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Inhibition of Calcium-Induced Insulin Secretion from Intact HIT-T15 or INS-1 β Cells by GTP Depletion

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ABSTRACT. Using intact rat islets, we previously observed that GTP depletion (achieved through the use of mycophenolic acid or other synthesis inhibitors) impedes nutrient- but not K^+-induced insulin secretion. It was concluded that a proximal nutrient-dependent step in stimulus–secretion coupling (but not the process of Ca^{2+}-induced exocytosis itself) is modulated by ambient GTP levels. To examine Ca^{2+}-dependent steps further in intact β cells, INS-1 cells (which synthesize GTP and ATP similarly to rat islets) and HIT-T15 cells (whose synthesis of purine nucleotides is different) were studied following cell culture for 1–18 hr in various concentrations of mycophenolic acid (MPA) or mizoribine (MZ). Both agents profoundly reduced GTP content (mean: −78%) and lowered the GTP/GDP ratio by an average of −73%; concomitantly, MPA or MZ reduced insulin secretion induced by 10 mM glucose, 30 or 40 mM KCl, or 100 μM tolbutamide, independent of any changes in cell viability, insulin content, ATP content, the ATP/ADP ratio, or cytosolic free Ca^{2+} concentrations. In INS-1 cells (which appear to have normal nucleobase transport and "salvage" pathway activities), guanine (but not adenine) restored GTP content, the GTP/GDP ratio, and Ca^{2+}-induced secretion. In HIT cells, the phosphoribosylation of exogenous guanine or hypoxanthine is defective; however, provision of 500 μM guanosine (but not adenosine) reversed the effects of MPA. We conclude that, at least in certain situations, a requisite role for GTP in the distal step(s) of exocytosis can be demonstrated. BIOCHEM PHARMACOL 53;12:1873–1882, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. GTP; insulin secretion; Ca^{2+}; exocytosis; guanine; adenine; mycophenolate

A role for GTP in insulin secretion from rat islets has been uncovered recently [1–3]. In intact rat pancreatic islets, GTP content can be decreased by 80% and the GTP/GDP ratio reduced by 65%, using any of several structurally dissimilar inhibitors of GTP synthesis; these include MPA‡ [1–3], MZ [4], tiazofurin [1], ribavirin [1], or 6-mercaptopurine [2]. Concomitantly, the insulin secretion induced by nutrients such as glucose, α-ketoisocaproic acid, or succinic acid can be potentially inhibited. Such effects on GTP and insulin secretion are reversed by the provision of guanine to fuel the "salvage" pathway of guanine nucleotide synthesis (through hypoxanthine-guanine phosphoribosyltransferase; Fig. 1), but not by adenine (which preserves the normal ATP content and ATP/ADP ratio). In contrast, secretion from normal rat islets induced by 40 mM K^+ is resistant to inhibition [1] or is even potentiated slightly [5] by MPA. Since high K^+-induced insulin secretion reflects a relatively pure model of exocytosis induced by influx of extracellular Ca^{2+} [6], we reasoned that the latter must be resistant (relatively or absolutely) to GTP depletion, at least at very high levels of Ca^{2+} influx, a formulation supported by recent studies of maximal Ca^{2+}-induced secretion from permeabilized cells [7, 8]. Therefore, the inhibitory effects on glucose, or other nutrient agonists, were taken to reflect purely the blockade of a more proximal step(s) in stimulus–secretion coupling. Indeed, in subsequent studies, fuel-induced activation of phospholipase C was found to be GTP dependent [5].

However, none of these studies involved the use of isolated pure β cells; additionally, since GTP content was not depleted completely, the possibility remained that Ca^{2+}-induced secretion might be GTP sensitive under other circumstances. Furthermore, although GTP modulates the storage and/or release or permeation of Ca^{2+} at several sites [9–11], none of the preceding studies included determinations of cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$). Lastly, secretion induced by a phorbol ester was found to be GTP sensitive [1], implying that GTP might have additional effects at distal exocytotic sites. The current studies broach these issues using HIT-T15 cells (a poorly granulated β cell line that also contains some glucagon and somatostatin [12, 13]) or INS-1 cells (a β cell line that is more normally granulated and lacks expression of the latter two paracrine modulators [14]). The synthesis

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‡ *Abbreviations:* MPA, mycophenolic acid; MZ, mizoribine; KRBH, Krebs-Ringer bicarbonate HEPES buffer; and IMPDH, inosine monophosphate dehydrogenase.

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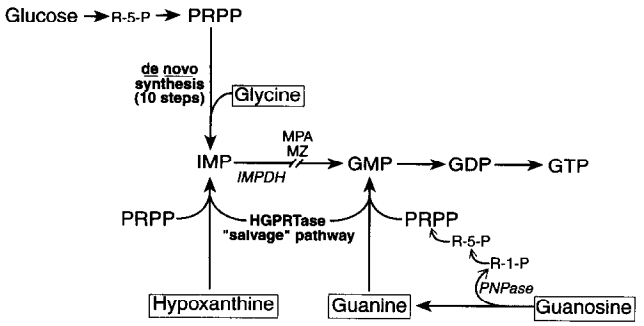

FIG. 1. Schematic representation of the biosynthetic pathways for guanine nucleotides. *De novo* synthesis is shown in the upper half of the diagram and "salvage" synthesis in the lower half. Compounds in rectangular boxes were added exogenously to study purine synthesis as described in the text. Key: R-5-P, ribose-5-phosphate; R-1-P, ribose-1-phosphate; PRPP, 5-phosphoribosyl-1-pyrophosphate; IMPDH, inosine monophosphate dehydrogenase; MPA; mycophenolic acid; MZ, mizoribine; and PNPase, purine nucleotide phosphorylate.

of GTP was necessarily also investigated, for the first time in detail, in these cells. An unequivocal role of GTP in Ca^{2+}-triggered secretion was unmasked, representing the first direct demonstration that ambient levels of endogenous GTP can modulate Ca^{2+}-induced exocytosis in intact cells.

