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Research Article

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Evidence of a Role for GTP in the Potentiation of Ca^{2+}-induced Insulin Secretion by Glucose in Intact Rat Islets

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Abstract

Glucose initiates insulin secretion by closing K^+-ATP channels, leading to Ca^{2+} influx (E_1); it also potentiates Ca^{2+}-induced secretion (E_2) when the K^+-ATP channel is kept open using diazoxide and depolarizing concentrations of K^+ are provided. To examine the roles of purine nucleotides in E_2, we compared the effects of glucose to those of the mitochondrial fuel monomethylsuccinate. Either agonist could induce E_2 accompanied by significant increases in ATP, ATP/ADP ratio, and GTP/GDP ratio; GTP increased significantly only with glucose. Mycophenolic acid (MPA), an inhibitor of cytosolic GTP synthesis, markedly inhibited glucose-induced E_2 (either in perifusions or in static incubations) and decreased GTP and the GTP/GDP ratio, but did not alter the ATP/ADP ratio. Provision of guanine (but not adenine) reversed these changes pari passu. In contrast, MPA had no effect on succinate-induced E_2, despite generally similar changes in nucleotides. A similar lack of effect of MPA on E_2 was seen with a second mitochondrial fuel, α-ketoisocaproic acid (KIC). However, in the absence of diazoxide and K^+, MPA blunted the secretory effects of either glucose, succinate, or KIC. These studies suggest that GTP plays a role in both glucose and succinate or KIC-induced insulin secretion at a step dependent on mitochondrial metabolism and the K^+-ATP channel. In addition to mitochondrial effects, glucose appears to have extramitochondrial effects important to its potentiation of Ca^{2+}-induced insulin secretion that are also dependent on GTP. (*J. Clin. Invest.* 1995. 96:811–821.) Key words: guanine nucleotides • adenine nucleotides • stimulus-secretion coupling • diazoxide • succinic acid

Introduction

Glucose is the major physiologic stimulator of insulin secretion from the pancreatic islets. Although the mechanism by which glucose triggers insulin release is still incompletely understood there is considerable evidence to support a central role for purine nucleotides. It is widely accepted that the process requires glucose metabolism leading to an increased ATP content or ATP/ADP ratio and closure of ATP-sensitive K^+ channels (1). Closure of K^+-ATP channels results in depolarization of the plasma membrane with subsequent influx of Ca^{2+} and the initiation of insulin release. This cascade of events is referred to subsequently as Effect 1 (E_1).[1] ATP may have other effects in exocytotic secretion as well (1). We have previously documented that GTP also plays at least a permissive role in physiologic insulin secretion (2). When the GTP content of isolated rat islets was selectively depleted using mycophenolic acid (MPA, a specific inhibitor of cytosolic GTP synthesis), subsequent nutrient-induced insulin secretion was inhibited (2). These effects (on GTP and insulin release) were reversed pari passu by provision of guanine, but not by provision of adenine (which restored ATP but not GTP content).

It has recently been shown by Gembal et al. (3, 4) and Aizawa et al. (5) that glucose can also promote insulin secretion even when the K^+-ATP channel is kept in the open state (using diazoxide [DZX]), provided that the intracellular Ca^{2+} concentration is raised using depolarizing concentrations of K^+ (3) or the Ca^{2+}-channel agonist BAY K 8644 (5). The exact mechanism of action of glucose in this "second effect" to potentiate Ca^{2+}-triggered insulin release (Effect 2 [E_2]) is unknown, although it also has been correlated with increments in the ATP/ADP ratio (4).

The present study asks three questions. First, can E_1 and E_2 potentially be explained by effects of glucose solely on mitochondrial oxidative metabolism? If so, E_1 and E_2 should both be inducible using pure mitochondrial agonists; for this purpose, we studied succinic acid methyl ester (SAME) which is metabolized intracellularly to succinic acid (6), a potent intermediate in the mitochondrial tricarboxylic acid cycle, as well as α-ketoisocaproic acid (KIC), a second mitochondrial fuel. Second, are the effects of glucose and SAME to induce E_2 accompanied by changes in ATP or GTP content, ATP/ADP ratio, and/or the GTP/GDP ratio? Third, is E_2 dependent on GTP content or the GTP/GDP ratio in islets? This question was studied through the use of MPA, which blocks cytosolic synthesis of guanine nucleotides at the level of inosine monophosphate dehydrogenase (2 and references therein).

Methods

Materials. MPA, SAME, succinic acid sodium salt, DZX, KIC, guanine, adenine, nucleotide standards, and monobasic ammonium phosphate (for the HPLC mobile phase) were purchased from Sigma Chemical Co. (St. Louis, MO). Pinacidil was purchased from Research Biochemicals Inc. (Natick, MA). The diluent used for making stock solutions of these drugs was ethanol (for MPA), DMSO (for DZX, pinacidil, guanine, and adenine), and water (for SAME, KIC); control tubes always

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1. *Abbreviations used in this paper:* AUC, area under the curve; df, degree of freedom; DZX, diazoxide; E_1, effect 1; E_2, effect 2; KIC, α-ketoisocaproic acid; MPA, mycophenolic acid; SAME, succinic acid monomethyl ester.

contained an equal amount of the relevant diluent as experimental tubes. [^{3}H(G)]hypoxanthine (17 Ci/mmol) was purchased from New England Nuclear (Boston, MA). HPLC columns were purchased from Alltech Associates Inc. (Deerfield, IL); PEI-cellulose sheets for TLC were obtained from Fisher Scientific Co. (Pittsburgh, PA). RPMI-1640 medium was purchased from GIBCO (Grand Island, NY).

Treatment of pancreatic islets for studies of insulin release. Intact pancreatic islets were isolated from adult male Sprague-Dawley rats as previously described (7), followed by double hand-picking under stereomicroscopic control to eliminate any contaminating acinar tissue. Islets were cultured overnight (18 h) in RPMI-1640 medium (containing 10% FBS, 100 U/ml penicillin, 100 μg/ml of streptomycin, and 11.1 mM glucose), and MPA where indicated. MPA was present during not only the culture period but also was included in islet isolation, wash, and preincubation steps the following day. However, MPA was excluded from the incubation period, to avoid any possible acute drug effects and to be consistent with our previous experiments. However, we have previously documented that the effects of MPA on insulin secretion are not reversible for at least 45 min after removal from the medium (2). 100 μM guanine or 150 μM adenine were added during the entire 18-h culture period in some studies; in others, where indicated, they were provided only during the last 4 h of the 18-h exposure to MPA. Static, batch-type insulin release studies (carried out the next day) consisted of a 45-min preincubation period at 37°C in KRB (pH 7.4 and gassed with 95% O_2/5% CO_2) containing 3.3 mM glucose, 25 μg/ml MPA, 150 μM adenine, or 100 μM guanine (where present), and 75 or 250 μM DZX (or 500 μM pinacidil) where present. The exposure to DZX or pinacidil was continued through the incubation period. Media for the subsequent 45-min incubation period was KRB (pH 7.4 and gassed with 95% O_2/5% CO_2) containing 3.3 mM glucose (for basal release), 16.7 mM glucose (for glucose-stimulated release) or 3.3 mM glucose plus 20 mM SAME or 20 mM KIC (as indicated for each experiment), and 40 mM KCl for E_2 studies. In studies using SAME, the drug was generally present in both the preincubation and incubation periods, since the time required for its uptake and hydrolysis intracellularly to succinate is unknown. However, in preliminary studies we observed that its effects on secretion persisted during such time periods; furthermore, its effects on nucleotides and insulin secretion under these conditions were similar to those seen after adding SAME only during the incubation period. The pH of these media containing SAME were adjusted to $\sim$ 7.4. In addition, 5 mM Hepes was also present in some studies using SAME in both the preincubation and incubation periods (unless otherwise noted) to help further stabilize the pH. Insulin content of media was measured by RIA as described (7).

