GBPs of eukaryotic peptide-secreting endocrine cells undergo such PTMs, or that there is a functional role for these modifications in exocytotic hormone release. In this study, we investigated this formulation in pancreatic islets. Despite the inherent difficulties in isolating sufficient amounts of tissue for study using normal rat islets, we principally studied this preparation for two reasons: first, we wished to avoid any possible abnormalities in *ras*-like GBPs that might exist in transformed β -cells (e.g. RIN or HIT cells); and second, we wanted to be able to study concomitantly insulin secretion from islets which respond normally to physiological secretagogues, especially glucose.

EXPERIMENTAL

Materials

RS-[2-¹⁴C]Mevalonolactone (50 mCi/mmol), [α -³²P]GTP (3000 Ci/mol), [1-¹⁴C]acetic acid (59 mCi/mol), *S*-adenosyl-L-[methyl-³H]methionine (73 Ci/mmol) and L-[methyl-³H]-methionine (72 Ci/mmol) were from New England Nuclear (Boston, MA, U.S.A.). Perillic acid (*p*-mentha-1,8 diene-7-carboxylic acid) was a gift from Dr. Michael Gould (Department of Human Oncology, University of Wisconsin, Madison, WI, U.S.A.). It was dissolved in 10% (w/v) BSA in Krebs/Ringer bicarbonate buffer, adjusted to pH 7.4 before use. Squalastatin 1 was a gift from Dr. B. M. Bain (Glaxo Group Research Ltd, Greenford, Middlesex, U.K.) and was solubilized in water. Mastoparan and mastoparan-17 were from Sigma (St. Louis, MO, U.S.A.) and Peninsula Laboratories (Belmont, CA, U.S.A.) respectively. Lovastatin (mevinolin), a gift from Dr. Alfred W. Alberts (Merck, Sharpe and Dohme Research Laboratories, Rahway, NJ, U.S.A.), was converted into the free acid before use. *N*-Acetyl-*S*-*trans,trans*-farnesyl-L-cysteine (AFC) and *N*-acetyl-*S*-*trans*-geranyl-L-cysteine (AGC) were synthesized as previously described [47]. Cerulenin (2,3-epoxy-4-keto-7,10-dodecadienamide) was from Calbiochem (San Diego, CA, U.S.A.). Homocysteine and 3-deaza-adenosine were purchased from Southern Research Institute (Birmingham, AL, U.S.A.); tunicamycin was from Sigma. In all studies involving pharmacological inhibitors, the appropriate diluent was added to all control tubes. Purified $\beta\gamma$ -subunits of transducin were a gift from Dr. Akio Yamazaki (Kresge Eye Institute, Detroit, MI, U.S.A.). HIT cells were obtained through the courtesy of Dr. Paul Robertson and Dr. Hui-Jian Zhang (Minneapolis, MN, U.S.A.) and human pancreatic islets courtesy of Dr. David Scharp (St. Louis, MO, U.S.A.).

Isolation and treatment of pancreatic islets for studies of insulin release

Intact pancreatic islets were isolated from adult male Sprague-Dawley rats using collagenase digestion and separation from acinar tissue and debris on Ficoll gradients, followed by hand-picking under stereomicroscopic control, as described previously [48,49]. For some studies, after isolation, islets were cultured overnight (for 18 h) in RPMI 1640 medium [containing 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 100 μ g/ml streptomycin and 11.1 mM glucose, except where specified otherwise]. The incubation medium for subsequent studies of insulin

release was Krebs/Ringer bicarbonate buffer (pH 7.4) containing 0.5% BSA and 3.3 or 16.7 mM glucose, and gassed with 95% O₂/5% CO₂. Albumin was excluded from tubes containing AFC or AGC because it binds these agents, and thereby lowers their effective concentration in the medium. Static batch-type incubations were carried out for 45 min so as to include contributions from both first and second phases of secretion. Test agents used in studies of isoprenylation (e.g. lovastatin, perillic acid, MVA, 25-hydroxycholesterol, squalastatin) were present not only during the overnight culture period but also during the islet picking, wash and preincubation steps on the following day, in order to avoid any attenuation of their effects over time; however, these drugs were excluded from the incubation period. In contrast, in studies of cultured or freshly isolated islets (using AFC, AGC or cerulenin), drugs were added only to the preincubation and incubation media. For studies involving pertussis toxin (PTx) pretreatment, rats received 1.5 μ g of PTx (List Biologic Laboratories, Campbell, CA, U.S.A.) intraperitoneally 24 and 12 h before being killed; the islets of the same rats were also exposed to 50 ng/ml PTx during the overnight culture period. Control rats and islets received PTx buffer (sodium phosphate) alone.

To assess insulin content, islets were extracted overnight as described [49] in 1 ml of acid alcohol (77% ethanol, 22% water and 1% 12 M HCl, by vol.) at 4 °C, the supernatant was removed, diluted (1:10) in PBS to a final dilution of 1:20, and assayed by radioimmunoassay as described [48,49].

Test agents (30 μ M lovastatin, 400 μ M MVA, 0.5–5.0 mM perillic acid, 100 μ M AGC, 100 μ M AFC, 90 μ M cerulenin or 200 μ M homocysteine plus 100 μ M deaza-adenosine) had no effect on the insulin radioimmunoassay.

Identification of GBPs and assessments of their PTMs

[α -³²P]GTP binding

To identify GBPs, the [α -³²P]GTP overlay technique was used, as described by Phillips et al. [50]. In brief, proteins in individual fractions were separated by SDS/PAGE (12.5% gels). The proteins were then transferred to nitrocellulose membranes using a Bio-Rad transblot apparatus. The blots were washed in a medium consisting of 50 mM Tris/HCl, 5 mM MgCl₂, 1 mM EGTA and 0.3% Tween 20, pH 7.4, for 10 min. The membranes were soaked in the above medium containing 1 μ Ci/ml [α -³²P]GTP for 1 h at room temperature followed by extensive washing in the same buffer to remove unbound label from the membranes. Radioactivity bound to the proteins was detected by autoradiography using X-OMAT AR film with exposure of 1–3 days at –70 °C. *M_r* values of the labelled proteins were determined using prestained standards (Bio-Rad, Richmond, CA, U.S.A.).