MATERIALS AND METHODS

Materials

MPA, azaserine, nucleotide standards, monobasic ammonium phosphate (for the HPLC mobile phase), guanine, adenine, guanosine, and adenosine were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). MZ was provided by Dr. N. Kazamatani (Tokyo Women's Medical College, Tokyo, Japan). The diluent used for making stock solutions of these drugs was ethanol (for MPA) or DMSO (for guanine and adenine); control tubes always contained an amount of the relevant diluent equal to that of the experimental tubes. [G-^{3}H]Hypoxanthine (17 Ci/mmol) and [U-^{14}C]glycine (110.5 mCi/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). HPLC columns were purchased from Alltech (Deerfield, IL, U.S.A.). RPMI 1640 medium and an RPMI 1640 Selectamine kit were purchased from Gibco (Grand Island, NY, U.S.A.).

Cell Culture

HIT-T15 cells (a gift from Drs. R. P. Robertson and H. Zhang, Minneapolis, MN, U.S.A.) and INS-1 cells (courtesy of Dr. C. B. Wollheim, Geneva, Switzerland) were cultured in 1.5 mL RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (from Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin. For INS-1 cells, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, and 10 mM HEPES were included in the culture medium (as described previously [14]). The experiments described were performed using cell passages 72–81 for HIT cells and 45–75 for INS-1 cells.

Insulin Secretion

HIT or INS-1 cells were seeded in 24-well culture plates and cultured for 3–4 days in RPMI 1640 medium as described above. Test agents were included in the culture medium for various times as specified. On the day of the experiment, the cells were washed with fresh KRBH. After a 30-min preincubation period at 37° in 0.5 mL KRBH (pH 7.4 and gassed with 95% O_2/5% CO_2), the medium was replaced by one containing secretagogues (0.5 mL) and the cells were incubated for 30 min. The supernatant was used for the measurement of insulin secretion, and the attached cells were extracted by 1 mL acid ethanol for the determination of insulin content. Insulin was measured by radioimmunoassay as previously described [15].

Measurement of $[Ca^{2+}]_i$

HIT cells were detached by trypsinization and kept in a spinner medium [16] for 3 hr. Thereafter, cells were loaded with Indo-1/AM (Molecular Probes, Eugene, OR, U.S.A.) in RPMI 1640 supplemented with 10 mM HEPES and 1% fetal bovine serum for 30 min at 37°. After washing, aliquots of cells (~2 × 10^6) were suspended in KRBH in a glass cuvette (2 mL), and the fluorescence ratio of two emission wavelengths (405/480 nm) was measured at an excitation wavelength of 355 nm using an SLM 8000c spectrofluorometer (SLM Instruments Inc., Urbana, IL, U.S.A.). $[Ca^{2+}]_i$ was calculated from the fluorescence data by the following equation [17]:

$$[Ca^{2+}]_i \text{ (nM)} = K_d \cdot (R_{max} - R)/(R - R_{min}) \cdot \beta$$

where K_d is the dissociation constant of Indo-1 (250 nM), R is the measured $F_{405/480}$ ratio, R_{max} is the maximum $F_{405/480}$ ratio when the dye is saturated with Ca^{2+} (in the presence of 5 μM ionomycin), R_{min} is the minimum $F_{405/480}$ ratio when the dye is free of Ca^{2+} (in the presence of 5 μM ionomycin and 10 mM EGTA), and β is the ratio of the fluorescence signals measured at emission of 480 nm in the absence of Ca^{2+} and at Ca^{2+} saturation (F_{480} free/F_{480} saturated). R_{max}, R_{min}, and β were determined to be 1.47, 0.11, and 1.47, respectively.

Determination of Nucleotide Content of HIT and INS-1 Cells

Studies done to measure nucleotides were performed using the same medium and times as described above for the insulin release studies. In some studies (where indicated), 2 μCi/mL [^{3}H]hypoxanthine or 10 μCi/mL of [^{14}C(U)]glycine was added to the culture medium to label the salvage and *de novo* pathways of purine nucleotide synthesis,

respectively, as previously described [2]. After the culture period (as indicated in the text), the culture medium was removed, 10 μL ITP and CTP (0.2 nmol; as internal standards) and 300 μL of ice-cold 0.6 N trichloroacetic acid were added to each well, and cells were allowed to stand on ice for 20 min. The medium was then transferred to a 1.5-mL microcentrifuge tube and centrifuged for 5 min at 9000 *g*. The supernatant was removed and subjected to ether extractions as previously described [1].

HPLC quantitation of nucleotide triphosphates was carried out as previously described [3]. Briefly, the compounds were separated using ammonium phosphate buffers (buffer A was 0.5 mM, pH 2.60; 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, in order to optimize separation of compounds. Separations were achieved using isocratic elution at a flow rate of 1.0 mL/min. Fifty microliters of extract was injected for the nucleotide triphosphates and 100 μL for the diphosphates. UV absorbance was monitored at 254 nm. The quantitation of mass of nucleotides was performed by UV spectroscopy using a Hewlett–Packard 3365 Series II Chemstation with an external standard curve and peak area integration. Standard curves were carried out each day. CTP and ITP were added as internal standards, as described above, in order to calculate (and correct for) recoveries [1]. ATP/ADP and GTP/GDP ratios were calculated from the mass determinations. Eluant trinucleotide HPLC fractions were collected every 30 sec (glycine label) or every 18 sec (hypoxanthine label) and were matched against the concomitant UV chromatograms as previously described [2]. Samples were counted for 5 min in 4 mL of scintillation fluid.

Other Methods

Protein content of cells was determined using a Bio-Rad assay described by Bradford [18], with BSA as standard. The MTS assay (reduction of a tetrazolium compound to colored formazan [19]) was performed using a kit purchased from Promega (Madison, WI, U.S.A.). In brief, 20 μL of a mixture of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxylmethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) and phenazine methosulfate (an electron coupling agent) was added to culture wells seeded with cells. After a 30-min incubation at 37°, the reaction was stopped by the addition of 25 μL of 10% SDS. The quantity of the formazan product was measured by the absorbance at 490 nm with a microplate reader.