Assessments of insulin release during perifusions. Groups of 100 islets were transferred into each of four perifusion chambers and were perifused using a previously described procedure (2). In brief, perifusion medium was pumped using a peristaltic pump (Manostat Corp., New York, NY) through Teflon tubing (Tygon; Fisher Scientific Co.) to in-line Swinney filter holders (Fisher Scientific Co.) containing polyethylene filters (62 μM pore size; Tetko Co., Briarcliff Manor, NY). The islets were first perifused for 45 min at 3.3 mM glucose to establish a stable basal rate of insulin secretion (unless otherwise noted). Where present, 250 μM diazoxide was included in the preincubation period as well as the ensuing incubation period. The incubation period was for 60 min. The perifusion medium consisted of KRB containing 0.2% BSA, gassed with 95% O_2, 5% CO_2, and the agonists (including 40 mM KCl) as indicated in Results. The medium was pumped at a flow rate of 1 ml/min. Samples of the effluent were collected every 2 min using a fraction collector (Gilson Medical Electronics Inc., Middleton, WI). The dead space of our system is $\sim$ 4 ml, and the data are corrected for this. The perifusate was analyzed for insulin using previously described methods (2).

Determination of nucleotide content of islets. 200 islets per condition were cultured overnight (18–20 h) in RPMI-1640 medium (containing 10% FBS, 100 U/ml penicillin, 100 μg/ml of streptomycin, 5 mM Hepes, 2 μCi/ml[^{3}H]hypoxanthine and 11.1 mM glucose) and MPA when indicated. We have previously shown that [^{3}H]hypoxanthine can be used to label both adenine and guanine nucleotides through the salvage pathway of purine nucleotide synthesis (8). After the culture period, islets were washed five times with 1 ml ice-cold KRB (pH 7.4, gassed with 95% O_2/5% CO_2, and containing 16.7 mM glucose and 0.2% BSA). The islets were then preincubated and incubated as in the insulin release studies (see above). In most studies, 20 mM succinic acid sodium salt (which does not enter the islet; reference 6) was used as the osmotic control for tubes containing SAME. In some studies 10 mM NaCl was used as the osmotic control; there was no difference in results using either method.

After incubation, the islets were placed immediately on ice, the media was removed, and 300 μl of ice-cold 0.6 N TCA was added to each tube. The islets were then extracted as previously described in detail (2). Samples were immediately analyzed by HPLC and TLC, or frozen at −70°C for future analysis within 2 wk. (No difference in results was discernible between the two handling procedures.)

HPLC for the nucleotide triphosphates was carried out as previously described (2). Briefly, the compounds were separated using ammonium phosphate buffers (buffer A was 0.5 mM, pH 2.6; buffer B was 0.65 M, pH 2.6) on an anion exchange column. For the nucleotide diphosphates, buffer A was 1 mM (pH 3.1) and buffer B was 0.65 M (pH 2.9) ammonium phosphate. Percentages of buffers A and B were varied each day as was the pH, to optimize separation of compounds. Separations were achieved using isocratic elution at 1.0 ml/min flow rate. Ultraviolet (UV) absorbance was monitored at 254 nm. 50 μl of extract was injected for the nucleotide triphosphates and 100 μl for the diphosphates. Quantitation of nucleotides was performed by a Hewlett-Packard 3365 Series II Chemstation (Palo Alto, CA) using an external standard curve and peak height integration. Standard curves for the di- and triphosphates were carried out each day. CTP and ITP were added as internal standards, as described previously, to calculate (and correct for) recoveries (2).

ATP/ADP and GTP/GDP ratios were determined by two methods. First, the mass (as determined by the UV absorbance of compounds after HPLC) was used to calculate ratios. Alternatively, 50 μl of extract, containing a mixture of 25 nmol each of unlabeled ATP, GTP, ADP, and GDP as carriers, was placed on PEI-cellulose TLC sheets. The sheets were placed in a TLC tank containing 200 ml of 0.75 M potassium phosphate buffer (pH 3.65). The TLC plates ran $\sim$ 3 h until the solvent front was $\sim$ 1 cm from the top. The sheets were then allowed to completely dry. The nucleotides were visualized under UV light and the spots were carefully marked. Spots containing tritiated nucleotides (derived from [^{3}H]hypoxanthine) were cut out and placed in glass scintillation vials containing 8 ml scintillation cocktail. The samples were vortexed vigorously for 30 s and counted for 5 min. Ratios were then calculated from the radioactivity counted in each nucleotide. These two methods gave comparable results, although ATP/ADP and GTP/GDP ratios obtained from TLC were generally smaller than those obtained from the HPLC data (see Results and Discussion). The NTP/NDP ratios reported in the Results section are from the TLC method unless otherwise stated. In addition, eluant trinucleotide HPLC fractions were collected every 18 s and were matched against the concomitant UV chromatograms as previously described (8) for calculation of specific activities. Samples were counted for 5 min in 4 ml of scintillation cocktail.

Data presentation and statistical analysis. Data are expressed as mean±SEM, with (*n*) representing the number of observations in a representative study, or the number of independent experiments, as indicated. Data are normalized to the number of islets per tube since, in preliminary studies, we observed that results were not altered by expressing that data in terms of protein content per tube. Total nucleotide content is expressed as picomole per islet; specific activity is expressed as disintegrations per min per picomole (using the HPLC fractions). Absolute insulin release is expressed as μU/45 min per 10 islets; incremental insulin release was calculated 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. Thus, incremental E_2 insulin release was calculated as the stimulated insulin release (e.g., at

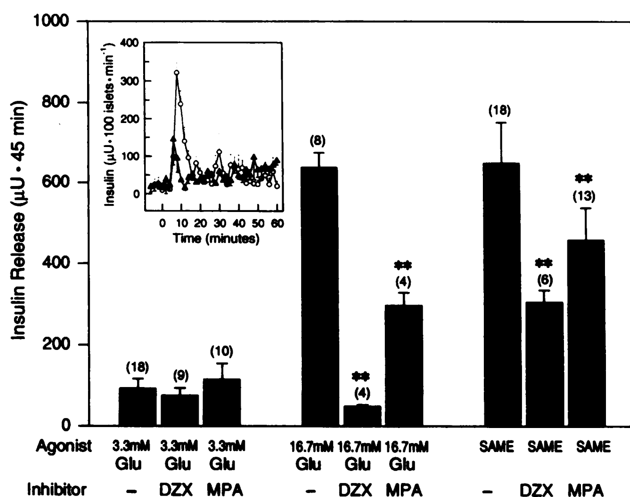

Figure 1. Effects of diazoxide (250 μM; DZX) or mycophenolic acid (25 μg/ml; MPA) on glucose-induced or succinic acid monomethyl ester (20 mM; SAME)-induced insulin secretion in static incubations. SAME was provided at 3.3 mM glucose. Data are expressed as the mean±SEM for the number of determinations (indicated in parentheses) from three separate experiments. * $P < .05$, ** $P < .001$ vs 16.7 mM glucose or SAME alone. Statistical analyses were performed on the incremental insulin secretion above basal. Inset shows insulin secretion induced by 20 mM SAME in perifusions in the absence (○——○) or presence of MPA (▲——▲). Data represent the mean±SEM of three separate perifusion experiments. Statistical analyses were performed on the incremental AUC as described in Methods.

16.7 mM glucose or 20 mM SAME) minus the mean control value (e.g. 3.3 mM glucose alone), all in the presence of DZX and K^+. In the perifusion studies, the integrated incremental areas under the curve (AUC) above the basal rate for insulin secretion was calculated using the mean of the last four basal values (at 3.3 mM glucose) and the trapezoid rule. Insulin secretion is expressed as μU·100 islets·min^{-1} in the perifusate. The data are expressed as mean±SEM with (n) representing the number of separate experiments or the number of determinations as indicated. Statistical analyses of insulin secretion were performed on the incremental insulin release, unless otherwise noted. Statistical analyses were by paired or nonpaired t tests, or the Mann-Whitney test, as appropriate.

Results

Glucose-induced insulin secretion. We first confirmed that glucose requires closure of the K^+-ATP channel to permit expression of its effects. For the purposes of this paper, we define E_1 as insulin release directly caused by closure of the K^+-ATP channel, leading to Ca^{2+} influx and subsequent insulin secretion. Diazoxide is a selective opener of islet K^+-ATP channels (9, 10) and was used to study insulin secretion dependent on closure of that channel (i.e., E_1). We define E_2 as the effect of glucose (or other fuels) to potentiate Ca^{2+}-induced secretion; the latter may either be subsequent to E_1 or to depolarizing concentrations of K^+ when the K^+-ATP channel is kept open with diazoxide. In these studies (using 45-min static incubations), the effects of glucose (or SAME) provided alone (i.e., in the absence of DZX and high K^+) therefore represents the combination of $E_1 + E_2$.

