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# Effects of Inhibitors of Guanine Nucleotide Synthesis on Membrane Potential and Cytosolic Free $Ca^{2+}$ Levels in Insulin-Secreting Cells

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**ABSTRACT.** Adenine nucleotides play an important role in the control of membrane potential by acting on ATP-sensitive $K^+$ ($K_{ATP}$) channels and, in turn, modulating the open probability of voltage-gated $Ca^{2+}$ channels in pancreatic islet β-cells. Here, we provide evidence that guanine nucleotides (GNs) also may be involved in the modulation of these events *in vivo*. GNs were depleted by treatment of HIT-T15 cells with mycophenolic acid (MPA). Resting membrane potential was more depolarized in cells treated for 3 and 6 hr with MPA than in control cells, and this effect was inhibited by diazoxide. After 6 hr of exposure to MPA, basal cytosolic free $Ca^{2+}$ concentrations ($[Ca^{2+}]_i$) were elevated by 20%. Increments in $[Ca^{2+}]_i$ induced by submaximal concentrations of $K^+$ (10–15 mM) or bombesin were enhanced by > 50%. Opening $K_{ATP}$ channels with diazoxide lowered basal $[Ca^{2+}]_i$ in MPA-treated cells to normal and abrogated the enhanced $[Ca^{2+}]_i$ responses. However, an L-type $Ca^{2+}$ channel blocker only abolished the enhanced $[Ca^{2+}]_i$ response to stimuli and had no effect on the elevated basal $[Ca^{2+}]_i$, in contrast to EGTA, which obliterated both, implying that the latter was due to $Ca^{2+}$ influx via non-L-type $Ca^{2+}$ channels. These effects on ion fluxes were attributable specifically to GN depletion, since guanosine, which restores GTP content and the GTP/GDP ratio, but not adenosine, prevented all MPA-induced ion changes; furthermore, the latter were mimicked by mizoribine (a structurally dissimilar GTP synthesis inhibitor). It is concluded that, in addition to adenine nucleotides, GNs might contribute to the modulation of $K_{ATP}$ channels in intact β-cells. In addition, GN depletion appeared to be able to reduce stimulated insulin secretion by a mechanism largely independent of the changes of ion fluxes observed above. BIOCHEM PHARMACOL **59**;5:545–556, 2000. © 2000 Elsevier Science Inc.

**KEY WORDS.** ATP-sensitive potassium channel; calcium; guanine nucleotides; insulin secretion; membrane potential; mycophenolic acid

Glucose-induced insulin secretion from pancreatic β-cells is triggered by an increase in $[Ca^{2+}]_i$[‖] mediated largely through voltage-gated $Ca^{2+}$ channels [1], consequent to closure of $K_{ATP}$ channels and, subsequently, membrane depolarization [2]. Glucose metabolism reduces cellular ADP levels and increases ATP concentrations [2, 3]. The open probability of $K_{ATP}$ channels is believed to be regulated mainly by the ATP/ADP ratio [2]. However, other nucleotides also may participate in the modulation of $K_{ATP}$ channels in insulin-secreting cells, based on studies using the patch-clamp technique [2, 4, 5]. Furthermore, these channels are modulated by receptor ligands, effects probably mediated by G-proteins [6, 7].

In addition to ANs, glucose metabolism generates other poorly defined factors that synergize with $Ca^{2+}$ in the stimulation of insulin secretion [8, 9]. Glucose increases cellular GN content and the GTP/GDP ratio in pancreatic islets [3, 10]. Our previous studies, using GN-depleting agents as probes, indicated that GNs may play an important role in the regulation of hormone release [11–15]. In treated cells, insulin secretion induced by glucose and other nutrients, including pure mitochondrial fuels, was inhibited.