Isoprenylation

Groups of 300 islets were incubated in the presence (or absence) of 15–30 μ M lovastatin, an inhibitor of HMG-CoA reductase [33,51], in order to deplete endogenous MVA and facilitate labelling using exogenous MVA [35]. Incubations were carried out in RPMI 1640 medium, containing 2.8 mM glucose and 10% (v/v) fetal calf serum, for 8 h in a metabolic chamber at 37 °C. This was followed by an additional 8–12 h incubation in the continued presence of 15–30 μ M lovastatin plus [¹⁴C]MVA (40 μ Ci/ml). In some studies, where indicated, lovastatin and labelled MVA were both present for the entire 20 h incubation. After labelling, cells were washed quickly with an isotonic medium consisting of 230 mM mannitol, 70 mM sucrose and 5 mM Hepes buffer, pH 7.4. Islets were homogenized in the same

medium and proteins were separated by SDS/PAGE; labelled proteins were identified by fluorography (using Fluoro-Hance; Research Products International, Mt. Prospect, IL, U.S.A.) as described above. Autoradiographic exposures of 6 weeks were generally required.

Carboxyl methylation

Protein carboxyl methylation was carried out in cell-free preparations, as well as in intact islets, using *S*-adenosyl-L-[methyl-³H]methionine and L-[methyl-³H]methionine respectively.

For cell-free preparations, islet fractions, singly or in combination, were incubated in 50 mM sodium phosphate buffer, pH 6.8, consisting of 1 mM EGTA in the presence of *S*-adenosyl-L-[methyl-³H]methionine (100 μ Ci/ml) for 60 min in the presence or absence of 10 μ M guanosine 5'-[γ -thio]triphosphate (GTP[S]), wherever indicated. Incubations were terminated by the addition of SDS/PAGE sample buffer; labelled proteins were subsequently identified by fluorography, generally after 4- to 6-week exposure period. The degree of incorporation of label into individual proteins was determined by densitometry using a Zeineh soft laser scanning/video densitometer (Biomed Instruments, Fullerton, CA, U.S.A.) coupled to an IBM PC-compatible computer. Test agents such as AFC were present as indicated in the text.

The α -carboxyl methyl group on prenyl-cysteine residues of modified GBPs are base-labile. To demonstrate that protein methylation in islets involves such sites specifically, methyl esters were quantified by a vapour-phase equilibration technique. Individual lanes of the dried gels were cut into 3 or 5 mm slices and were placed in 1.5 ml Eppendorf tubes (without caps) containing 100 or 150 μ l of 1 M NaOH. Tubes were placed in 20 ml scintillation vials containing 3 ml of scintillation cocktail (Ultima Gold; Packard Instrument Co., Meriden, CT, U.S.A.) and the vials were then capped and left at room temperature overnight to facilitate the base-catalysed hydrolysis of the methyl esters and the equilibration of released [³H]methanol. After the incubation, the tubes were gently removed from the vials and the sides of the tubes were rinsed (into the vials) with an additional 5 ml of the scintillant and the radioactivity was determined by scintillation spectrometry.

For intact cells, groups of 100 islets were incubated in an isotonic Krebs/Ringer bicarbonate medium in a metabolic chamber (at 37 °C) for 4 h in the presence of [methyl-³H]-methionine (40–100 μ Ci/ml). Where present, cycloheximide (5 μ M) and actinomycin D (2 μ M) were added 1 h before provision of labelled methionine. Preincubation with methionine was necessary to prelabel the endogenous *S*-adenosylmethionine pools which form the substrate for methylation reactions. After prelabelling, cells were washed once with an isotonic medium to remove the label, and various modulators were added (as indicated in the text) and islets were incubated for an additional 30 min. Labelled proteins were separated by SDS/PAGE (pH 8.8).

Analysis of acetate and MVA metabolism by islets

Metabolism of acetate

Groups of 400 islets were cultured overnight in the presence or absence of 30 μ M lovastatin. The next morning, the medium was carefully removed and cells were incubated for an additional 4 h in a fresh medium containing [¹⁴C]acetic acid (30 μ Ci/ml) in the presence or absence of 30 μ M lovastatin. Lipids were extracted and separated by t.l.c. as described below.

Metabolism of MVA

Groups of 300 islets were cultured for 16 h in the presence or absence of 30 μ M lovastatin. The cells were then labelled with [³H]MVA (50 μ Ci/ml) for an additional 4 h at 37 °C in the presence or absence of lovastatin (30 μ M) as indicated in the text. Lipids were extracted with chloroform/methanol and separated by t.l.c. as described below.

T.l.c.

Lipids were extracted and separated by one-dimensional t.l.c. for sterols and non-sterol isoprenoids using a hexane/diethyl ether/acetic acid (140:60:3, by vol.) solvent system. A mixture of authentic standards was added to islet lipid extracts, spotted on to silica gel 60 plates (0.2 mm thickness; EM Science, Gibbstown, NJ, U.S.A.) and lipid spots were developed in an iodine chamber. Relative migration (R_f) values were determined for authentic standards on at least three occasions to study the reproducibility of the separation by this method. Radioactivity in individual lipid spots was quantified by scintillation spectrometry.

Miscellaneous

For subcellular fractionation, islets were homogenized in buffer containing 230 mM mannitol, 70 mM sucrose and 5 mM Hepes, pH 7.4, and subcellular fractions were isolated using a differential centrifugation method, as previously described [52,53]. Lactate dehydrogenase was quantified spectrophotometrically using a kit from Sigma. Formation of NADH was monitored from exogenously added NAD⁺ and lactate. Protein concentration was quantified colorimetrically using a dye-binding method described by Bradford [54].