Data Presentation and Statistical Analysis

Data are expressed as means ± SEM, with (N) representing the number of determinations. Total nucleotide content is expressed as nanomoles per well; specific activity is expressed as disintegrations per picomole (using the HPLC fractions). Insulin secretion data are expressed as percent of control or as fractional insulin release, calculated using the formula: insulin released/initial insulin content. Statistical analyses were by non-paired *t*-tests, or the Mann–Whitney test, as appropriate.

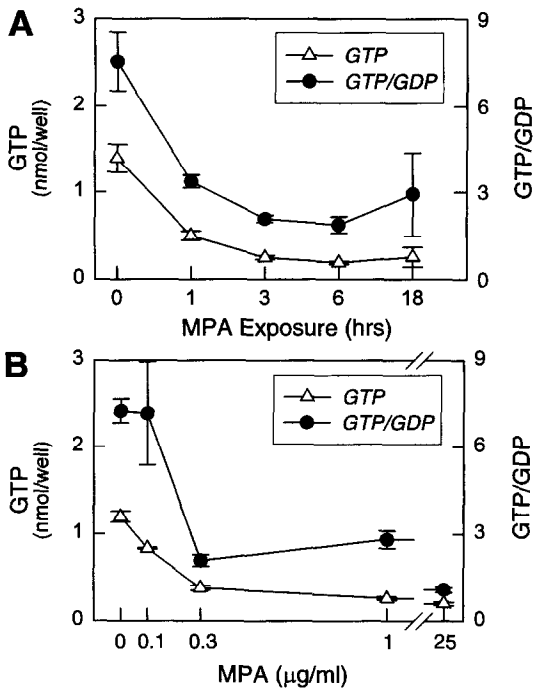

FIG. 2. Time–course and concentration–response curves for the effect of MPA on guanine nucleotides in HIT-T15 cells. (A) HIT cells were cultured for various times in the presence of 1 μg/mL MPA. (B) HIT cells were cultured for 6 hr at various concentrations of MPA. Data are the means ± SEM from 3–5 separate experiments.

RESULTS

Effect of MPA on Purine Nucleotides in HIT and INS-1 Cells

We first examined the effects of MPA on purine nucleotides, to document that the drug is able to block the synthesis of GTP (and to a lesser extent ATP) in HIT and INS-1 cells as it does in rat islets [1–3]. MPA treatment of HIT cells resulted in a concentration- and time-dependent decrease in purine nucleotides (Figs. 2 and 3). As would be expected from an inhibitor of IMPDH, guanine nucleotides were more sensitive to the effects of MPA than adenine nucleotides. Significant decreases in GTP content and the GTP/GDP ratio occurred by 1 hr ($P < 0.001$, Fig. 2A) and by 0.3 μg/mL MPA ($P < 0.05$, Fig. 2B). Significant decreases in ATP content required 1 μg/mL MPA ($P = 0.004$, Fig. 3B) and a 3-hr exposure ($P < 0.001$, Fig. 3A). Consistent with our previous observations in islets [3], the ATP/ADP ratio was unchanged (or even increased) at any concentration of MPA. Maximal decrements in GTP content (−83%), and the GTP/GDP ratio (−86%) were seen

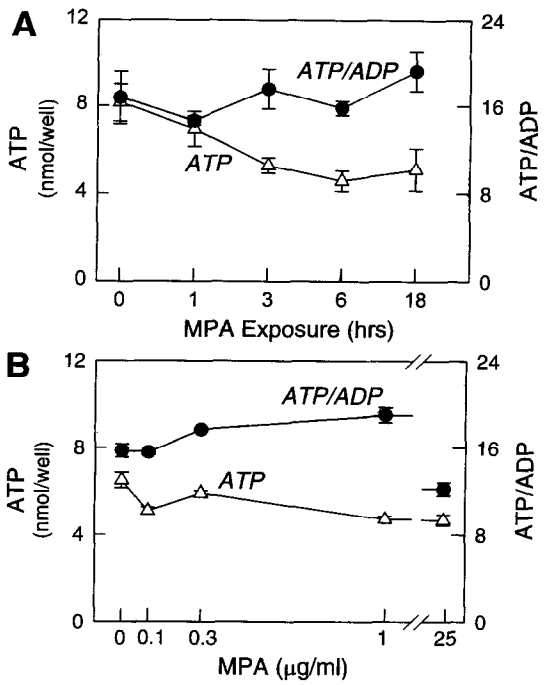

FIG. 3. Time–course and concentration–response curves for the effects of MPA on adenine nucleotides in HIT-T15 cells. (A) HIT cells were cultured for various times in the presence of 1 μg/mL MPA. (B) HIT cells were cultured for 6 hr at various concentrations of MPA. Data are the means ± SEM from 3–5 separate experiments.

at 25 μg/mL MPA provided over a 6-hr culture (Fig. 2). Marked decreases in purine nucleotides were observed as early as 1 hr after exposure to 1 μg/mL MPA with maximal decreases in GTP content, ATP content, and the GTP/GDP ratio observed by 6 hr of MPA (1 μg/mL) treatment (Figs. 2 and 3). Thus, the effect of MPA on purine nucleotides in HIT cells resembles that seen in islets, although HIT cells appear more sensitive to the drug, as near-maximal effects on nucleotides were observed by 6 hr with only 1 μg/mL MPA in HIT cells (effects that require 25 μg/mL MPA and an 18-hr exposure in islets).

In islets, the effects of MPA can be prevented by guanine [1], which restores GTP content via synthesis through the salvage pathway, thus bypassing the blockade of IMPDH imposed by MPA (Fig. 1). In contrast to results in islets, in HIT cells 100 μM guanine was unable to restore significantly GTP content or the GTP/GDP ratio (Table 1). Likewise, 150 μM adenine could not totally restore ATP content. However, addition of 500 μM guanosine was able to restore GTP to 75% of basal values; concomitantly, GTP/GDP was restored to 81% of basal, and ATP content was restored completely (Table 1). Provision of 500 μM adenosine had no restorative effect on GTP content or the GTP/GDP ratio, although it did increase ATP content significantly (Table 1).