Since $Ca^{2+}$ is a critical trigger of insulin secretion, the aim of this study was to examine whether $[Ca^{2+}]_i$ is also affected in GN-deficient cells. To examine this question, we used two immunosuppressive drugs, MPA and mizoribine, which specifically deplete cellular GNs by inhibition of IMP dehydrogenase, with insignificant effects on ANs [11, 15]. The results showed that GN-depleted cells displayed partial depolarization, probably mediated via the closure of $K_{ATP}$ channels, and thus had an augmented $[Ca^{2+}]_i$ rise in

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‖ *Abbreviations*: ANs, adenine nucleotides; $[Ca^{2+}]_i$, cytosolic free $Ca^{2+}$ concentrations; GNs, guanine nucleotides; $K_{ATP}$ channels, ATP-sensitive $K^+$ channels; KRBH, Krebs–Ringer–bicarbonate–HEPES buffer; MPA, mycophenolic acid; and MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium.

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response to submaximal stimulation by depolarizing agents. These findings suggested that GNs might participate in the maintenance of $[Ca^{2+}]_i$ homeostasis, perhaps mediated by modulation of the activity of $K_{ATP}$ channels. We also provide further evidence [15] for a role of GNs in maintaining insulin secretion distal to any $[Ca^{2+}]_i$ rise.

## MATERIALS AND METHODS

### *Materials*

MPA, bombesin, thapsigargin, and adenosine, guanosine, and purine nucleotides were purchased from the Sigma Chemical Co.; RPMI 1640 and fetal bovine serum were obtained from Gibco/BRL; and indo-1/acetoxymethyl ester and bis-(1,3-diethylthiobarbituric acid)trimethineoxonol (bisoxonol) were purchased from Molecular Probes. Mizoribine was a gift from Dr. N. Kazmatani (Tokyo Women's Medical College). MPA and mizoribine were dissolved in ethanol and distilled water, respectively.

### *Cell Culture*

Insulin-secreting HIT-T15 cells (passage 73–80; provided by Drs. R. P. Robertson and H-J. Zhang, University of Minneapolis) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum as described previously [16].

### *Measurement of Nucleotides*

HIT cells were cultured in 24-well plates and treated with various concentrations of MPA for 6 hr. Purine nucleotides were extracted after the MPA treatment in culture medium (RPMI 1640 containing 11.1 mM glucose) and quantitated by HPLC as described in detail previously [11, 15].

### *Determination of $[Ca^{2+}]_i$*

Cytosolic free calcium ($[Ca^{2+}]_i$) was determined as described previously [16]. HIT cells were treated with MPA or other agents for various periods in culture medium, as well as during a 3-hr recovery period in spinner medium [16] after detachment from the flasks by trypsinization. Subsequently, the cells were loaded with 1 μM indo-1/acetoxymethyl ester for 30 min at 37°. After washing, about $2 \times 10^6$ cells in 2 mL of glucose-free KRBH were transferred to a cuvette. No MPA was present in the cuvette, since the compound quenches indo-1 fluorescence. All stimuli were added to the cuvette after equilibration of cells at 37° for about 20 min. Fluorescence was recorded using a spectrofluorometer (Perkin Elmer LS-50B), with excitation and emission wavelengths of 355 and 405 nm, respectively. Unless specified, the extracellular indo-1 fluorescence was assessed by adding 50 μM $Mn^{2+}$ (which then was chelated by 100 μM diethylenetriaminepentaacetic acid) and was subtracted accordingly from each trace. The calibration of $[Ca^{2+}]_i$ was carried out by using an equation described in [17]:

$$[Ca^{2+}]_i = (F - F_{min})/(F_{max} - F) \cdot K_d,$$

where F denotes the intracellular fluorescence and $K_d$ is 250 nM for indo-1. The maximal fluorescence ($F_{max}$) was obtained by adding 5 μM ionomycin (a $Ca^{2+}$ ionophore), and the minimal fluorescence ($F_{min}$) was achieved by introducing 8 mM EGTA plus 60 mM Tris in the presence of 5 μM ionomycin.