Data presentation and statistical analysis

Insulin secretion is expressed as μ units/10 islets per 45 min (means $\pm$ S.E.M.) for static incubations. Incremental release is expressed as the value for insulin released under stimulated conditions minus the mean value for basal release (i.e. at 3.3 mM glucose) in that same study. Insulin content is expressed as μ units of insulin per 10 islets (assessed at the end of the incubation period). Fractional release was calculated by the formula:

$$\frac{\text{insulin released}}{\text{initial insulin content}}$$

where the denominator equals the insulin content remaining in islets at the end of the incubation period plus that insulin released during the incubation period. Fractional release is expressed as percentage of initial insulin content released per 45 min. Statistical comparisons were made using paired or non-paired *t* testing, as appropriate.

RESULTS

Isoprenylation

In order to verify that islets have the ability to synthesize and metabolize MVA endogenously, conversion of labelled acetate into compounds co-migrating with authentic standards was assessed. Synthesis of cholesterol, lanosterol, squalene, dolichols and ubiquinone was evident; lovastatin (30 μ M) reduced their synthesis by 48–93% (results not shown). In addition, islets converted exogenously provided labelled MVA into similar compounds; in the presence of lovastatin, exogenous MVA was converted more efficiently (242% of control) into labelled

Figure 1 (a) Isoprenylation of low- M_r GBPs in normal rat islets and (b) effect of lovastatin on the subcellular distribution of these GBPs

(a) Islets (300–350) were labelled with [^{14}C]MVA in the absence or presence of lovastatin ($30\text{ }\mu\text{M}$) as described in the Experimental section. Labelled proteins were separated by SDS/PAGE and identified by autoradiography (exposure for 4 weeks at $-70\text{ }^\circ\text{C}$). In the gel on the left, cells were exposed concurrently (for 20 h) to lovastatin and [^{14}C]MVA; in the gel on the right, lovastatin alone was present for 8 h, followed by lovastatin plus [^{14}C]MVA for the next 12 h (b) Islets (300–350) were cultured for 18 h in the absence (C) or presence (L) of $15\text{ }\mu\text{M}$ lovastatin. The total membranous (M) and soluble (S) fractions were isolated by centrifugation, and GBPs were identified by the [$\alpha\text{-}^{32}\text{P}$]GTP overlay method, as described in the text. The top autoradiograph was exposed (at $-70\text{ }^\circ\text{C}$) for 4 h, and the lower one for 16 h in order to augment visibility of less strongly labelled bands. Numbers to the right are used to identify individual labelled bands. The redistribution to the cytosol was seen in each of five separate experiments.

products, presumably indicating a reduction in dilution of isotopic label and thus providing indirect evidence of depletion of endogenous MVA by lovastatin. Additionally, when intact islets were incubated with lovastatin to deplete cellular stores of MVA, exogenous [^{14}C]MVA labelled at least five proteins (Figure 1a) of M_r (20000–27000) similar to the low- M_r GBPs labelled via the [$\alpha\text{-}^{32}\text{P}$]GTP overlay technique (Figure 1b). (The relative abundance of the GBPs using the two techniques cannot be compared as the GTP overlay technique does not label all such GBPs.) Islets not treated with lovastatin showed very little incorporation of labelled MVA (not shown). In lovastatin-treated islets, there was also a shift in the subcellular localization of these GBPs (assessed by GTP overlay), with an increase in their relative cytosolic labelling being visible in each of five experiments (Figure 1b), a finding confirmed by cutting the nitrocellulose membranes and directly counting radioactivity and/or by densitometric analysis of the labelled bands on autoradiographs. There was a small concomitant reciprocal decrease in the relative labelling of the proteins in the membrane fraction (and possibly a slight retardation in their migration), although the loss from membrane fractions was less obvious than the accumulation in the cytosol (Figure 1b; see the Discussion section). Thus pancreatic islets have the enzymic machinery to synthesize isoprenoid groups endogenously and to prenylate candidate GBPs, facilitating their association with membranes.

Islets incubated overnight in the presence of lovastatin ($15\text{--}30\text{ }\mu\text{M}$) demonstrated a subsequent impairment (by 46–57 %) of absolute insulin secretion in response to 16.7 mM glucose (Figure 2). Lovastatin did not alter the insulin content of islets, and thus fractional insulin-release rates were also reduced (Table 1 and Fig. 2, inset). The inhibitory effect of lovastatin was visible

Figure 2 Effect of lovastatin and/or MVA on incremental glucose-induced insulin release

The latter is calculated as stimulated release minus basal release. MVA was provided as the lactone or as the sodium salt, as indicated. Inset: effects in terms of fractional release (lovastatin was provided at $15\text{ }\mu\text{M}$ and mevalonolactone at $100\text{ }\mu\text{M}$; symbols are the same as for the absolute release data immediately under the inset). The number of determinations in each case is shown in parentheses. *Significantly reduced compared with control value.

Table 1 Effect of lovastatin on fractional (glucose-induced) insulin secretion

Secretion is expressed as fractional release in response to 16.7 mM glucose over a 45 min incubation period; basal values at 3.3 mM glucose, which were unaltered by lovastatin, were subtracted. The numbers of individual experiments, each comprised of multiple determinations, are given in parentheses.