As these responses to purine bases were different in HIT cells compared with our previous observations in islets [1], we further investigated the purine nucleotide synthetic pathways in HIT cells. Cells were cultured with either [^{14}C]glycine (to label the *de novo* pathway) or [^{3}H]hypoxanthine (to label the salvage pathway; Fig. 1). Consistent with the findings (above) using unlabeled guanine and adenine, very little radioactivity was incorporated into either GTP or ATP when HIT cells were cultured in the presence of [^{3}H]hypoxanthine. This was reflected in total disintegrations per minute measured in ATP or GTP (Table 2). In contrast, [^{14}C]glycine was readily incorporated into either GTP or ATP (Table 2). In this setting, addition of 1 μg/mL MPA resulted in an 82% decrease in mass and a 79% decrease in labeling, indicating that the majority of GTP appears to be synthesized via the *de novo* pathway in HIT cells (Table 2). These results are different from our previous results in islets, which documented predominance of the salvage pathway of purine nucleotide synthesis [2]. To confirm this finding, we cultured HIT cells overnight in 100 μM azaserine, a selective inhibitor of the *de novo* pathway [20]. Azaserine treatment resulted in significant decreases in ATP content (8.54 ± 0.22 to 2.65 ± 0.15 nmol/well, $P < 0.001$), GTP content (1.49 ± 0.1 to 0.46 ± 0.06 nmol/well, $P < 0.001$), the ATP/ADP ratio (18.24 ± 1.12 to 12.99 ± 0.05, $P = 0.002$), and the GTP/GDP ratio (8.29 ± 1.27 to 4.73 ± 0.86, $P = 0.04$) compared with control. Incorporation of [^{14}C]glycine into either ATP or GTP was decreased significantly by 98% (data not shown), confirming that virtually all of the ATP and GTP in HIT cells was synthesized via the *de novo* pathway. Again, this is in contrast to data in islets, where azaserine did not reduce significantly nucleotide levels or insulin secretion [2]. As in islets, the effects of MPA on nucleotides in HIT cells are not restricted to that pharmacologic probe. Provision to HIT cells of 75 μg/mL MZ (a structurally dissimilar IMPDH inhibitor) for 6 hr resulted in a 77% decrease in GTP content ($P < 0.001$ vs control; df = 6), a 68% decrease in the GTP/GDP ratio ($P < 0.001$ vs control; df = 6), and no significant changes in ATP content or the ATP/ADP ratio. These effects on guanine nucleotides were fully reversible with co-provision of 500 μM guanosine (data not shown).

MPA (25 μg/mL for 18 hr) was also able to decrease significantly GTP, ATP, and the GTP/GDP ratio in INS-1 cells (Table 1). However, in contrast to HIT cells, addition of 100 μM guanine restored GTP content and the GTP/GDP ratio in MPA-treated INS-1 cells (Table 1). In keeping with these findings, incorporation of [^{3}H]hypoxanthine into both GTP and ATP was much greater than that of [^{14}C]glycine (Table 2). Thus, a comparison of radiolabel incorporation between the two transformed β cell lines shows much greater incorporation of hypoxanthine into INS-1 cells and greater incorporation of glycine into HIT cells (Table 2), indicating a predominance of salvage pathway activity in INS-1 cells (as in islets) and *de novo* pathway activity in HIT cells. Consistent with this formulation, addition of azaserine (100 μM) in INS-1 cells resulted in only a 20–30% decrease in total ATP or GTP

TABLE 1. Effect of MPA on mass of purine nucleotides in HIT and INS-1 cells in the presence or absence of guanine, adenine, guanosine, or adenosine

	ATP (nmol/well)	ATP/ADP	GTP (nmol/well)	GTP/GDP
HIT cells*				
Control	7.37 ± 0.33 (4)	17.55 ± 0.33 (4)	1.33 ± 0.06 (4)	7.38 ± 0.19 (4)
MPA	4.71 ± 0.22† (4)	19.84 ± 0.36‡ (4)	0.15 ± 0.01† (4)	1.60 ± 0.10† (4)
MPA + guanine	5.58 ± 0.26† (4)	21.35 ± 0.56‡ (4)	0.18 ± 0.01† (4)	1.80 ± 0.06† (4)
MPA + adenine	6.42 ± 0.31 (4)	20.91 ± 0.31‡ (4)	0.15 ± 0.05† (4)	1.35 ± 0.08† (4)
Control	5.43 ± 0.07 (6)	16.47 ± 1.16 (5)	0.92 ± 0.01 (6)	7.29 ± 0.60 (5)
MPA	4.57 ± 0.22‡ (6)	20.71 ± 1.30‡ (6)	0.20 ± 0.02† (6)	2.60 ± 0.26† (6)
MPA + guanosine	5.79 ± 0.12‡ (6)	17.48 ± 0.91 (6)	0.69 ± 0.05‡ (6)	5.92 ± 0.97 (6)
MPA + adenosine	6.29 ± 0.11† (6)	23.77 ± 1.53‡ (5)	0.21 ± 0.02† (6)	3.01 ± 0.55† (5)
INS cells*				
Control	2.52 ± 0.19 (17)	17.40 ± 0.9 (11)	0.50 ± 0.03 (17)	5.97 ± 1.38 (10)
MPA	1.22 ± 0.22† (16)	19.00 ± 1.13 (10)	0.077 ± 0.01† (16)	2.33 ± 0.77‡ (10)
MPA + guanine	1.59 ± 0.17† (17)	16.30 ± 0.86 (10)	0.97 ± 0.08† (17)	14.03 ± 3.13‡ (10)
MPA + adenine	1.78 ± 0.30‡ (17)	22.60 ± 1.28‡ (10)	0.08 ± 0.01† (17)	2.09 ± 0.64‡ (11)

* HIT cells were cultured for 6 hr with 1 μg/mL MPA in the presence or absence of 500 μM guanosine or 500 μM adenosine. In other studies, HIT and INS-1 cells were cultured for 18 hr in 1 and 25 μg/mL, respectively, MPA in the presence or absence of 100 μM guanine or 150 μM adenine. Values are the means ± SEM for the number (in parentheses) of determinations for each condition.