As shown in Fig. 1, DZX had no effect on basal insulin secretion at 3.3 mM glucose. Insulin secretion increased 10-fold in islets stimulated by 16.7 mM glucose; this stimulation was blocked virtually totally in the presence of DZX (Fig. 1). Therefore, the data imply that (a) glucose initiates insulin secretion via K^+-ATP channel closure (E_1); and (b) in the absence of E_1 (i.e., an increased cytosolic Ca^{2+} concentration), glucose has little or no effect to promote insulin release.

We next isolated E_2, by providing 40 mM KCl (in the presence of DZX) to increase cytosolic Ca^{2+} levels and bypass the effect of the K^+-ATP channel (3). The addition of 40 mM KCl to islets incubated in the presence of 3.3 mM glucose and 250 μM DZX resulted in the initiation of insulin secretion—control levels of 93±24 rose to 728±58 μU insulin/45 min ($P < .001$; degree of freedom (df) = 7). This confirms earlier findings that DZX is without direct effect on Ca^{2+}-induced insulin secretion (3). When high glucose (16.7 mM) was added to this effect of K^+, insulin secretion increased an additional 96%, confirming the presence of the "second effect" (E_2) of glucose to potentiate insulin secretion even when the K^+-ATP channel is kept open (Fig. 2 A).

Similar effects were seen when insulin secretion was assessed using perifusions. Addition of 40 mM K^+ alone increased insulin secretion at 3.3 mM glucose in the presence of diazoxide (Fig. 2 B). Addition of 16.7 mM glucose induced an additional 150% increase in insulin secretion above control responses (i.e., E_2; Fig. 2 B). The integrated area under the curve for the insulin response to 3.3 mM glucose was 9,162±434 μU and increased to 23009±1277 μU at 16.7 mM glucose ($P < .001$, df = 5). This E_2 effect was sustained over the entire 60 minutes of incubation in high glucose.

Effects of glucose on purine nucleotides. Numerous studies (1, 11, 12) suggest glucose can acutely increase ATP and ATP/ADP, and, with less certainty, GTP and the GTP/GDP ratio, albeit with considerable variability in the degrees observed. In preliminary studies, we confirmed that glucose by itself increases each of these parameters after 45 min stimulation (ATP increased 18%, ATP/ADP by 91%, GTP/GDP by 21%; $P < .05$ for each; GTP increased insignificantly by 10%, $P = .12$; df = 6; data not shown). However, the current studies focus on the role of the nucleotides in E_2. In islets incubated with diazoxide and high K^+ and stimulated with 16.7 mM glucose, ATP content increased 62% and the ATP/ADP ratio increased by 110%, compared to findings at 3.3 mM glucose in the presence of K^+ and DZX (Fig. 3 A). Total GTP content increased significantly by 41% and the GTP/GDP ratio was stimulated by 76% over that seen at 3.3 mM glucose (Fig. 3 B). Addition of 13.4 mM 3-O-methylglucose to 3.3 mM glucose failed to reproduce these stimulatory effects of 16.7 mM glucose (data not shown), indicating that the stimulation of nucleotides by glucose was not a nonspecific effect of a hexose or of changes in osmolarity.

Role of GTP in glucose-induced insulin release. We previously demonstrated that there is (at least) a permissive role for GTP in glucose-induced insulin release (2), using MPA and similar pharmacologic probes. Since GTP and the GTP/GDP ratio increased during glucose-induced E_2, we next studied the role of GTP (and the GTP/GDP ratio) in E_1 and E_2 using MPA, in both static incubations and perifusions. The data depicted in Fig. 1 demonstrate that 25 μg/ml MPA had no effect on basal insulin secretion. MPA reduced by 68% the insulin secretion induced by 16.7 mM glucose (without diazoxide and K^+) (Fig. 1), confirming our previous studies (2). MPA had no effect on control insulin secretion at 3.3 mM glucose in the

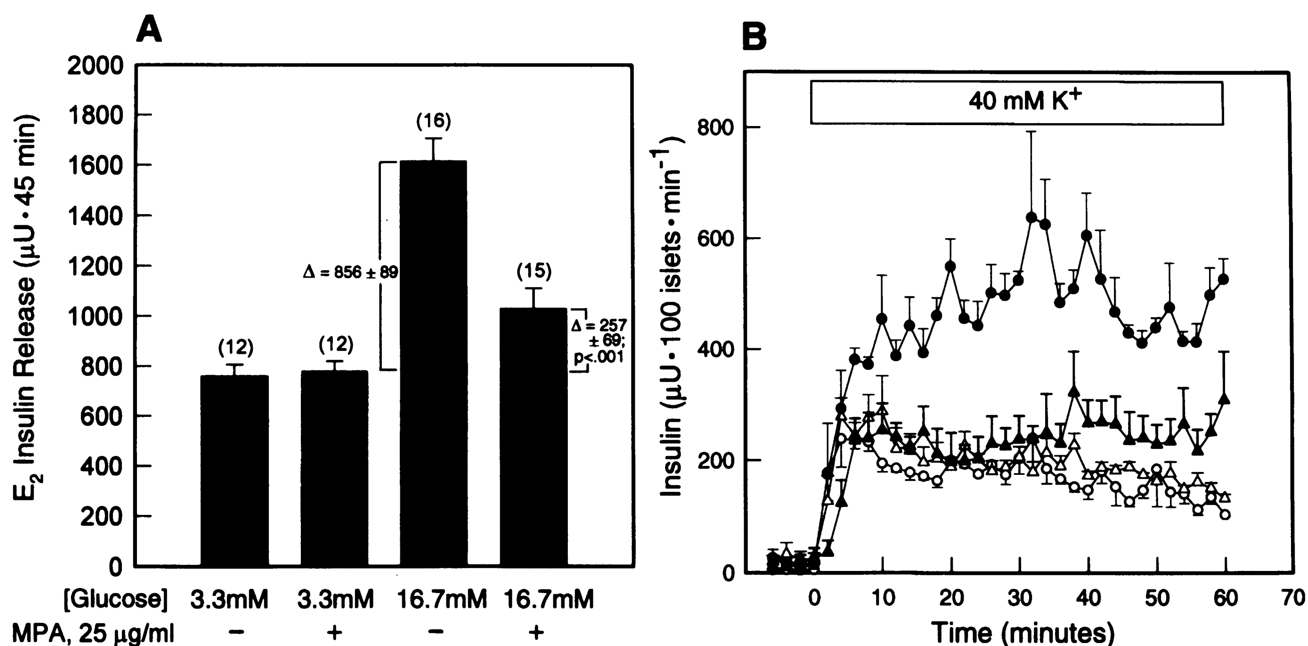

Figure 2. Effect of MPA on glucose-induced insulin secretion in the presence of 250 μM diazoxide and 40 mM KCl (E_2 condition). Data from static incubations shown in *A* are expressed as the mean±SEM for the number of determinations (indicated in parentheses) from two separate experiments. Statistical analyses were done on the incremental insulin secretion above respective basal values (= Δ). *B* shows the insulin response obtained during perifusions. 3.3 mM glucose (○——○); 3.3 mM glucose + MPA (△——△); 16.7 mM glucose ((●——●); 16.7 mM glucose + MPA (▲——▲). Each point represents the mean±SEM of four separate perifusion experiments. Statistical analyses were performed on the incremental AUC as described in Methods.

presence of DZX and K^+; thus, MPA does not block the effects of 40 mM K^+ alone (confirming our previous studies which showed a lack of effect of MPA on K^+-induced insulin secretion in the absence of DZX; reference 2). However, glucose-induced E_2 insulin secretion was reduced by 70%, suggesting that guanine nucleotides play a role in E_2 (Fig. 2 *A*). Additionally, these data imply a role for GTP in E_1 alone as well, since the inhibition of E_2 was quantitatively similar to that seen in the simultaneous presence of E_1 plus E_2 (see below).