Lovastatin concentration (μ M)	Inhibition (%)
0	0
1	-32% (2)
5	-43 $\pm$ 12% (3)
15	-46 $\pm$ 8% (4)
30	-48 $\pm$ 4% (6)
75	-28% (2)

Figure 3 Effects of increasing concentrations of perillic acid on incremental insulin release (a), insulin content (b) and fractional insulin secretion (c)

Perillic acid was provided during an 18 h culture period. Agonists were 16.7 mM glucose (●) or 15 mM α -oxo-4-methylpentanoic acid (○). These agonists were provided during the 45 min static incubation period subsequent to the overnight exposure to the drug. Values to the right of each panel (□) represent the mean ($\pm$ S.E.M.) of the data from the number of experiments indicated in parentheses using glucose or α -oxo-4-methylpentanoate as agonist.

at a concentration of the drug of 1 μ M or less, and was maximal at 15–30 μ M (Table 1); at higher concentrations, the inhibitory effect became somewhat less (Table 1). Lovastatin had no acute inhibitory effect, i.e. the effect occurred only after a 45 min preincubation period (results not shown), compatible with the formulation (see the Introduction) that isoprenylation is not an acute regulatory event in cell function. Furthermore, the lovastatin effect was totally prevented by co-provision of 100 or 200 μ M mevalonolactone or sodium mevalonate (Figure 2), which by themselves had little effect on glucose-induced secretion (Figure 2). In contrast with its effect on glucose, lovastatin did not reduce the effects of a phorbol ester [12-*O*-tetradecanoylphorbol 13-acetate (TPA), 2 μ M] (fractional secretion: -4%; n = 2) and only minimally blunted the effects of 50 mM K^+ (-31 $\pm$ 6%; n = 4) or 15 mM α -oxo-4-methylpentanoic acid (-19 $\pm$ 3%; n = 3).

These studies suggested that MVA, or a product of MVA, is required for maximal exocytotic secretion induced by glucose. Provision during the culture period of 1.25–12.5 μ M 25-hydroxycholesterol (a weaker inhibitor of HMG-CoA reductase which decreases cholesterol synthesis but does not impede isoprenylation [34,55]) failed to impair glucose-stimulated

Figure 4 Stimulation by GTP[S] of protein carboxyl methylation in rat islets: effects of AFC and AGC

(a) Islet homogenates were incubated with 100 μ Cl/ml Sadenosyl[3 H]methionine, at pH 6.8, in the presence or absence of 10 μ M GTP[S] for 45 min at 33 $^{\circ}$ C. AFC (100 μ M) or AGC (100 μ M) were also present as depicted. The degree of incorporation of methyl groups into islet proteins was monitored by SDS/PAGE followed by fluorography. Arrows indicate two GTP[S]-sensitive proteins of M_r approx. 21 000 and 23 000 and an additional band of M_r < 10 000 (putatively γ -subunits of heterotrimeric GBPs). (b) Assessment of base-labile carboxyl methylation of GBPs (as assessed by the release of [3 H]methanol; see the Experimental section) in islet or HIT cell homogenates in the presence of diluents alone, 10 μ M GTP[S] or GTP[S] plus 100 μ M AFC. Data are expressed as percentage of diluent controls; numbers in parentheses are the numbers of individual experiments. Absolute control (basal) values were 14.3 $\pm$ 1.08 nmol/mg of total homogenate for islets and 41.4 $\pm$ 5.3 nmol of [3 H]methanol/mg for HIT cells.

secretion in four experiments. Furthermore, squalstatin 1 (a selective inhibitor of squalene synthase, the first committed step in the cholesterol-synthetic limb; see the Discussion section) failed to inhibit glucose-induced insulin release at 0.1–500 μ M (results not shown). Dolichols are MVA-derived lipids required for N-linked glycosylation of proteins; if the effects of lovastatin were mediated via inhibition of dolichol synthesis, one might expect similar effects from a known inhibitor of N-linked glycosylation, such as tunicamycin. However, tunicamycin (6 or 12 μ M) actually increased insulin release by 52 and 123% respectively.

The site of action of lovastatin was further studied using perillic acid, a recently described [56] purportedly selective inhibitor of the prenylation of low- M_r GBPs. Perillic acid (0.5–5 mM) led to a dose-responsive reduction in absolute insulin release in response to glucose or the amino acid metabolite α -oxo-4-methylpentanoic acid (Figure 3); it also markedly reduced

Table 2 Effects of inhibitors of protein carboxyl methylation or AGC (an inactive analogue) on glucose- or amino acid-induced insulin release

Values are means $\pm$ S.E.M. for (*n*) determinations. Values for basal release (i.e. at 3.3 mM glucose) were subtracted from stimulated values; none of the three test agents altered basal release. ***P* < 0.001 compared with control. **P* < 0.01–0.025 compared with control.

	Absolute release (μ units/ml)	Change (%)	Fractional release (%)	Change (%)
Experiment 1				
Control (16.7 mM glucose)	1231 $\pm$ 76 (6)		9.80 $\pm$ 0.74 (6)	
+ AFC (100 μ M)	551 $\pm$ 60 (6)	–55**	4.30 $\pm$ 0.19 (6)	–56**
+ AGC (100 μ M)	1108 $\pm$ 83 (6)	–10	9.50 $\pm$ 0.67 (6)	–3
+ Homocysteine (200 μ M) and 3-deaza-adenosine (100 μ M)	822 $\pm$ 72 (5)	–35*	6.8 $\pm$ 0.67 (5)	–31*
Experiment 2				
Control (16.7 mM glucose)	985 $\pm$ 45 (6)		5.75 $\pm$ 0.42 (6)	
+ AFC (100 μ M)	382 $\pm$ 26 (6)	–61**	2.21 $\pm$ 0.20 (6)	–62**
Control (15 mM α -oxo-4-methylpentanoic acid) [†]	1155 $\pm$ 81 (5)		9.42 $\pm$ 0.77 (6)	
+ AFC (100 μ M)	412 $\pm$ 30 (6)	–68**	1.42 $\pm$ 0.48 (5)	–85**
+ Homocysteine (200 μ M) and 3-deaza-adenosine (100 μ M)	487 $\pm$ 46 (4)	–62**	2.16 $\pm$ 0.24 (4)	–77**

[†] α -oxo-4-methylpentanoic acid was added to a substimulatory glucose concentration (3.3 mM).

the insulin content of islets (Figure 3). However, fractional insulin release (i.e. exocytosis) was also clearly inhibited by 5 mM perillic acid (Figure 3). Similar results with perillic acid were seen whether islets were cultured at 4.4 mM or 11.1 mM glucose (results not shown). In contrast (but in confirmation of results with lovastatin), the secretory response to TPA was not inhibited, with fractional release being reduced by only 6% (not significant; average of two experiments). Perillic acid, like lovastatin, did not alter glucose- or K^+ -induced insulin release (or insulin content) when added only to the preincubation media. In a preliminary study, the effect of perillic acid on secretion (but not on insulin stores) seemed to be readily dissipated upon removal of the drug from the medium after the culture period. Perillic acid (5 mM) did not induce detectable release of lactate dehydrogenase into the overnight culture (or preincubation) medium (results not shown). Although limonene, the precursor of perillic acid, is said to share its ability to inhibit isoprenylation [56], we did not pursue the use of limonene because of difficulties with solubilization, and recent reports of its possible toxicity (see the Discussion section).