† $P \leq 0.001$ compared with control.

‡ $P \leq 0.05$ compared with control.

content, a 30% decrease in the GTP/GDP ratio, and no change in the ATP/ADP ratio (data not shown).

Effect of MPA on Insulin Secretion from HIT-T15 Cells and INS-1 Cells

We next validated that glucose-induced insulin secretion could be inhibited by MPA in HIT-T15 cells as it is in rat islets [1–3]. MPA treatment resulted in a time-dependent decrease in glucose-induced insulin secretion. No effects of MPA on insulin secretion were seen at exposures of 1 hr or less, but insulin secretion was decreased markedly by 60–80% after MPA (1–10 μg/mL) treatment of 3–18 hr (Fig. 4, a and b). This degree of inhibition of insulin secretion is comparable to that of our previous data using islets treated with 25 μg/mL MPA. Thus, glucose-induced insulin secretion in HIT cells is inhibited by MPA, as in islets, but HIT cells appear to be more sensitive to the effects of MPA than islets. Insulin content was decreased by 32 ± 3% ($N = 9$, $P < 0.01$) after 18 hr in HIT cells, but was not affected after 6 hr of MPA treatment. Since the inhibition of fractional insulin secretion was similar at 6 or 18 hr, the majority of subsequent studies on HIT cells employed a 6-hr exposure to MPA.

In rat islets, MPA inhibits secretion induced by nutrients or a phorbol ester, but has no effect on K^+-induced insulin secretion [1, 3]. However, in contrast to islets, MPA treatment of HIT cells resulted in a time- and concentration-dependent inhibition of K^+-induced insulin secretion, even in the absence of glucose (Fig. 4). This inhibition of K^+-induced insulin secretion was slightly more resistant to the effects of MPA than glucose, requiring at least 1 μg/mL for significant inhibition of insulin release, whereas glucose-induced secretion was inhibited by 0.3 μg/mL MPA. The inhibition of K^+-induced insulin secretion by MPA was not altered by inclusion of glucose (0.2 or 10 mM) or the cyclic AMP-raising agent forskolin (data not shown). MPA inhibited K^+-induced insulin secretion not only over a 30-min incubation but also during early time points; fractional insulin secretion during the first 10 min of K^+ stimulation was decreased by 32% ($P < 0.05$) in MPA-treated cells (1 μg/mL for 6 hr). Insulin release induced by tolbutamide (100 μM), which closes ATP-sensitive K^+ channels and thereby promotes cell depolarization and Ca^{2+} influx, was also inhibited by 52% ($P < 0.01$) after a 6-hr MPA treatment. As in islets [1], the effects of MPA on insulin secretion in HIT cells do not appear to be non-

TABLE 2. Effect of MPA on incorporation of [^{3}H]hypoxanthine or [^{14}C]glycine into purine nucleotides in HIT-T15 or INS-1 cells*

	ATP		GTP	
	dpm (per well)	Mass (nmol/well)	dpm (per well)	Mass (nmol/well)
HIT cells				
[^{3}H]Hypoxanthine				
−MPA	5,880 ± 490 (4)	5.62 ± 0.25 (4)	660 ± 70 (4)	1.12 ± 0.05 (4)
+MPA	5,090 ± 310 (4)	3.90 ± 0.22† (4)	290 ± 30† (4)	0.32 ± 0.02† (4)
[^{14}C]Glycine				
−MPA	104,700 ± 4,350 (9)	8.64 ± 0.17 (9)	19,830 ± 1,800 (9)	1.41 ± 0.05 (9)
+MPA	59,370 ± 5,860‡ (9)	4.64 ± 0.22† (9)	4,260 ± 1,250‡ (9)	0.26 ± 0.04† (9)
INS cells				
[^{3}H]Hypoxanthine				
−MPA	54,500 ± 3,870 (4)	2.42 ± 0.12 (4)	8,080 ± 390 (4)	0.52 ± 0.01 (4)
+MPA	13,520 ± 1,230‡ (4)	0.81 ± 0.03† (4)	570 ± 50‡ (4)	0.06 ± 0.04† (4)
[^{14}C]Glycine				
−MPA	6,160 ± 800 (9)	2.44 ± 0.35 (10)	1,160 ± 180 (9)	0.77 ± 0.08 (10)
+MPA	6,670 ± 980 (9)	1.0 ± 0.14‡ (10)	290 ± 30‡ (10)	0.16 ± 0.04† (10)

* HIT or INS-1 cells were cultured for 18 hr with 2 μCi/mL [^{3}H]hypoxanthine or 10 μCi/mL [^{14}C]glycine in the presence or absence of 1 μg/mL MPA. Values are the means ± SEM for the number (in parentheses) of determinations for each condition.
† $P < 0.05$ compared with no MPA.
‡ $P < 0.001$ compared with no MPA.

specific effects of that drug, since treatment of HIT cells for 6 hr with 25 μg/mL MZ, a structurally dissimilar IMPDH inhibitor, also blocked glucose-induced insulin secretion by 75% ($P < 0.01$) and K^+-induced insulin secretion by 29% ($P < 0.05$) (data not shown).