Similar results were obtained in perifusion studies in the presence of diazoxide and 40 mM K^+ (Fig. 2 *B*). MPA again had no effect on control insulin release at 3.3 mM glucose, providing further evidence that MPA does not block K^+-induced insulin secretion (integrated AUC for control = 9,162±434 μU vs 9,346±538 μU in the presence of MPA). However, in the presence of 16.7 mM glucose, MPA inhibited insulin secretion throughout the 60-min incubation, resulting in an inhibition of net insulin release of 52% (23,009±1,277 μU in the absence of MPA vs 10975±1784 μU in the presence of MPA; P = .002, df = 6).

The corresponding nucleotide data using MPA in the presence of DZX and K^+ are shown in Fig. 3. In this setting, MPA

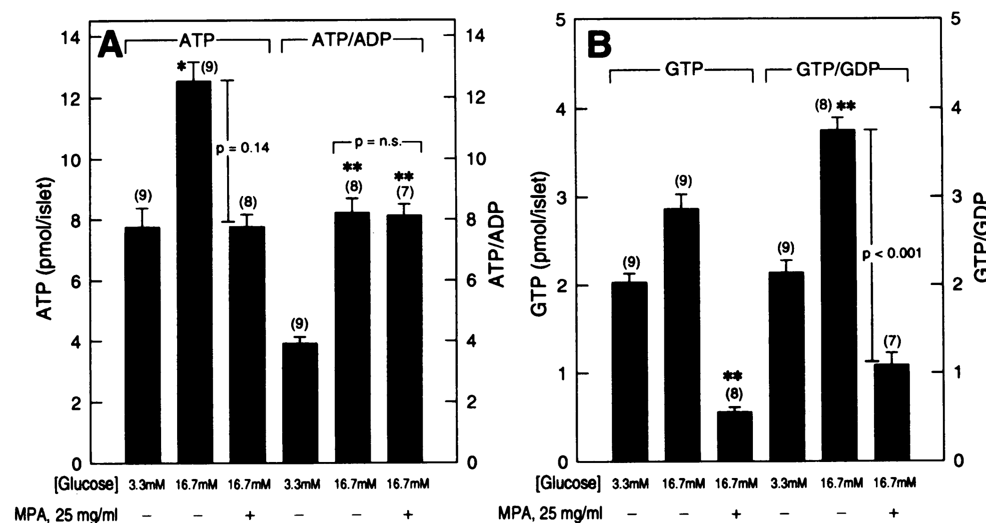

Figure 3. Effect of MPA on ATP and ATP/ADP ratio (*A*) and GTP and GTP/GDP ratio (*B*) in the presence of 16.7 mM glucose, 250 μM DZX, and 40 mM KCl. Data are expressed as the mean±SEM for the number of determinations (indicated in parentheses) from three separate experiments. ATP/ADP and GTP/GDP ratios were calculated from TLC (see Methods) $*P < .05$, $**P < .001$ vs 3.3 mM glucose alone.

Table I. Specific Activities of [^{3}H]GTP and [^{3}H]ATP Stimulated by Glucose or SAME, in the Presence of 250 μM Diazoxide and 40 mM K^+, and in the Presence or Absence of MPA Pretreatment

Condition	ATP	GTP
	dpm/pmol	*dpm/pmol*
3.3 mM glucose	151±6	122±15
3.3 mM glucose + MPA	121±7*	48±6*
16.7 mM glucose	143±8 (9)	104±11 (9)
16.7 mM glucose±MPA	111±4 (8)‡	42±8§ (8)
20 mM SAME	130±7 (8)	91±6 (9)
20 mM SAME + MPA	98±6§ (6)	31±5§ (6)

[^{3}H]hypoxanthine (2 μCi/ml) was present during 18-h culture period only; MPA (25 μg/ml) was present throughout the culture, preincubation, and incubation periods. Number in parenthesis represents number of determinations (mean±SEM) from four separate experiments. *$P < .05$ from 3.3 mM glucose alone; $^‡P < .05$ from 16.7 mM glucose alone; $^§P < .001$ from 16.7 mM glucose or 20 mM SAME alone.

reduced total ATP content by 38%; however, there was no significant change in the ATP/ADP ratio compared to control (16.7 mM glucose without MPA; Fig. 3 *A*). In contrast, MPA reduced not only GTP content (−80%) but also decreased the GTP/GDP ratio significantly (−71%; Fig. 3 *B*). Thus, the GTP/GDP ratio, but not the ATP/ADP ratio, was correlated with the decrease in insulin secretion seen under these conditions. MPA also reduced the specific activities of ATP and GTP (Table I), and of ADP and GDP (data not shown), indicating that its effect was principally, if not totally, on cytosolic pools of purine nucleotides (i.e., synthesized de novo and via the salvage pathway), the synthesis of which is accessible to inhibition by MPA. These data are consistent with our previous experience with MPA in the absence of DZX and K^+ (8).

To examine further the importance of guanine nucleotides in insulin secretion in the presence of DZX and K^+, islets were cultured with MPA in the presence of 100 μM guanine or 150 μM adenine either for the entire 18-h culture period or for the last 4 h of culture. Data regarding insulin secretion subsequently stimulated by 16.7 mM glucose are shown in Fig. 4. Addition of guanine for 18 h prevented the inhibition of insulin secretion induced by MPA by restoring glucose-induced insulin release back to 76% of that in control islets (P = NS; Fig. 4). In contrast, adenine had no restorative effect on MPA-inhibited insulin secretion. Similar results were obtained when adenine or guanine were present only for the last 4 h of MPA exposure (glucose-induced insulin secretion in the presence of 100 μM guanine for 4 h was restored to 77% of that in control islets; P = NS vs no MPA; df = 10), suggesting that guanine could not only prevent, but also reverse, the effects of MPA.

Furthermore, the addition of guanine (for 4 h) not only restored GTP content to 177% of that seen with control islets, but also restored the GTP/GDP ratio to 165% of control (Table II) and restored insulin secretion, as indicated above. Addition of adenine restored ATP content to 88% of control and maintained the ATP/ADP ratio at 103% of control (Table II), but did not restore secretion at all. When guanine or adenine was present for the entire 18-h culture period, GTP levels were restored to 112% and ATP levels were restored to 107% of control, respectively (data not shown), which were similar to

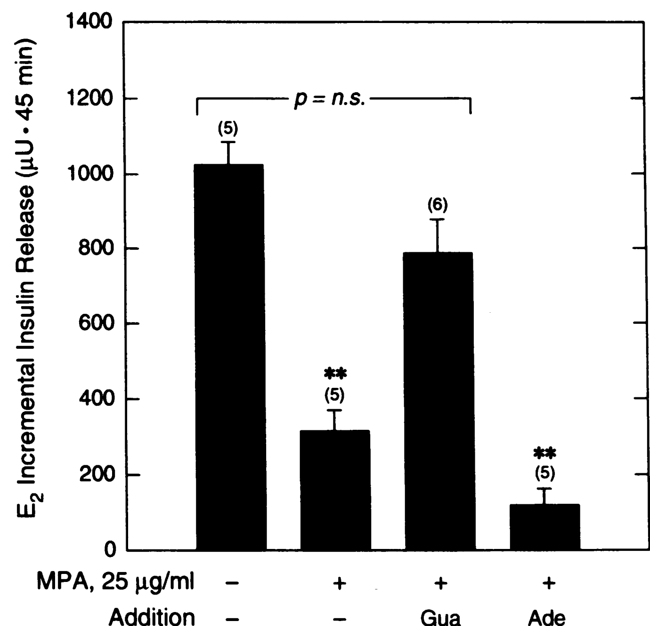

Figure 4. Effect of MPA in the presence or absence of guanine (100 μM; *Gua*) or adenine (150 μM; *Ade*) on subsequent glucose-induced insulin secretion under E_2 conditions. Guanine and adenine were present during the 18-h culture period as well as in preincubation period the following day. Data are expressed as the incremental insulin response above basal (3.3 mM glucose alone) and represent the mean±SEM for the number of determinations (indicated in parentheses). ** $P < .001$ vs 16.7 mM glucose alone.

the results seen at 4 h (except for the lack of overshoot of GTP). Again, insulin secretion was restored by guanine but not adenine, despite the preservation by the latter of ATP content and the ATP/ADP ratio. Thus changes in GTP content and/or the GTP/GDP ratio, but not in ATP content or the ATP/ADP ratio, corresponded to the effects of guanine (and adenine) on the inhibition of insulin secretion induced by MPA. It can be noted that both the ATP/ADP and GTP/GDP ratios as measured by mass (see Table II) tended to be numerically higher than those calculated by the dpms from TLC (see Fig. 3 and Discussion). However, ratios measured by either technique changed similarly with the different experimental conditions.