Carboxyl methylation

Islet fractions were incubated alone or in combination with *S*-adenosyl-L-[methyl- 3 H]methionine as methyl donor, in the presence or absence of AFC, a selective inhibitor of the α -carboxyl methyl esterification of low- M_r GBPs [38,57,58]. Virtually no labelling was seen with cytosol alone (in the presence or absence of GTP[S]). The membrane fraction alone showed some activity, but the two fractions together showed the greatest activity. At least four (groups of) proteins (M_r < 10000, 21000–23000, approx. 50000, > 100000; Figure 4a) were labelled. The first band probably includes the γ -subunits of heterotrimeric G-proteins (as it co-migrated with the γ -subunits of transducin) and the second appeared to contain at least two low- M_r GBPs of about 21000 and 23000 (Figure 4a). The latter bands were also detected when intact islets were exposed to [methyl- 3 H]methionine, whether or not protein synthesis inhibitors were present (results not shown). Similar data were also obtained using homogenates of human islets or using transformed

β (HIT) cells (A. Kowluru and S. Metz, unpublished work). The bands at M_r 21000–23000 were not attributable to the N-methylation of calmodulin [59], as label incorporated into exogenous calmodulin had a greater mobility and was not stimulated by GTP[S] (not shown). The identity of the higher- M_r labelled proteins was not investigated further.

Inclusion of 10 μ M GTP[S] increased (to nearly triple the basal values) the labelling of the 21000–23000- M_r proteins (Figure 4a) in islets, as previously observed in other cells [44,60]. GTP[S] (10 μ M) also increased the carboxyl methylation of putative low- M_r GBPs in HIT cell homogenates (to 407 $\pm$ 26%; *n* = 5). As incorporation of label persisted after isolation by SDS/PAGE (Figure 4), type II methylation cannot be involved [61] (see also the Discussion section). Furthermore, this methylation of low- M_r GBPs was base-labile. In each of ten experiments using islets or HIT cells (Figure 4b), base treatment released [3 H]methanol from the GBP fraction; GTP[S] stimulated base-labile carboxyl methylation to 302 $\pm$ 37% of basal (*n* = 10; Figure 4b). Inclusion of 10 μ M AFC markedly inhibited total labelling of the bands with M_r of 23000 and < 10000 in rat and human islets and HIT cells in each of six experiments using broken cell preparations (cf. Figure 4a). AFC also reduced methylation of the low- M_r GBPs in each of three experiments using intact islets labelled with [3 H]methionine (not shown). Additionally, AFC eliminated GTP[S]-stimulated [3 H]methanol release from the 21000- and 23000- M_r GBP fractions (to 84 $\pm$ 22% of the no-GTP control value; *n* = 6; Figure 4b), and markedly reduced methylation of the putative γ -subunit-containing band (results not shown). In contrast, 100 μ M AGC, which is ineffective in other systems as an inhibitor of protein carboxyl methylation (K_i approx. 1 mM; [57]), had no effect on protein carboxyl methylation in islets (Figure 4a).

As carboxyl methylation is subject to rapid regulation [39,57], insulin secretion was studied using islets that were freshly isolated, as well as islets cultured overnight (in the absence of AFC). Acute exposure to AFC (100 μ M) reduced (16.7 mM) glucose-stimulated insulin release by 41–62% and (15 mM) α -oxo-4-methylpentanoate-stimulated release by 68–85% (Table 2). This inhibitory effect began at least as low as 50 μ M AFC and rose progressively to 100 μ M, concentrations in accord with the K_i of

AFC as well as its K_m as substrate for rodent methyltransferases [47,57,58]. The effects of AFC were observed to be time-dependent. Thus these concentrations probably do not reflect true thresholds for inhibitory effects, as preincubations of greater than 30–45 min in the absence of BSA have detrimental effects on islet function and could not be used in these studies. In contrast, AGC had no significant effect on secretion (Table 2). In addition, 200 μ M homocysteine plus 100 μ M 3-deaza-adenosine, which inhibit *S*-adenosylmethionine-dependent methyltransferases [35,61,62], also inhibited glucose- or α -oxo-4-methylpentanoate-induced insulin release (Table 2).

In contrast, AFC (like lovastatin and perillid acid) had only minimal effects on K^+ -induced secretion, in both freshly isolated islets (results not shown) and islets cultured overnight (in the absence of drug) and then studied in the same way as freshly isolated islets (94 % of control; difference not significant). The effects of TPA were not only resistant to inhibition by AFC (as with lovastatin or perillid acid) but in several experiments were actually potentiated by AFC (Table 3; Experiments 1 and 2)

Table 3 Effects of AFC and lovastatin on insulin release induced by nutrients, TPA or mastoparan, in the presence or absence of PTx pretreatment

Values are means $\pm$ S.E.M. for (*n*) determinations. *P* values compare the presence and absence of AFC (or lovastatin or PTx) for each agonist. Freshly isolated islets were used for Experiment 1. For Experiments 2 and 3, islets were cultured overnight (in the presence or absence of lovastatin or PTx where indicated). OMP, α -oxo-4-methylpentanoate.