The inhibitory effects of MPA on insulin secretion in HIT cells parallel the effects on nucleotides described above, both in time–course and concentration–response (cf. Figs. 2–4). As in islets [1], a decrease of nearly 80% of the total GTP content must be attained before significant decreases in insulin secretion are seen. The inhibitory effects of MPA on insulin secretion in HIT cells appear to be due to the decrement in GTP content (or GTP/GDP ratios) as it is in islets. Addition of 500 μM guanosine was able to reverse totally the effects of MPA on K^+-induced insulin secretion (Fig. 5), whereas treatment of cells with guanosine alone did not alter their secretory profiles (fractional insulin secretion 8.4 ± 1.2% in 40 mM KCl alone vs 9.5 ± 1.2% in 40 mM KCl plus 500 μM guanosine; $P = 0.1$). As expected, 100 μM guanine was unable to reverse the effects of MPA on K^+-induced insulin secretion in HIT cells (Fig. 5), since it did not restore GTP content. Five hundred micromolar adenosine insignificantly reversed the MPA effect (Fig. 5), and adenine (150 μM) had no effect on the inhibitory actions of MPA in HIT cells (not shown). Likewise, 500 μM adenosine alone (in the absence of MPA) had no significant effect on K^+-induced insulin secretion (fractional insulin secretion 8.4 ± 1.2% in KCl alone vs 9.6 ± 0.6% in KCl plus 500 μM adenosine; $P = 0.14$).

The inhibitory effects of MPA do not appear to be due to an effect on cell viability. There was no significant difference between control and MPA-treated (6 hr) cells when the trypan-blue exclusion test was performed (only 2–5% of HIT cells stained in either control or experimental conditions). There was no change in the contents of DNA and protein between control and MPA-treated (1 μg/mL) cells after up to 12 hr (data not shown). The MTS test, which is based on the amount of formazan produced, reflects the state of nutrient metabolism in viable cells [21]; MPA-treated (6 hr) cells induced a similar amount of formazan production as that produced by control cells (data not shown).

To determine whether the decrease in K^+-induced insulin secretion was unique to HIT cells, studies were also performed in INS-1 cells. Similar effects of MPA on insulin secretion were seen in INS-1 cells, although higher concentrations (i.e. similar to those needed to inhibit glucose-induced insulin release in islets) were required. Provision of 25 μg/mL MPA for 18 hr resulted in significant inhibition of both glucose plus forskolin- or K^+-induced insulin secretion in INS-1 cells (Table 3). Similar to the findings in islets [1], but in contrast to HIT cells, MPA only slightly decreased insulin content in INS-1 cells (95 ± 4% of

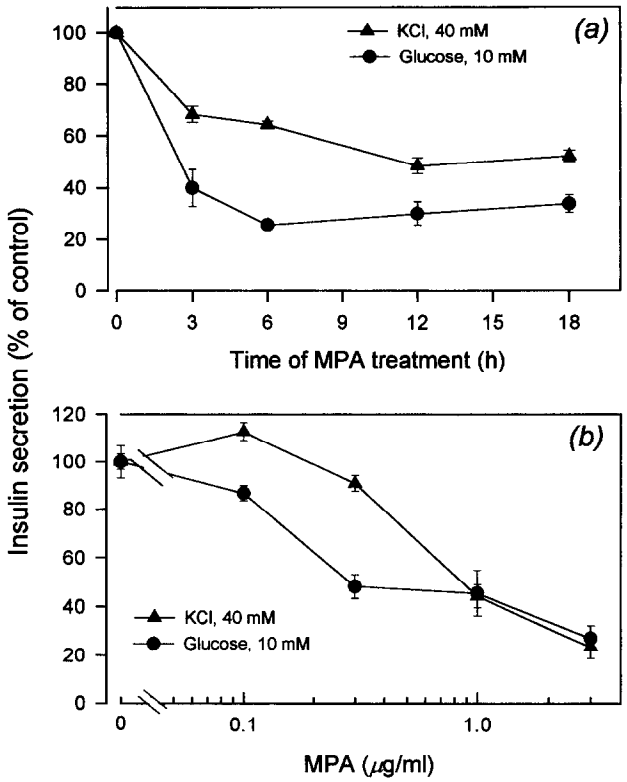

FIG. 4. Time–course and concentration–response curves of the effects of MPA on subsequent 10 mM glucose or 40 mM K^+-induced insulin secretion in HIT-T15 cells. (a) HIT cells were cultured in the presence of 1 μg/mL MPA for various times. (b) A 6-hr pretreatment with various concentrations of MPA. Values (expressed as percent of control secretion in the absence of MPA) are the means ± SEM for 6–9 determinations from 2–3 separate experiments. All KCl incubations were performed in the absence of glucose. Absolute values (expressed as fractional secretion of insulin) for panel A were: Glucose controls: 3 hr, 3.53 ± 0.23; 6 hr, 5.64 ± 0.26; 12 hr, 4.10 ± 0.36; 18 hr, 3.63 ± 0.50. KCl controls: 3 hr, 7.12 ± 0.34; 6 hr, 6.25 ± 0.38; 12 hr, 4.41 ± 1.8; 18 hr, 4.76 ± 0.03. Absolute values for panel B were: Glucose control, 6.56 ± 1.25; KCl control, 11.02 ± 2.66.

control; $P > 0.05$) and, thus, clearly inhibited exocytotic release (i.e. fractional secretion). INS-1 cells were also similar to islets in that addition of 100 μM guanine in MPA-treated cells prevented the inhibitory effects on insulin secretion. As with all cell types studied, 100 μM adenine had no effect on the inhibition of insulin secretion induced by MPA (Table 3).