### *Measurement of Membrane Potential*

Membrane potential of HIT cells was monitored using a voltage-sensitive fluorescent probe, bisoxonol, as described previously [16]. The anionic dye binds to the plasma membrane; its fluorescence is increased when cells are depolarized and is decreased when cells are hyperpolarized. Cells were treated with MPA for various periods in the same way as for $[Ca^{2+}]_i$ determinations. After washing, about $2 \times 10^6$ cells in glucose-free KRBH buffer were placed in a cuvette. Bisoxonol (100 nM) was added for equilibration of cells at 37° for about 20 min. Fluorescence was recorded with excitation and emission wavelengths of 540 and 580 nm, respectively. For comparison and statistical analysis, the bisoxonol fluorescent signals were normalized by expression of results as the percentage of a near-maximal depolarization achieved by 40 mM KCl in each trace. A higher concentration (60 mM) of KCl only slightly increased the signal, by a further 5%.

### *Assessment of Nutrient Metabolism by MTS Assay*

Nutrient metabolism of the cells was monitored using an MTS assay kit (CellTiter 96$^{tm}$) developed by Promega. The MTS assay [18] has a major advantage over the older, widely used MTT assay [19], since the formazan formed during the MTS assay is soluble and exits the cells without a need for disruption. In addition, the nutrient-generated signal over basal is much larger, about five times greater than with the MTT test. Our studies indicated that the reducing equivalents measured by the MTS test probably reflect NAD(P)H generated by glucose metabolism in HIT cells from both glycolysis (major effect) and mitochondrial oxidation (more minor effect) [20].

HIT cells were seeded onto 96-well plates and cultured in the presence of test agents for various periods. A mixture of MTS and PMS (phenazine methosulfate; an electron coupling reagent) (final concentrations 333 and 25 μg/mL, respectively) was added, and cells were incubated for 30 min at 37°. The reaction was stopped by the addition of 10% SDS if samples were not subjected immediately to the determination of absorbance. Formazan formed from reduction of MTS [18] was quantitated by measurement of absorbance of the medium at 490 nm using a microplate reader. All data have been corrected for background signals.

**TABLE 1. Effects of MPA treatment on the content of GNs and ANs**

| MPA (μg/mL) | GTP (nmol/well) | ATP (nmol/well) | GTP/GDP ratio | ATP/ADP ratio |
|---|---|---|---|---|
| Control | 1.20 ± 0.06 | 6.52 ± 0.35 | 7.24 ± 0.41 | 15.73 ± 0.62 |
| 0.1 | 0.83 ± 0.01* | 5.11 ± 0.17 | 7.16 ± 1.80 | 15.56 ± 0.22 |
| 0.3 | 0.38 ± 0.03† | 5.93 ± 0.10 | 2.08 ± 0.20† | 17.67 ± 0.16 |
| 1 | 0.23 ± 0.02† | 4.72 ± 0.14† | 2.81 ± 0.30† | 19.00 ± 0.69* |
| 3 | 0.22 ± 0.01† | 5.48 ± 0.32 | 1.08 ± 0.09† | 16.75 ± 1.13 |
| 25 | 0.20 ± 0.02† | 4.69 ± 0.25† | 1.04 ± 0.20† | 12.27 ± 0.58* |

HIT cells were treated with various concentrations of MPA for 6 hr. Then the cellular nucleotides were extracted and separated by HPLC. Values are means ± SEM of three separate experiments.

*$P < 0.05$ vs controls.

†$P < 0.01$ vs controls.

### *Measurement of Insulin Secretion*

HIT cells were seeded on 24-well plates and cultured for 2–3 days. Test agents were added to the culture medium for various periods. After one wash, the cells were preincubated for 30 min in glucose-free KRBH buffer at 37°. Then the medium was replaced with stimulating solution and incubated for 30 min. MPA and other agents used for pretreatment also were included during the preincubation and incubation periods, when appropriate. The supernatants were removed for measurement of released insulin, and the attached cells were extracted with acid ethanol for determination of insulin content. Insulin was measured by double-antibody radioimmunoassay, using rat insulin as a standard [21].