Experiment	Insulin (μ units/45 min)	
No. 1		
(a) 3.3 mM glucose	273 $\pm$ 61 (3)	
(b) 3.3 mM glucose + AFC	256 $\pm$ 18 (4)	
(c) 3.3 mM glucose + TPA (2 μ M)	930 $\pm$ 92 (5)	
(d) 3.3 mM glucose + TPA + AFC	1529 $\pm$ 117 (6)	(<i>P</i> < 0.01)
(e) 3.3 mM glucose + mastoparan (30 μ M)	2493 $\pm$ 224 (5)	
(f) 3.3 mM glucose + mastoparan + AFC	5581 $\pm$ 358 (4)	(<i>P</i> < 0.001)
(g) 3.3 mM glucose + OMP (15 mM)	1654 $\pm$ 133 (6)	
(h) 3.3 mM glucose + OMP + AFC	1151 $\pm$ 112 (6)	(<i>P</i> < 0.02)
No. 2		
(a) 3.3 mM glucose	92 $\pm$ 25 (3)	
(b) 3.3 mM glucose + lovastatin (15 μ M)	31 $\pm$ 12 (3)	
(c) 3.3 mM glucose + AFC (100 μ M)	33 $\pm$ 11 (3)	
(d) 3.3 mM glucose + mastoparan (30 μ M)	438 $\pm$ 49 (6)	
(e) 3.3 mM glucose + mastoparan + lovastatin	889 $\pm$ 141 (5)	(<i>P</i> < 0.02)
(f) 3.3 mM glucose + mastoparan + AFC	1867 $\pm$ 212 (5)	(<i>P</i> < 0.01)
(g) 3.3 mM glucose + mastoparan-17 (30 μ M)	89 $\pm$ 12 (6)	
(h) 3.3 mM glucose + mastoparan-17 + lovastatin	53 $\pm$ 16 (5)	
(i) 3.3 mM glucose + mastoparan-17 + AFC	96 $\pm$ 17 (5)	
No. 3		
(a) 3.3 mM glucose	62 $\pm$ 9 (3)	
(b) 3.3 mM glucose + PTx	101 $\pm$ 17 (3)	
(c) 3.3 mM glucose + mastoparan (30 μ M)	818 $\pm$ 75 (7)	
(d) 3.3 mM glucose + mastoparan + PTx	317 $\pm$ 26 (7)	(<i>P</i> < 0.001)

under conditions in which the effects of glucose and α -oxo-4-methylpentanoate were inhibited.

Effects of PTM inhibition on mastoparan-induced insulin release

These latter findings suggested the possibility that AFC also altered the function of an inhibitory GBP. To examine this hypothesis further, we studied the effects of mastoparan, a peptide from wasp venom which by-passes proximal effects of nutrients or receptor activation by directly activating heterotrimeric [63] or low- M_r [64] GBPs. Mastoparan increased insulin release progressively from a concentration at least as low as 10 μ M to at least as high as 50 μ M (Table 3 and additional results not shown). This effect seemed to be specific, as mastoparan-17 (a structurally similar peptide which does not activate GBPs [63]) had no effect even at 30 (Table 3) or 40 μ M (results not shown). The effects of mastoparan, like those of K^+ or TPA, were not blocked by AFC, perillid acid or lovastatin. In fact, absolute insulin released by 30 μ M mastoparan was potentiated by lovastatin and AFC (Table 3, Experiments 1 and 2) as was fractional secretion induced by 30 or 40 μ M mastoparan (not shown), suggesting that, in addition to its stimulatory effects, mastoparan might simultaneously activate one or more inhibitory GBPs which require carboxyl methylation. The stimulatory effect of mastoparan might be mediated (at least in part) through the simultaneous activation of a stimulatory GBP, as in each of seven experiments, the effects of mastoparan (at 10, 20, 25 or 30 μ M) were reduced (by 63 $\pm$ 7 %) by pretreatment of islets with PTx (see Table 3, Experiment 3).

Fatty acylation

Cerulein is a potent time-dependent inhibitor of protein acylation [65–67] at concentrations of 45–134 μ M. As over longer time periods this probe can inhibit fatty acid, sterol and protein synthesis [65–67], and because it is chemically unstable [65], cerulein, like AFC, was provided to islets only acutely. The potential for protein acylation to serve an acute reversible regulatory role in cell events [37,68] provided a rationale for this approach. Cerulein at 22–45 μ M had no effect, whereas at a concentration of 90 μ M it inhibited nutrient-induced insulin release (by 38–43 %; Table 4 and additional results not shown) in freshly isolated islets, or using islets cultured overnight first (in the absence of drug). [As with AFC, the inhibitory effects of each concentration of cerulein are probably underestimated, as such effects appeared to be time-dependent and progressive (even after its removal from the medium, possibly reflecting a delay in transport to its site of action after uptake from the medium).] A

Table 4 Effects of cerulein on glucose (16.7 mM)-induced insulin release

Data are expressed as μ units of insulin secreted over 45 min (means $\pm$ S.E.M. for the number of determinations indicated in parentheses).

Cerulein concentration (μ M)	Incremental insulin response (above basal)	Change (%)
0	887 $\pm$ 57 (7)	
22	878 $\pm$ 104 (6)	–1
45	934 $\pm$ 78 (6)	+5
90	537 $\pm$ 32 (5)	–39 (<i>P</i> < 0.001)
134	328 $\pm$ 33 (5)	–63 (<i>P</i> < 0.001)

higher concentration (134 μM) substantially inhibited absolute or fractional rates of insulin secretion induced by 16.7 mM glucose or 15 mM α -oxo-4-methylpentanoate (glucose: -63 to -88% ; α -oxo-4-methylpentanoate: -73 to -100% ; Table 4 and additional results not shown). In contrast, cerulenin did not reduce the effects of 30 μM mastoparan (cerulenin plus mastoparan = 105% of mastoparan alone).