Effect of MPA on $[Ca^{2+}]_i$

Since Ca^{2+} plays a crucial role in the stimulation of insulin secretion [6], the inhibitory effects of MPA on K^+-induced insulin release might be due to an impairment in the rise of $[Ca^{2+}]_i$ induced by depolarization of the plasma membrane. To address this possibility, $[Ca^{2+}]_i$ in HIT cells was measured with a fluorescent probe, Indo-1, both in control cells and after a 6-hr treatment with 1 μg/mL MPA. Basal $[Ca^{2+}]_i$ levels were increased slightly, but significantly,

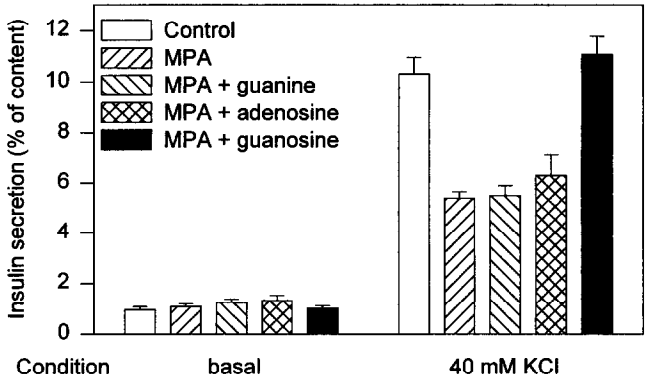

FIG. 5. Reversal by guanosine of MPA-induced inhibition of 40 mM K^+-induced insulin secretion in HIT cells. HIT cells were cultured for 6 hr in the presence of 1 μg/mL MPA alone, or MPA combined with 100 μM guanine, 500 μM guanosine, or 500 μM adenosine. Data are the means ± SEM for 7–9 determinations from 3 separate experiments.

(control 101 ± 3 nM vs MPA-treated 116 ± 5; $P < 0.05$) in MPA-treated cells. However, neither the $[Ca^{2+}]_i$ peak nor the plateau concentration evoked by 40 mM K^+ was altered in these cells (Fig. 6). Moreover, the $[Ca^{2+}]_i$ rise induced by 100 μM tolbutamide, which closes ATP-sensitive K^+ channels and depolarizes cells, also was not affected by MPA treatment (Fig. 6). These results indicate that both ATP-sensitive K^+ channels and voltage-gated Ca^{2+} channels are intact in MPA-treated cells and that a step(s) in stimulation of insulin secretion distal to the rise in $[Ca^{2+}]_i$ must be impeded by GTP depletion.

DISCUSSION

Previously there were no data regarding biosynthetic pathways for GTP or ATP in transformed, cultured β cells. Therefore, we first defined these parameters, as we had for intact islets [2] using mass and radiolabeling of synthetic pathways as end points. [^{3}H]Hypoxanthine was used to mark the "salvage" pathway and [^{14}C]glycine to mark *de novo* synthesis (Fig. 1; [2]). Normal rat islets have both pathways, but the former clearly predominates [2]. In contrast, as reported for several other tumoral (or rapidly proliferating) cells, transformed β cells appear to have a very active *de novo* synthetic pathway; this was most evident in HIT cells which proliferate more rapidly than INS-1 cells [22]. While INS-1 cells incorporated [^{3}H]hypoxanthine avidly, as do islets, an unexpected finding was the negligible incorporation of [^{3}H]hypoxanthine by HIT cells (a finding confirmed by the inability of guanine to "salvage" the effects of MPA on GTP or insulin secretion) at concentrations that are effective in islets or INS-1 cells. However, since provision of guanosine (which provides not only intracellular guanine but also ribose-1-phosphate, following hydrolysis via purine nucleoside phosphorylase) was able to prevent the effects of MPA, these cells do appear to have a functional salvage pathway. In theory, HIT cells might have an absence of the nucleobase trans-

TABLE 3. Effect of MPA treatment in the presence or absence of guanine or adenine on subsequent insulin secretion from INS-1 cells

Condition*	Insulin content (%)			
	Control	25 μg/mL MPA-treated	25 μg/mL MPA + 100 μM adenine	25 μg/mL MPA + 100 μM guanine
Basal	2.34 ± 0.10 (12)	2.31 ± 0.24 (12)	2.03 ± 0.08 (6)	2.61 ± 0.17 (9)
15 mM Glucose	3.07 ± 0.19† (9)	2.13 ± 0.20‡ (9)	2.18 ± 0.15‡ (3)	3.08 ± 0.22 (6)
15 mM Glucose + 1 μM Forskolin	8.53 ± 0.47† (9)	5.01 ± 0.37‡ (9)	5.01 ± 0.54‡ (3)	9.87 ± 0.42 (6)
40 mM KCl	6.56 ± 0.41† (9)	4.07 ± 0.36‡ (9)	4.58 ± 0.38‡ (6)	7.45 ± 0.36 (6)

Results are expressed as percent of insulin content (i.e. fractional secretion). Values are the means ± SEM for the number (in parentheses) of determinations for each condition.
* MPA and other agents were provided for 18 hr prior to stimulation.
† $P < 0.01$ compared with basal (2 mM glucose).
‡ $P < 0.01$ compared with control.

port mechanism [23], a shortage of substrate (5-phosphoribosyl-1-pyrophosphate) or, more remotely, might secrete the guanine degradation enzyme guanase into the medium [24]. These possibilities were not pursued further in the current studies.

HIT cells were more sensitive to the inhibitory effects of MPA on nucleotides than were INS-1 cells or islets. Whereas in islets and INS-1 cells, MPA inhibited GTP content by approximately 80% at 25 μg/mL after an 18-hr culture, in HIT cells the sensitivity to MPA (but not the overall efficacy) was shifted to the left, such that maximal effects of MPA were seen at 1 μg/mL MPA provided for only 6 hr. This differential sensitivity to MPA coincides with the differences in nucleotide synthetic pathways between the cell types, implying that the *de novo* pathway might be more sensitive to the effects of MPA. Alternatively, HIT cells might contain an IMPDH isoform that is more sensitive to inhibition to MPA, as has been described in other cells [25, 26].