We have previously shown that the pyrimidine nucleotide UTP is increased by MPA treatment, most likely due to increased availability of its precursor 5-phosphoribosyl-1-pyrophosphate (2). In the current studies, UTP was also stimulated (+83%) by 16.7 mM glucose as compared to 3.3 mM glucose in the E_2 setting (Table III). Addition of MPA at 16.7 mM glucose resulted in a further increase in UTP of 110% (Table III). This effect of MPA on UTP content was reversed completely by addition of guanine and was partially reversed by adenine (Table II). Thus under E_2 conditions (as previously observed in the absence of DZX and K^+), UTP itself does not bear any evident relationship to insulin secretion.

Succinate-induced insulin secretion. When islets were incubated in the presence of 20 mM SAME, insulin secretion was stimulated from 75±22 to 632±98 μU insulin/45 min ($P < .001$, df = 28), a similar response as that seen with glucose. Coprovision of 250 μM DZX to islets stimulated by SAME resulted in a 72% decrease in insulin secretion (Fig. 1). Thus SAME, like glucose, also exerts the majority of its effect to

Table II. *Effect of Guanine or Adenine on Nucleotide Content and Ratios in the Presence of MPA, 250 μM DZX, and 40 mM K+*

Condition	ATP	ATP/ADP	GTP	GTP/GDP	UTP
	pmol/islet		*pmol/islet*		*pmol/islet*
16.7 mM glucose	10.47±0.3 (9)	11.10±0.7 (6)	2.39±0.1 (9)	4.02±0.2 (5)	2.40±0.1 (9)
16.7 mM glucose + MPA	7.85±0.3* (8)	160.86±0.9 (5)	0.68±0.03* (8)	2.38±0.4‡ (5)	5.25±0.1* (9)
16.7 mM glucose + MPA + guanine	9.61±0.4 (9)	11.51±1.3 (6)	4.22±0.2* (9)	6.62±0.8‡ (6)	2.61±0.09 (9)
16.7 mM glucose + MPA + adenine	9.18±0.4 (9)	11.40±0.7 (6)	0.60±0.01* (9)	1.94±0.2* (6)	3.40±0.09* (9)

Islets were cultures 18° in presence of 25 μg/ml MPA (except for control condition) alone. During the last 4° of culture, 100 μM guanine or 150 μM adenine were added. MPA, guanine, and adenine were also present during the preincubation and incubation periods. Number in parentheses represents number of determinations (mean±SEM) from three separate experiments. ATP/ADP and GTP/GDP ratios were calculated from mass measurements from HPLC (see Methods). $*P < .001$ from 16.7 mM glucose alone; $^{‡}P < .05$ from 16.7 mM glucose alone.

stimulate insulin secretion ($E_1 + E_2$) via processes requiring closure of the K^+-ATP channel. In the presence of DZX and K^+, 20 mM SAME increased insulin secretion by 58% over control values at 3.3 mM glucose alone (i.e. E_2; Fig. 5 *A*). This was also true when studied using perifusions (Fig. 5 *B*). In the presence of DZX and K^+, SAME potentiated insulin secretion by 69% ($P < .001$, df = 4), a somewhat lesser response than with 16.7 mM glucose. SAME potentiated insulin secretion within the first 2 min and maintained an augmentation of insulin release throughout the remainder of the perifusion. In contrast, SAME in the $E_1 + E_2$ condition has been reported primarily to increase the first phase of insulin secretion (13). Indeed, when 20 mM SAME was provided to islets in the absence of DZX and K^+, the majority of insulin release occurred during the first 14 min (Fig. 1, *inset*). Taking into account the need for SAME to permeate β cells (and specifically their mitochondria) and to be hydrolyzed to succinate, this likely represents a stimulation of first phase insulin release. In contrast, very little stimulation of second phase insulin release was observed. Thus SAME shares the ability of glucose to potentiate Ca^{2+}-induced insulin secretion when the K^+-ATP channel is blocked (E_2), including sharing a similar time course for that effect; however, it may not be able to fully mimic glucose's effect to induce a second phase of secretion.

Diazoxide is felt to be a relatively specific opener of K^+-ATP channels. However, high concentrations of DZX have been reported to inhibit succinate dehydrogenase (14, 15), the glycerol phosphate shuttle (16), and mitochondrial Ca^{2+} accumulation (17), which could provide alternative explanations for some of the results above. Therefore, to confirm the results with DZX, we studied a structurally dissimilar, selective opener of the K^+-ATP channel, pinacidil (10, 18). Pinacidil (500 μM), like DZX, had no effect on basal insulin secretion at 3.3 mM glucose; however, pinacidil blocked SAME-induced insulin secretion by 98% (Table IV). When depolarizing concentrations of K^+ were added to islets incubated with pinacidil, SAME was again able to potentiate insulin secretion (Table IV). We also performed some studies using 75 μM DZX, a concentration well below that reported to alter mitochondrial metabolism (15, 17). 75 μM DZX reduced SAME-induced insulin secretion by 98% (control 554±14 μU insulin/45 min vs 12±8 μU insulin with 75 μM DZX; $P < .001$; df = 11). Addition of SAME to 75 μM DZX plus KCl resulted in additional insulin secretion (330±22 μU insulin/45 min control to 1,270±48 μU insulin; $P < .001$, df = 22); the latter value was not significantly different from that seen using the higher concentrations (250 μM) of DZX. Thus, the analysis of E_1 and E_2 using DZX appear to be appropriately specific for its effect on the K^+-ATP channel.

Effect of SAME on nucleotides. Preliminary data during stimulation of islets with SAME alone ($E_1 + E_2$) did not indicate a clear increase in either total ATP or GTP content at 45 min; however, there was a 54% increase in the ATP/ADP ratio ($P = .05$; df = 6) and a 19% increase in the GTP/GDP ratio ($P = .089$; df = 6). In the presence of diazoxide and K^+, SAME significantly increased both total ATP content (+39%) and the ATP/ADP ratio (+91%; Fig. 6 *A*). There was a small, insignificant increase in GTP content (+12%), but a significant 35% increase in the GTP/GDP ratio (Fig. 6 *B*). Thus, like glucose, SAME stimulates both nucleotide ratios in the E_2 setting. Interestingly, SAME tended to stimulate ATP content and the ATP/ADP ratio more than did glucose; conversely glucose stimulated GTP and the GTP/GDP ratio more than did SAME (see Discus-

Table III. *Effect of Glucose and SAME in the Presence and Absence of MPA Pretreatment on UTP Levels, All in the Presence of 250 μM DZX and 40 mM K+*

Condition	UTP
	pmol/islet
3.3 mM glucose	1.51±0.13 (6)
16.7 mM glucose	2.77±0.21* (9)
16.7 mM glucose + MPA	5.22±0.48‡ (8)
20 mM SAME	2.48±0.25* (9)
20 mM SAME + MPA	5.71±0.64‡ (6)

MPA (25 μ/ml) was present throughout the 18-h culture, preincubation and incubation periods. Number in parentheses represents number of determinations (mean±SEM) from four separate experiments. $*P < .001$ vs 3.3 mM glucose; $^{‡}P < .001$ vs agonist without MPA.