### *Statistical Analysis*

Data are expressed as means ± SEM, and statistical analysis was carried out using Student's two-tailed *t*-test. The number of observations reported refers to the number of replicate determinations from at least three independent experiments.

## RESULTS

### *Effect of MPA Treatment on Purine Nucleotide Content*

Table 1 shows the effect of 6-hr MPA treatment on the content of purine nucleotides in HIT cells. Both GTP content and the GTP/GDP ratio were reduced markedly (by 80 and 86%, respectively), with the maximal effect caused by 1 μg/mL of MPA. The effect of MPA on ANs was much smaller and less potent, but complex. ATP content was decreased by about 15% at 1 μg/mL or higher concentrations of MPA. ATP/ADP was increased marginally at 1 μg/mL of MPA and slightly decreased at a higher MPA concentration (25 μg/mL); there were no changes at other concentrations of MPA. The MPA-induced decrease in GTP content at 6 hr could be blocked by guanosine but not adenosine [15]. The marked decrease of the GTP/GDP ratio induced by MPA also was prevented by co-culture with guanosine (500 μM), being reduced to 81% of the control, whereas there was no substantial change in the ATP/ADP ratio under the above conditions. In contrast, the presence of 500 μM adenosine did not prevent the MPA-induced reduction of the GTP/GDP ratio and had no major effect on the ATP/ADP ratio compared with MPA treatment alone [15]. Thus, MPA mainly alters the GN content and the GTP/GDP ratio, and these alterations are reversed specifically by guanosine but not adenosine. Shorter treatment (1–3 hr) with MPA (1 μg/mL) also induced a marked decrease of GTP and the GTP/GDP ratio in HIT cells while not changing ATP/ADP and inducing a slight drop in ATP content [15]. All biological effects described below were seen under the above experimental conditions.

### *Effect of Depletion of GNs on $[Ca^{2+}]_i$*

MPA (1 μg/mL) treatment for 3 hr failed to increase basal $[Ca^{2+}]_i$ levels significantly (186 ± 11 in control and 197 ± 11 nM in treated cells; $P = 0.47$, N = 17 each). $[Ca^{2+}]_i$ rises in response to submaximal $K^+$ or bombesin also were not altered significantly (data not shown). Glucose-induced $[Ca^{2+}]_i$ rises also were not affected (308 ± 27 in control and 301 ± 14 nM in treated cells; N = 4 each; $P > 0.05$).

However, changes in $[Ca^{2+}]_i$ clearly occurred after a 6-hr MPA treatment. From studies of eight batches of cells, basal $[Ca^{2+}]_i$ was elevated significantly by 20% ($P < 0.001$), from 179 ± 6 nM (N = 22) in control cells to 215 ± 8 nM (N = 23) in MPA-treated (1 μg/mL for 6-hr) cells. Co-exposure of cells to 500 μM guanosine restored basal $[Ca^{2+}]_i$ to control levels (183 ± 5 nM, N = 8; $P = 0.68$ vs control), whereas 500 μM adenosine was unable to prevent the MPA effect (235 ± 11 nM, N = 8; $P < 0.01$ vs control).

$[Ca^{2+}]_i$ responses to several stimuli also were examined in these cells. The $[Ca^{2+}]_i$ increment evoked by submaximally effective (10 and 15 mM) $K^+$ concentrations was enhanced significantly in GTP-depleted, MPA-treated cells (Fig. 1B). At 10 mM $K^+$, the $[Ca^{2+}]_i$ increment was augmented by 79% (29 ± 3 in control and 51 ± 4 in MPA-treated cells; N = 3 in each case; $P = 0.01$), and by 54% at 15 mM $K^+$ (139 ± 11 in control and 213 ± 17 in