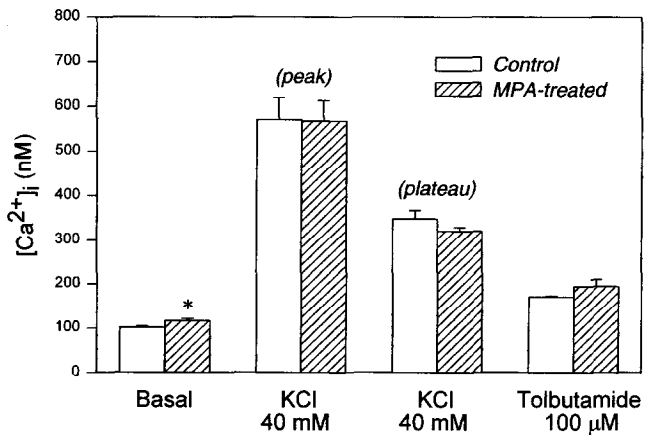

FIG. 6. Effect of MPA on cytosolic free Ca^{2+} concentrations in HIT cells. Cells were detached by trypsinization and left in a spinner for 3 hr. Thereafter, cells were loaded with 1 μM Indo-1/AM for 30 min. After washing, aliquots of cells were suspended in a glass cuvette, and the fluorescence was recorded. Stimulus was added after 20 min equilibrium in glucose-free KRBH at 37°. $[Ca^{2+}]_i$ was calculated as described in Materials and Methods. When present, MPA (1 μg/mL) was included during the last 2.5 hr in culture, the 3 hr in spinner, and the 30 min of Indo-1 loading. MPA was not present in KRBH during recording fluorescence because of its quenching effect. The $[Ca^{2+}]_i$ plateau was measured at 3 min after the addition of high K^+. Data are the means ± SEM for 3–7 determinations. Key: (*) $P < 0.05$ compared with control.

As in normal islets, MPA inhibited glucose-induced insulin release in both HIT and INS-1 cells. Of note, however, was the new observation that MPA inhibited insulin release induced by high K^+ in either cell line. In HIT cells, significant inhibition of insulin secretion was seen by 3 hr and was essentially maximal by 6 hr. This paralleled the effects of MPA on GTP content and the GTP/GDP ratio, except that significant (albeit submaximal) effects on nucleotides were seen at 1 hr and thus appeared to slightly precede in time the effects on secretion. Likewise, MPA inhibited K^+-induced insulin secretion in INS-1 cells with concomitant inhibitory effects on guanine nucleotides. Additionally, MZ (a structurally dissimilar IMPDH inhibitor [4]) reduced GTP and the GTP/GDP ratio to similar degrees as did MPA and also inhibited K^+-induced insulin release. As in islets, reversal of the effects of MPA on guanine nucleotides, by guanine in INS-1 cells and guanosine in HIT cells, also reversed the inhibitory effects of MPA on insulin secretion. The ATP/ADP ratio was not decreased by either MPA or MZ in HIT or INS-1 cells and reversal of the decrements specifically in ATP content (using purine bases or nucleosides) had no, or only minor, effects on the inhibitory action of MPA on insulin secretion. Thus, as with islets, it is clear that the effects of MPA (or MZ) on guanine (but not on adenine) nucleotides explain most, if not all, of the observed effects on secretion.

The acute effects of GTP depletion on secretion do not reflect a depletion of β cell insulin content, since the latter was only seen after 18 hr in HIT cells and was not observed in INS-1 cells even after 18 hr of exposure. Consequently, at earlier time points, fractional insulin secretion was clearly inhibited. It is also clear that MPA (or GTP depletion) does not globally interfere with β cell viability at

such early times, since insulin content, DNA content, protein content, and trypan blue exclusion were unaffected during the 6-hr MPA exposure in HIT cells. Furthermore, GTP depletion did not alter $[Ca^{2+}]_i$ levels achieved after high K^+, suggesting that depolarization of the β cell plasma membrane, and the resultant calcium influx, remained intact. However, interestingly, we note in passing that MPA slightly, but consistently, elevated basal $[Ca^{2+}]_i$ with a tendency towards elevations in basal insulin release; while the etiology of this effect remains to be determined, it could well represent blockade of GTP-dependent loading of intracellular Ca^{2+} stores [viz. 9–11].

The formulation that GTP is required for the full exocytotic response to Ca^{2+} receives some support in the literature. In permeabilized non-endocrine cells [27], mast cells [28], and β cells [7, 8], GTP or its analogs sensitize the contractile or secretory apparatus to Ca^{2+}. This may occur mostly at submaximal $[Ca^{2+}]_i$, since GTP has been reported recently to also *inhibit* the insulin secretory response to a maximal Ca^{2+} challenge [7]. Indeed, in the intact islet, GTP depletion slightly potentiated the early secretory response to 40 mM K^+ [5]. Our studies do not elucidate the reason why GTP depletion does not impair inhibited K^+-induced insulin release in islets [1, 5] as it does in single β cells. However, it is not explicable by a relative deficiency of cyclic AMP (due to the absence or deficiency of glucagon) in the latter, since MPA decreased insulin secretion even in the presence of forskolin. The relative magnitude of reductions in GTP content and the GTP/GDP ratio are equivalent between HIT cells, INS-1 cells, and islets. However, the alterations in GTP biosynthetic pathways in the transformed cells (described above), or the absence of other physiologic paracrine relationships, merit investigation in this regard, as does the possibility of an altered subcellular localization of the GTP measured in the whole cell. Interestingly, in the studies of Detimary and colleagues [29], azide (a mitochondrial poison) inhibited glucose-induced insulin secretion at lower concentrations than it inhibited K^+-induced insulin release; these effects were felt to reflect a differential sensitivity to the depletion of ATP. However, in our studies [3] as well as those of Detimary *et al.* [30], ATP depletion induced a parallel depletion in GTP. Thus, it is possible that changes in GTP actually mediate some of the postulated effects of ATP in exocytotic secretion, and that blockade of Ca^{2+}-induced secretion requires lower levels of GTP (or more prolonged depletion) than does blockade of nutrient-induced release.

MPA (and other GTP synthesis inhibitors) has been very useful in defining a role for guanine nucleotides in insulin secretion; in addition, our results may have clinical implications. MPA is the active compound in the newly approved immunosuppressive drug mycophenolate mofetil, which is being used currently in organ transplantation to prevent graft rejection [31, 32]. Given the deleterious effects of MPA on insulin secretion *in vitro*, concern about potential impairment of insulin secretion *in vivo* seems warranted. Conversely, MPA could be quite useful in the treatment of certain types of hyperinsulinemia, such as insulinoma.

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