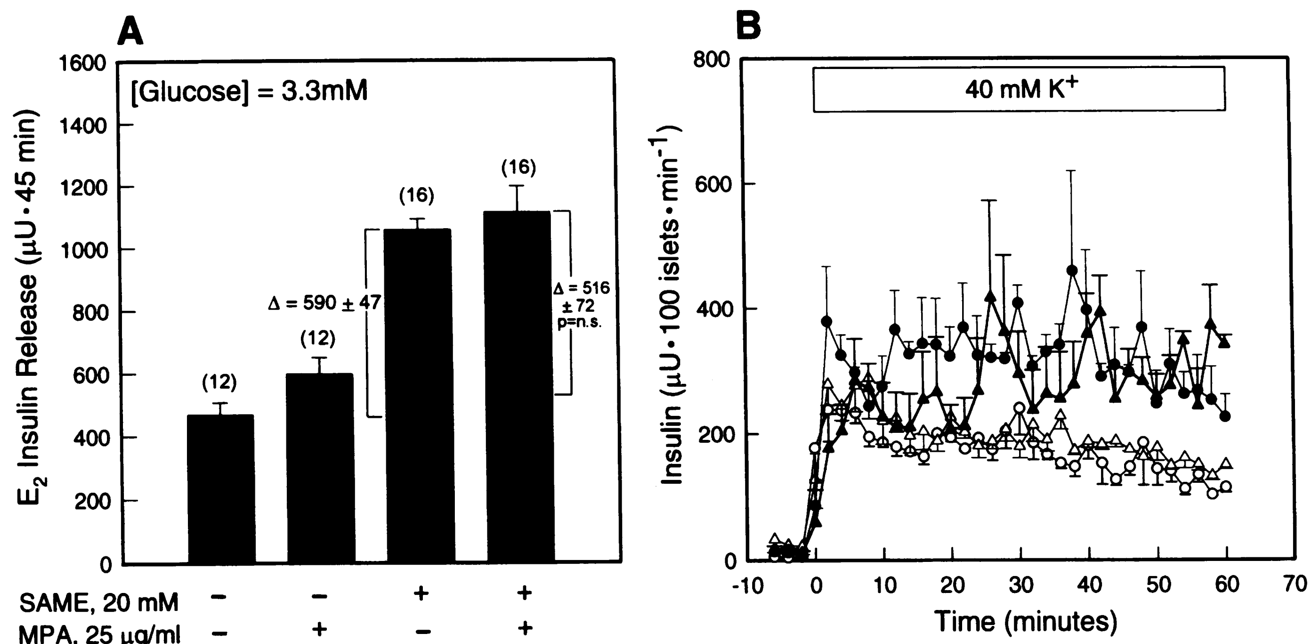

Figure 5. Effect of MPA on SAME-induced insulin secretion in the presence of 250 μM DZX and 40 mM KCl. Data from static incubations shown in *A* are expressed as the mean±SEM for the number of determinations (indicated in parentheses) from two separate experiments. Statistical analyses were done on the incremental insulin secretion above basal (=Δ). *B* shows the insulin response to obtained during perifusions. 3.3 mM glucose (○——○); 3.3 mM glucose + MPA (△——△); 20 mM SAME (●——●); 20 mM SAME + MPA (▲——▲). Each point represents the mean±SEM of three separate perifusion experiments. Statistical analyses were performed on the incremental AUC as described in Methods.

sion). However, these differences did not reach statistical significance.

Role of GTP in SAME-induced insulin release. To explore a possible role for GTP in SAME-induced insulin secretion, MPA was again provided during the culture and preincubation periods first in the absence of DZX and K^+. SAME-induced insulin secretion was inhibited 51% by MPA in static incubations (Fig. 1). MPA also reduced the effect of SAME (by 45%) in perifusions, albeit mostly during the first 14 min (Fig. 1, *inset;* integrated AUC SAME alone = 1,582±109 vs AUC SAME + MPA = 457±199 during 0–14 min; P = .008; df = 4). These effects were somewhat less than the inhibition by MPA of glucose's effects under E_1 + E_2 conditions (−67%; Fig. 1). However, in the presence of DZX and K^+, addition of MPA had no effect on subsequent SAME-induced insulin secretion in static incubations (Fig. 5 *A*) or in perifusions (Fig. 5 *B;* integrated AUC for SAME: 15,514±1397 vs AUC for SAME + MPA = 13,010±1,331; P = .264, df = 4). This is in marked contrast to the 71% inhibition of glucose-induced insulin secretion (E_2) produced by MPA. Since MPA did not reduce succinate-induced E_2 at all, but reduced E_1 plus E_2 by 51%, one can infer that MPA reduces E_1, a conclusion consonant with the perifusion results carried out in the absence of DZX and K^+. These findings imply a role for GTP in the events triggered by K^+ channel closure.

Table IV. Effect of 500 μM Pinacidil on Basal and SAME-induced Insulin Secretion

Experimental condition	Insulin release
	μU/45 min
(1*a*) 3.3 mM glucose alone (12)	72±14
(*b*) 3.3 mM glucose + pinacidil (12)	39±8
(2*a*) SAME (20 mM) alone (24)	562±42
(*b*) SAME + pinacidil	13±7*
(3*a*) 3.3 mM glucose + pinacidil + 40 mM KCl (18)	32±9
(*b*) 20 mM SAME + pinacidil + 40 mM KCl (18)	408±28*

Batches of 10 islets were incubated 45 min in KRB medium containing 3.3 mM glucose alone or ±20 mM SAME, ±500 μM pinicidil, ±40 mM KCl as indicated. Values are the mean ±SEM for the number of determinations (in parentheses) from two separate experiments. *P < .001.

The effect of MPA on nucleotides (in the presence of DZX, K^+, and SAME) are shown in Fig. 6. MPA significantly decreased total ATP content by 25%; however, there was no effect of MPA on the ATP/ADP ratio. These results are very similar to (and not statistically different from) the results seen with MPA treatment of glucose-stimulated islets. Islet GTP content was significantly decreased by MPA treatment (−72%) and the GTP/GDP ratio was reduced by 57%. As with glucose, these ATP/ADP and GTP/GDP ratios, and their response to MPA as assessed by TLC, were confirmed using mass ratios from HPLC (data not shown). Again, the mass ratios tended to be numerically higher than those calculated from TLC but changed similarly under the different experimental conditions (see Discussion). These effects of MPA on guanine nucleotides stimulated with SAME tended to be less than the effects of MPA on glucose-stimulated islets; however, these differences did not achieve statistical significance. As with glucose as agonist, the specific activities of both the guanine and adenine nucleotides in the presence of SAME were reduced by MPA (Table I).

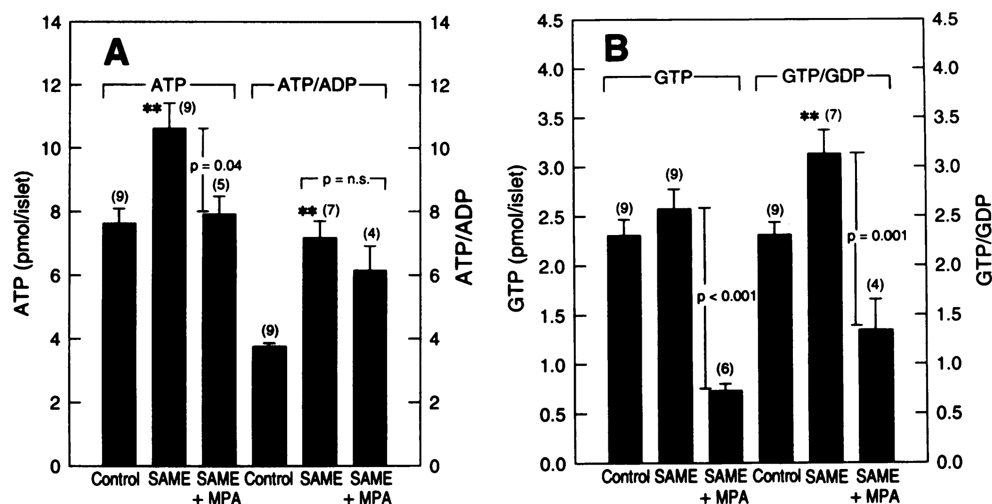

Figure 6. Effect of MPA on ATP and ATP/ADP ratio (*A*) and GTP and GTP/GDP ratio (*B*) in the presence of 20 mM SAME, 250 μM diazoxide, and 40 mM KCl. Data are expressed as the mean±SEM for the number of determinations (indicated in parentheses) from three separate experiments. ATP/ADP and GTP/GDP ratios were calculated from TLC (see Methods) $*P < .05$, $**P < .001$ vs control condition (20 mM succinic acid sodium salt in 3.3 mM glucose) alone.