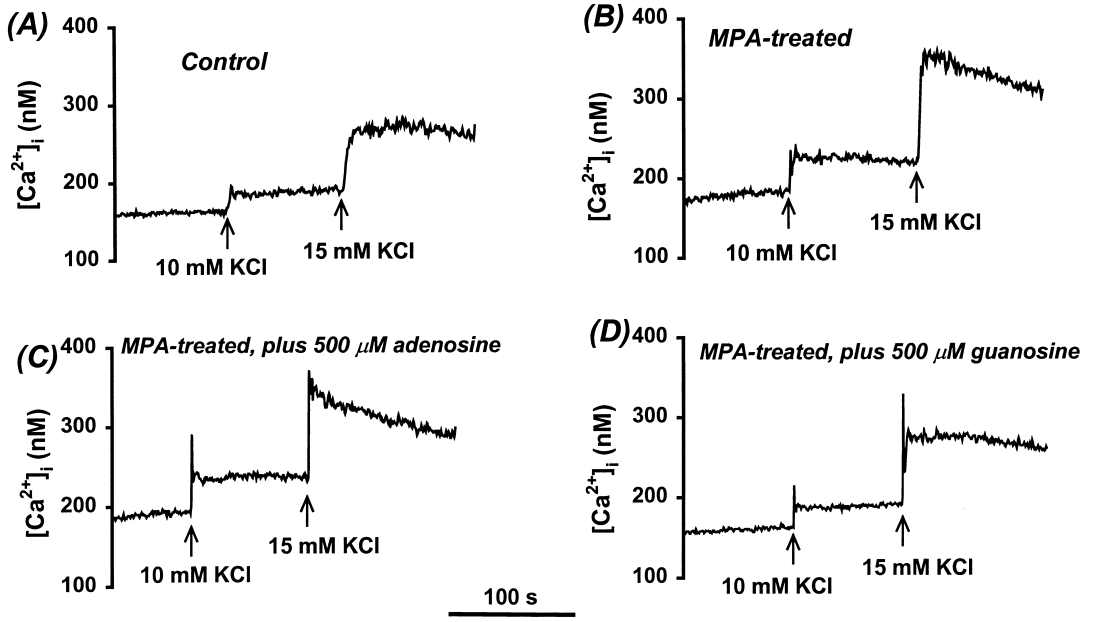


**FIG. 1. Enhancement of increments in $[Ca^{2+}]_i$ induced by submaximal concentrations of extracellular $K^+$ subsequent to MPA treatment. HIT cells were treated with 1 μg/mL of MPA, alone or combined with either adenosine or guanosine, for 6 hr. After loading with indo-1/AM (1 μM) for 30 min, the fluorescent signal in cells was recorded using a spectrofluorometer. Cells were washed and transferred into a glass cuvette containing 2 mL of KRBH buffer. Stimulus was added after about 15 min of equilibration of cells in the cuvette at 37°. Extracellular fluorescent signal was assessed by $Mn^{2+}$ quenching and subtracted before calibration of $[Ca^{2+}]_i$, which was calculated by an equation described in Materials and Methods. The basal $K^+$ concentration was 6 mM. All KCl additions in the figure are the final concentrations in the medium. All traces are representative of at least four observations in each case.**

MPA-treated cells; N = 5 in each case; $P < 0.01$). However, the $[Ca^{2+}]_i$ rise induced by a higher $K^+$ (23 mM) or a saturating concentration (40 mM) of $K^+$ was not affected by MPA treatment (data not shown). The elevated basal $[Ca^{2+}]_i$ and the enhanced $[Ca^{2+}]_i$ responses to depolarizing $K^+$ could be prevented by co-provision of guanosine (Fig. 1D), but not by adenosine (Fig. 1C), even at 2 mM (not shown). Identical changes in $[Ca^{2+}]_i$ also were observed after exposure (6 hr) of cells to a second, chemically different but mechanistically identical, GN-depleting agent, mizoribine (25 μg/mL; data not shown).