DISCUSSION

Many low- M_r GBPs (as well as the γ -subunits of some trimeric GBPs [30,32,34,39]) are post-translationally modified (see the Introduction); this renders them increasingly hydrophobic [41], and thereby facilitates a tight association with vesicular membranes and possibly increases the ability of receptor proteins to recognize the GBPs. Such modifications permit at least some GBPs to direct vesicular traffic or to subserve other roles [30,46]. Although others [8–11] have also reported the presence of low- M_r GBPs in islet cells, the current data are the first to demonstrate, in addition, the isoprenylation and GTP-dependent carboxyl methylation of such proteins in islets. Such GBPs exist in the β -cells of the islet (at least in part), as they were also localized in transformed β -cells (A. Kowluru and S. Metz, unpublished work) [9,10]; however, further work is needed to determine their exact distribution within the various cells of the islet. The current findings are complementary to our other studies (A. Kowluru and S. Metz, unpublished work) [20,21] in which we observed that the secretory granules of normal rat islets contain at least five low- M_r GBPs (M_r 20000–27000) and at least one heterotrimeric GBP; these proteins thus are in a strategic location to direct the traffic of secretory granules to the plasma membrane where they dock, fuse and release their contents into the extracellular space.

In the current studies, inhibitors of prenylation, methyl esterification or protein acylation impeded nutrient-induced insulin release. Admittedly, such studies are pharmacological and one cannot totally exclude unforeseen, non-specific or toxic effects of the probes. However, the internal consistency of the effects of five distinct chemical classes of inhibitors (which are structurally and mechanistically dissimilar), and the use of both cultured and freshly isolated islets, argue in the aggregate for an involvement of such modifications in nutrient-induced insulin release. Furthermore, these drugs were concordant in their differential effects on insulin secretagogues with differing sites and mechanisms of action. However, rigorous proof of such a relationship must await specific identification of each of the GBPs found in islets [8–14,20,21] and an elucidation of the cell biology of each. Furthermore, we cannot exclude a contribution from other unidentified effector proteins which might be post-translationally modified similarly to GBPs [30,32,36,69].

Lovastatin, which reduced the metabolism of acetate to non-sterol and sterol metabolites in islets in the current study, also inhibited glucose-induced insulin release by about 50%; this effect was reversed by exogenous MVA, provided in order to bypass the lovastatin-induced synthetic block. At the same time, lovastatin caused an accumulation in cytosol of GBPs and a probable retardation of the mobility on gels of membrane-associated GBPs; similar phenomena have been reported in other cell types [30,34–37,43] including transformed β -cells [11]. These data suggest that inhibition of post-translational prenylation impedes the maturation, membrane association and biological effect of (at least a subgroup of) these proteins. The resistance of other agonists (K^+ , TPA or mastoparan) to inhibition by lovastatin suggests that prenylation might be required

at a step proximal to physiological insulin release, rather than directly in late steps in the exocytotic pathway. As expected, lovastatin did not markedly deplete the GBPs in the membrane fraction. Each of the protein bands on one-dimensional SDS/PAGE probably contains multiple GBPs [9,10,17] and it is probable that only one (or a minority) of these proteins accumulated in the soluble fraction during a relatively short exposure to lovastatin. An increase in a few GBPs in cytosol would be considerably easier to visualize than their loss from membranes, as these proteins are considerably enriched in membranes compared with the cytosol in the untreated state. Additionally, the period of lovastatin exposure was relatively brief in comparison with the half-life of some GBPs that are already isoprenylated [30,34,35,37,70] and thus a discernible decrease in the particulate fraction over short periods only occurs with certain GBPs [11,70]. Furthermore, lovastatin is probably unable to abrogate the process of isoprenylation totally, as this branch of the MVA metabolic pathway is preferentially conserved compared with the sterol branch [33,34,71] when HMG-CoA reductase is partially inhibited. Additionally, serum contains small amounts of preformed MVA [71]. For these reasons, we speculate that an incomplete inhibition of isoprenylation (as well as offsetting effects on a putative inhibitory GBP; see below) may contribute to the subtotal inhibition by lovastatin of glucose- or α -oxo-4-methylpentanoate-induced secretion.

It seems unlikely that MVA-derived lipids other than those used for isoprenylation can explain these findings. The fetal bovine serum present during culture provides both free and lipoprotein-bound sterols and non-sterol metabolites [36,72]. Furthermore, neither 25-hydroxycholesterol (which inhibits sterol synthesis but with little or no inhibition of isoprenylation [34,55,71]) nor squalenyl ester 1 (an inhibitor of cholesterol synthesis at the level of squalene synthase [73]) impede secretion, even when provided at concentrations several orders of magnitude above their reported K_i [73]. Dolichols serve a role chiefly in N-linked glycosylation; however, tunicamycin, which inhibits the latter process, failed to mimic the effects of lovastatin. Additionally, even a major reduction in ubiquinone content of cells does not impede its role in mitochondrial function [36,72]. Nonetheless, for confirmation, we also studied perillal acid, a recently described supposedly selective inhibitor of the isoprenylation of GBPs but not of other proteins such as nuclear lamins [56]. Perillal acid, like lovastatin, reduced insulin release induced by glucose but not that induced by mastoparan or a phorbol ester. The fact that perillal acid (unlike lovastatin) substantially reduced secretion due to α -oxo-4-methylpentanoate is not unexpected because, as indicated above, lovastatin probably cannot completely inhibit the synthesis of non-sterol isoprenoids required for prenylation [33,34,71], whereas perillal acid is said to directly block the isoprenylation process itself. The decrease in insulin content caused by perillal acid might be due to a blockade of GBPs involved in the trafficking of newly synthesized proteins or in secretory granule biogenesis [7], or, alternatively, to an unidentified toxic effect of the drug. Whatever the reason, the effect of perillal acid on exocytotic secretion was dissociable from that on insulin content, as only the former seemed to be rapidly reversible. Furthermore, perillal acid inhibited fractional release. Non-specific effects of this pharmacological probe on exocytosis cannot be excluded but seem unlikely to be critical, as the response to the phorbol ester, TPA, was resistant to inhibition by perillal acid; we have demonstrated that the effects of TPA on insulin release are attributable to exocytotic secretion and are readily inhibited [74].