SAME (20 mM), in the E_2 setting, increased UTP content by 64% which was similar to 83% increase produced by glucose (Table III). Addition of MPA resulted in a further 130% increase in UTP which, again, was not different from that found with glucose as agonist (Table III).

The inability of MPA to block SAME-induced insulin secretion in the K^+ (ATP) channel–independent setting was confirmed using another pure mitochondrial fuel, KIC. We have previously demonstrated that KIC-induced insulin secretion is significantly decreased by MPA in the $E_1 + E_2$ setting (2). In the E_2 setting, KIC (20 mM), like SAME, also produced (during static incubations) a potentiation of insulin release (610±51 μU insulin/45 min at 3.3 mM glucose vs 1,030±51 μU insulin with 20 mM KIC; $P < .001$; df = 10). Pretreatment with MPA had no effect on KIC-induced insulin secretion (E_2 = 1,030±51 vs 1,078±24 μU insulin/45 min; P = NS; df = 10), verifying the results obtained with SAME. These results using KIC were confirmed in preliminary perifusion studies as well (data not shown).

Discussion

The present studies were designed to examine further the role of purine nucleotides in both the initiation of insulin secretion via the K^+-ATP channel (E_1) and, for the first time, to extend these studies to the potentiation of Ca^{2+}-induced insulin release (distal to the K^+-ATP channel; E_2) by glucose. Since glucose stimulates both glycolysis in the cytosol and oxidative metabolism in the mitochondria, we compared the effects of glucose not only to those of succinate, but also to those of KIC, a second pure mitochondrial agonist. Our studies of GTP content and the GTP/GDP ratio not only reveal new aspects of E_1 and E_2, but also unmask a difference in the role of these nucleotides when islets are stimulated by glucose as compared to the mitochondrial fuels.

All three agonists are able to induce first-phase insulin secretion through closure of the K^+-ATP channel, since diazoxide (or pinacidil) inhibited their induction of acute insulin release. Thus, the effects of glucose on closure of the K^+-ATP channel can be mimicked by pure mitochondrial fuels. The simplest conclusion is that mitochondrially derived ATP is the major source of ATP which closes the K^+-ATP channel. This is compatible with effects observed with azide (19), or with amytal (20), which are mitochondrial inhibitors, or with bongkrekic acid (21), an inhibitor of the mitochondrial ATP/ADP translocase. Conversely, an electron donor couple (*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine + ascorbate) can decrease K^+ channel currents (22).

Our results also confirm previous studies (3–5) showing the ability of glucose to potentiate insulin secretion in the presence of diazoxide and depolarizing concentrations of K^+ (E_2). We observed in addition that a similar effect is exerted by succinate or KIC, implying that mitochondrially derived signals are sufficient to potentiate Ca^{2+}-dependent insulin secretion distal to the K^+-ATP channel. During the course of these studies, Kelley et al. (23) also reported that succinate can mimic glucose's effect to induce insulin secretion under E_2 conditions. As in our perfusion studies, SAME rapidly induced insulin secretion in the E_2 setting and maintained insulin release over the entire perifusion period; note that SAME-induced E_2 was less potent than that of glucose (compare with Figs. 2–5). Correspondingly, in the current studies (and those of others, 13), in the absence of DZX and K^+, SAME induced primarily first-phase insulin secretion and only a small second phase. These data imply that glucose provides a signal to generate both E_2 and second-phase secretion which is greater than the comparable signal induced by the pure mitochondrial agonist succinate. In the current studies, stimulation with either agonist in the presence of DZX and K^+ (E_2 conditions) resulted in statistically significant increments in total ATP, the ATP/ADP ratio, and the GTP/GDP ratio. Total GTP levels were significantly increased by glucose and tended to increase with SAME as well, although these changes did not reach statistical significance. (However, this small difference between the two agonists may be functionally relevant to quantitative differences in E_2 and second-phase induced by the two agonists, as well as to the resistance of SAME-induced insulin secretion to inhibition by MPA; see below). The results of glucose's effect on adenine nucleotides are in agreement with those found by Gembal et al. (4); however, our studies explore for the first time the effects of these fuels on guanine nucleotides under E_2 conditions.

In general, GTP levels and the GTP/GDP ratio tend to parallel changes in ATP levels and the ATP/ADP ratio, presumably due to the action of nucleoside diphosphokinase (24) (For

example, in preliminary studies with the mitochondrial poison antimycin, GTP content and the GTP/GDP ratio fell as much as ATP and the ATP/ADP ratio [Meredith, M., M. Rabaglia, and S. Metz, unpublished results]. Other investigators [25, 26] have observed that GTP and GTP/GDP ratio closely follow ATP and the ATP/ADP ratio.). Thus it is difficult to separate the effects of the adenine nucleotides from those of the guanine nucleotides and, in fact, GTP might be the proximate effector of some of the actions of ATP in stimulus-secretion coupling. To specifically examine the role of GTP (and/or the GTP/GDP ratio), we used MPA to selectively manipulate guanine nucleotide synthesis, in the presence and absence of guanine and adenine. We have previously documented that a 70–80% decrease in total GTP content, to absolute levels < 0.7–0.8 pmol/islet, resulted in up to a 70% inhibition of subsequent glucose-induced insulin secretion (in the $E_1 + E_2$ setting) during static incubation. Insulin secretion produced by another fuel, KIC, was also inhibited, whereas K^+-induced insulin secretion was unaffected (2). Although ATP content was decreased up to 40% in those studies, the inhibition of insulin secretion could only be reversed by restoration of GTP with guanine, but not by restoration of ATP with adenine (2).

The present studies confirm the ability of MPA to inhibit glucose-induced insulin secretion when the K^+-ATP channel is functional. However, a new finding is that this effect of MPA was also seen with SAME, implying that GTP plays a role in the initiation of insulin secretion which is pursuant to closure of the K^+-ATP channel (especially since MPA did not inhibit SAME-induced E_2; see below). The site of action of GTP in this response remains unclear. It might not involve a direct action on the K^+-ATP channel, since extant studies do not suggest an inhibitory effect of GTP on this channel (27). It is also not likely that GTP affects Ca^{2+} influx or Ca^{2+}-induced insulin release subsequent to closure of K^+-ATP channels and depolarization of the β cell, since MPA does not block K^+-induced insulin secretion (reference 2 and current studies). In addition, provision of GTPγS to permeabilized β cells principally acts in a Ca^{2+}-independent manner (28), suggesting a lack of effect of GTP-binding proteins on Ca^{2+}-induced exocytosis. GTP could conceivably have actions on calcium influx at Ca^{2+}-channels specifically activated by nutrients, or on depolarization of the membrane induced specifically by fuels. However, our recent studies suggest that GTP might be acting distal to calcium influx, specifically on Ca^{2+}-induced phospholipase C activation (Vadakekalam, J., and S. Metz, manuscript in preparation).

Our data also show that MPA, in addition, can inhibit glucose-induced insulin secretion even when the K^+-ATP channel is kept open with DZX and a depolarizing concentration of K^+ is provided. This implies that GTP plays a second role (i.e., in the potentiation of Ca^{2+}-induced insulin secretion) independent of the K^+-ATP channel. The nucleotide data, which show a large decrease in total GTP and the GTP/GDP ratio induced by MPA, support this conclusion. Total ATP was significantly reduced, but the ATP/ADP ratio was unchanged with MPA. Restoration of GTP with guanine (but not restoration of ATP with adenine) reversed the inhibitory effects of MPA on insulin secretion. Thus, only the changes in guanine nucleotides appeared to correlate with the effects of MPA on glucose-induced insulin secretion, in contrast to changes in ATP or the ATP/ADP ratio. This finding is consistent with studies of Detimary et al. (19), suggesting that the K^+-ATP channel–independent action of glucose (i.e., E_2) is relatively resistant to decreases in the ATP/ADP ratio produced using azide. In their previous study, Gembal et al. (4) postulated that the ATP/ADP ratio, or a substance that varied in parallel to the ATP/ADP ratio, was the stimulus-secretion coupling factor. It is thus tempting to speculate that GTP or the GTP/GDP ratio ultimately mediates the effects of ATP (or the ATP/ADP ratio) in stimulus-secretion coupling induced by glucose at a site distal to the K^+-ATP channel. Indeed, Jonas and colleagues (29) have recently directly demonstrated using permeabilized HIT-T15 cells that GTP potentiates Ca^{2+}-induced insulin secretion.