The addition of 10 mM glucose increased $[Ca^{2+}]_i$ by ~70 nM in control cells. In cells treated with 1 μg/mL of MPA for 6 hr, the $[Ca^{2+}]_i$ response to glucose was not enhanced significantly above control values; the $[Ca^{2+}]_i$ increment over basal was 67 ± 16 in control and 51 ± 17 in MPA-treated cells (N = 4 each; $P = 0.55$).

Bombesin is a receptor agonist that causes partial depolarization of the membrane potential and increases $[Ca^{2+}]_i$, both by mobilization of intracellular stores and by promotion of $Ca^{2+}$ entry into HIT cells [16]. Bombesin-induced depolarization is probably due to the closure of $K_{ATP}$ channels, similar to the effect of vasopressin in RINm5F cells [7], as it can be reversed or blocked by diazoxide [16]. The effects of MPA pretreatment on the $[Ca^{2+}]_i$ response to this agonist are shown in Figs. 2 and 3. Bombesin (100 nM) elicited a large $[Ca^{2+}]_i$ spike followed by a small plateau elevation in control cells (Fig. 2A). The $[Ca^{2+}]_i$ response to bombesin was enhanced in MPA-treated cells, with a higher initial peak and a marked increase of the later phase (Fig. 2B). The incremental rise in $[Ca^{2+}]_i$ at 1 min after the addition of bombesin was doubled in MPA-treated cells (Fig. 2F). The enhanced $[Ca^{2+}]_i$ rises appeared not to be due to augmented $Ca^{2+}$ stores in inositol 1,4,5-trisphosphate-sensitive intracellular sites, since the effect of thapsigargin, which depletes these $Ca^{2+}$ stores, on increments in $[Ca^{2+}]_i$ was not amplified in MPA-treated cells (Fig. 2, B and F). The very early $[Ca^{2+}]_i$ rise (first 20 sec), which is due mainly to $Ca^{2+}$ mobilization from intracellular stores, was elevated by 30% (average increment: 238 ± 11 nM in MPA-treated cells vs 183 ± 14 nM in control; N = 6 each, $P < 0.01$), whereas the later (100 sec after the initial) elevation was increased markedly by 2.4-fold (average increment: 78 ± 7 nM in MPA-treated cells vs 23 ± 4 nM in control; N = 6 each; $P < 0.01$) in GN-depleted cells. In addition, bombesin-evoked $[Ca^{2+}]_i$ rises in MPA-treated cells were enhanced to the same extent in the presence of 10 mM glucose in KRBH solution, compared with the absence of glucose (data not shown). Thus, it seems that the effect of MPA on ion fluxes is independent of glucose concentrations. Furthermore, the L-type $Ca^{2+}$ channel blocker verapamil abolished the enhanced $[Ca^{2+}]_i$ rises (mainly acting on the later phase) induced by bombesin (Fig. 2, E and F), implicating extracellular $Ca^{2+}$ entry via voltage-sensitive channels. However, the elevated basal $[Ca^{2+}]_i$ levels in the GN-depleted cells were not affected by verapamil (Fig. 2E), but were blocked when extracellular $Ca^{2+}$ was chelated by EGTA (see below). The enhanced