These findings with isoprenylation inhibitors were extended to the use of blockers of protein carboxyl methylation (AFC; also,

3-deaza-adenosine plus homocysteine) and protein acylation (cerulenin). It has been previously reported that 3-deaza-adenosine and homocysteine reduce insulin release [62]; although this was attributed to a blockade of phospholipid methylation [62], the current studies suggest a new interpretation of studies using such inhibitors, which retard protein methylation as well [35,61]. Pancreatic islets contain a protein methyltransferase which is stimulated by glucose and isobutylmethylxanthine [75]. The finding that GTP[S] promoted base-labile methylation of several islet proteins in an AFC-sensitive fashion in islets (as in other cells; [44,60]), and that acute inhibition of this process retards physiological insulin release, is in accord with the formulation that carboxyl methylation (unlike isoprenylation) is a rapid and potentially reversible [39,40] regulatory step. However, once again, unexpected effects of the pharmacological inhibitors on unrelated processes cannot be totally excluded from contributing to their effects.

PTM inhibitors reduced secretion induced by physiological nutrient agonists, but were ineffective against the direct effects of a rise in intracellular Ca^{2+} (induced by 50 mM K^{+}), a stimulator of protein kinase C (TPA) or mastoparan (a direct activator of GBPs). In fact, AFC and lovastatin potentiated many of these responses. Therefore we speculate that at least one additional inhibitory GBP requiring isoprenylation and carboxymethylation may retard late steps in stimulus–secretion coupling. If so, there would be two classes of GBP involved at two sites, a stimulatory class acting proximally in stimulus–secretion coupling (perhaps at the effects of nutrients on ion channels or phospholipases) and an inhibitory class acting more distally. Such a concept would be in accord with our recent studies using neutrophils and platelets, in which AFC inhibited early receptor-mediated events but not the effects of bypassing receptors using phorbol ester or ionophore [76,77]. PTx-sensitive heterotrimeric GBPs (G_i and G_o) exist in islets and mediate inhibition of insulin release [12,78,79] induced by receptor agonists such as adrenaline. AFC might reduce the methylation of certain γ -subunits of the trimeric GBPs that are carboxyl methylated [80] but not acylated [81]. Indeed, AFC seemed to reduce the methylation of a protein in islets tentatively identified as γ -subunit(s) (Figure 4a); such a blockade would be expected to alter either the membrane attachment [82] of such GBPs or the high-affinity interactions of G-protein $\beta\gamma$ -subunits with α -subunits or their effectors [83]. The results might be direct interference with the activity of an inhibitory GBP. [However, the potentiation of distally acting agonists (TPA, mastoparan) induced by AFC was not reduced by prior PTx pretreatment; furthermore, AFC pretreatment failed to block the inhibitory effects of adrenaline (S. Metz and M. Rabaglia, unpublished work). Therefore PTx and AFC appear to act on different proteins, suggesting the possible presence of another inhibitory GBP, in addition to G_i or G_o , acting distally in exocytosis (' G_{ei} ').] Alternatively, interference with carboxyl methylation might lead to loss from the membrane of α - [84] or $\beta\gamma$ -subunits, which might then directly activate stimulatory effector mechanisms [85] for secretion.

It has been suggested that guanine nucleotides may also directly activate secretion via a putative ' G_E ' [25,86], presumably at terminal steps in exocytosis. G_E might be activated by mastoparan, which (like GTP[S]) directly activates many GBPs. Mastoparan has been reported to stimulate release in insulin-secreting cells; this effect has been said to be either blunted in normal islets [87] or potentiated in transformed β -cells [88] by pretreatment with PTx. In our hands also, mastoparan promoted secretion from islets, whereas mastoparan-17, a chemical analogue that is inactive on GBPs, did not. This secretion was resistant to inhibitors of PTM, but was blunted by PTx. Thus

a stimulatory heterotrimeric GBP might partially mediate the insulin-releasing properties of mastoparan. However, PTx failed to blunt the stimulatory effects of any other agonist (A. Kowluru and S. Metz, unpublished work) [78], raising doubts about the physiological importance of the putative G_E , at least when based solely on observations using mastoparan. If mastoparan does activate G_E , the latter would appear to function despite inhibitors of PTM. One candidate for such a putative GBP might be *rab3a* as (1) *rab3a* may not require the full cascade of PTM to interact with membranes or several specific target proteins [42,89–91]; and (2) an effector domain of *rab3a* stimulates exocytosis [22,23] even though lacking the structural domain required for PTM.

Since this paper was submitted for publication, Li and colleagues [92] reported effects of lovastatin in a different *in vitro* preparation of insulin-secreting cells. Their findings are similar to ours in that lovastatin caused a subcellular redistribution of low- M_r GBPs and a MVA-sensitive inhibition of insulin release. However, their findings differ in that they studied a cultured transformed β -cell line rather than intact rat islets, and found that bombesin- and vasopressin-potentiated secretion were reduced, whereas the direct effect of a mixture of nutrients (glucose, leucine and glutamine) was resistant to inhibition by lovastatin. In their studies [92], lovastatin also had only minimal inhibitory effects on the secretion provoked by TPA, Ca^{2+} or GTP[S] in permeabilized cells (the latter two perhaps being analogous to the use of K^{+} and mastoparan in our studies of intact islets). Lovastatin did not appear to have significant non-specific effects on cytosolic Ca^{2+} levels or receptor function. However, limonene had a number of toxic effects. Inhibitors of carboxyl methylation or acylation were not studied. Despite some differences between the two reports (presumably due in part to the different preparations studied), they are both consistent with the conclusion that inhibition of the function of GBPs in β -cells may retard a proximal step(s) in physiological insulin release, while leaving relatively intact or even potentiating the more distal events in stimulus–secretion coupling.

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