In our studies, MPA inhibited glucose-induced insulin secretion in both the presence (E_2) and the absence of diazoxide and K^+ ($E_1 + E_2$). Our previous studies also demonstrated that both the first phase and the second phase of glucose-induced insulin secretion are inhibited by MPA in the absence of DZX. The current studies, in the E_2 setting, also demonstrate that MPA inhibits glucose-induced insulin secretion throughout the perifusion period; the time course of this insulin secretion fits temporally with second-phase secretion. Therefore, it is possible that E_1 corresponds to first phase secretion and that E_2 corresponds to second phase insulin release. However, since these phases are operationally defined, and since the ultimate mechanisms of phasic insulin release (or of E_1 and E_2) are not yet biochemically elucidated, their equivalence cannot be unequivocally established.

MPA exerts its effects on GTP synthesis by inhibiting cytosolic production of guanine nucleotides (at the level of inosine monophosphate dehydrogenase). Furthermore, GDP and GTP are not able to enter the mitochondria via a known translocase (30, 31). Thus, the site of action of GTP in mediating the K^+-ATP channel–independent action of glucose is most likely in the cytosol as well. In fact, MPA reduced the labeling of GTP (disintegrations per min) disproportionately to the inhibition of mass of GTP (i.e., decreased the specific activity of GTP), by inhibition of the metabolism of hypoxanthine to GMP (a step which is confined to the cytosol). Presumably the mitochondrial (and perhaps secretory granule) compartment(s) contain relatively inert pools of GTP which contribute to its mass, but only poorly to its labeling. Indeed, purine nucleotides in secretory granules of islets (32) and platelets (33) are not readily labeled. Thus MPA would be expected to inhibit GTP labeling more than the total cell mass of GTP, as was observed. This would be compatible with the fact that NTP/NDP ratios were numerically higher when measured by mass that when calculated by disintegrations per min (from TLC), although they responded similarly to MPA; this quantitative difference would be compatible with the presence of poorly labeled pools of GTP and ATP. This formulation receives further support by the inability of MPA to block the secretory effects (under E_2 conditions) of the mitochondrial fuel succinate (or KIC; see below), which presumably increases GTP and ATP largely by synthesis within the mitochondria. The sensitivity of glucose-induced E_2 to inhibition by MPA therefore suggests that glucose might stimulate a specific cytosolic pool of GTP. One pathway might involve cytosolic nucleoside diphosphokinase converting GDP to GTP using ATP as phosphoryl donor (34, 35). It has been suggested (36) that the cytosolic generation of reducing equivalents promotes the mitochondrial conversion of ADP to ATP, via the glycerol phosphate shuttle. This ATP might then exit the mitochondria and form GTP in the cytosol via the action of islet nucleoside diphosphokinase (35). Another possible site might

be the use of GDP as phosphoryl acceptor by glycolytic enzymes (37). It has recently been shown that GTP in fact might be produced in glycolysis at the level of either phosphoglycerate kinase or pyruvate kinase, at least in rod outer segments (38). Both of these enzymes are in the central part of the glycolytic pathway and have been documented to exist with high activity in islets (39). Alternatively, glucose might selectively increase the synthesis of cytosolic guanine nucleotides by increasing availability of 5-phosphoribosyl-l-pyrophosphate, which is itself derived from glucose via ribose-5-phosphate. Many studies (39–41) have suggested that metabolism of glucose through the glycolytic pathway may contribute to glucose-induced insulin secretion, although the metabolic signal generated is unknown. MacDonald (39) has speculated that the glycolytic pathway between the triose phosphates and phosphoenolpyruvate either interacts with mitochondrial metabolism and/or independently produces messengers that complement those produced by the mitochondria. Our data support the idea that glycolysis is important in glucose-induced insulin secretion and suggest that this role may involve GTP.

As with glucose, MPA was able to inhibit SAME-induced insulin secretion in the absence of diazoxide and K^+ ($E_1 + E_2$) during static incubations. Correspondingly, MPA inhibited first-phase insulin secretion generated by SAME in perifusions. However, in marked contrast to glucose, MPA had no effect on SAME-induced insulin secretion in the presence of diazoxide and high K^+ in either static incubations or perfusions, despite changes in ATP, the ATP/ADP ratio, GTP, and the GTP/GDP ratio statistically indistinguishable from those seen with glucose. Thus, the role of GTP (or the GTP/GDP ratio) in insulin secretion produced by pure mitochondrial fuels (SAME or KIC) appears to be restricted largely to the proximal steps in insulin secretion that are dependent on the K^+-ATP channel; the current studies provide little or no evidence to suggest a role for GTP in the ability of mitochondrial fuels to potentiate Ca^{2+}-induced insulin secretion (E_2) or to induce a small second-phase secretion (although we cannot exclude the possibility that still greater decrements in GTP content or GTP/GDP ratio, if achievable, might blunt the latter events). How then can one reconcile the similarity of the changes in nucleotides in the presence of glucose and succinate, under E_2 conditions, with their different secretory responses to MPA in both static incubations and perifusions? One possibility is that measurements of total islet nucleotide content (and their ratios) are not sensitive enough indicators of these parameters, and that small changes in critical pools not quantifiable by these techniques are the more relevant factors. Indeed, glucose tended to induce greater increases in total GTP content and the GTP/GDP ratio, which in turn tended to fall more in the presence of MPA, than did succinate. Alternatively, it is possible that levels of purine nucleotides differed between glucose- versus succinate-stimulated islets, but that these differences were no longer apparent by the end of the 45-min static incubations. Finally, glucose might modify some process critical to secretion, rendering it dependent on GTP but not necessarily altering GTP levels in the cytosol. Future studies will be required to examine these possibilities. Whatever the explanation, it does not seem surprising that pure mitochondrial agonists would work directly via the ATP/ADP ratio, a parameter not blocked by MPA. Conversely, it is clear that all of glucose's effect cannot be readily ascribed to its effects in the mitochondria (It should be noted that provision of succinate exogenously might not faithfully mimic glucose's effect in the mitochondrion, since the tricarboxylic acid cycle provides succinyl CoA whereas exogenous succinate enter the cycle distal to this metabolite. However, KIC is converted to α-ketoglutarate which does yield succinyl CoA.).

In conclusion, both glucose and pure mitochondrial fuels can initiate insulin secretion through closure of the K^+-ATP channel, as evidenced by their sensitivity to diazoxide or pinacidil. Insulin secretion induced by both fuels is also blunted by MPA, implying a role for GTP (or the GTP/GDP ratio) in early events in stimulus-secretion coupling when the K^+-ATP channel is functional. Both fuels also share a common second effect, to potentiate Ca^{2+}-induced insulin secretion, in a K^+-ATP channel–independent manner. GTP is involved in both the proximal, K^+-ATP channel–dependent steps of glucose-induced insulin secretion as well as in the more distal, K^+-ATP channel–independent steps when glucose (but not succinate or KIC) is agonist. The exact biochemical site(s) of action of GTP in these effects remain to be elucidated. Interestingly, methyl esters of succinic acid have recently been shown to enhance insulin release from hyperglycemic rats and are proposed as possible therapeutic agents in non–insulin-dependent diabetes mellitus (42). This leads one to speculate that one defect in glucose-induced insulin secretion seen in non–insulin-dependent diabetes may reside in an inability of glucose to potentiate Ca^{2+}-induced insulin release distal to the K^+-ATP channel; if this defect can indeed be shown to be bypassed by mitochondrially derived fuels, then the possibility of a defect in the cytosolic production of GTP in some forms of diabetes merits investigation.

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