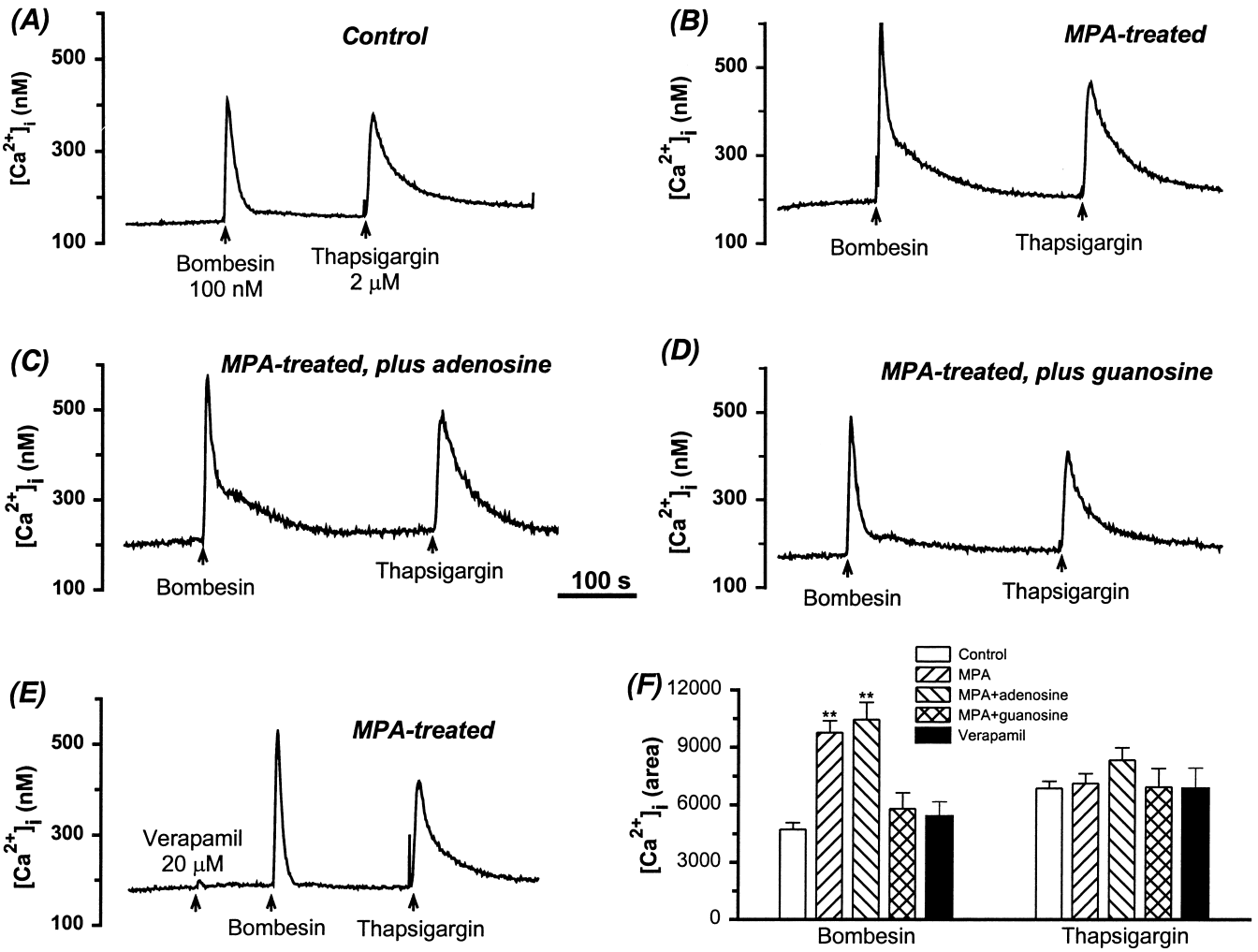


**FIG. 2. Enhancement by MPA of bombesin-induced $[Ca^{2+}]_i$ rises via opening L-type $Ca^{2+}$ channels, without affecting $Ca^{2+}$ release from intracellular stores. HIT cells were treated with 1 μg/mL of MPA for 6 hr. Final concentrations of additions: bombesin, 100 nM; verapamil, 20 μM; and thapsigargin, 2 μM. All traces are representative of at least four observations. The $[Ca^{2+}]_i$ area in (F) was calculated by integrating the increment during 60 sec after adding a stimulus, and the values are means ± SEM of 4–8 observations. Key: (**) $P < 0.01$ vs control.**

$[Ca^{2+}]_i$ responses to bombesin in MPA-treated cells were prevented by co-provision of guanosine (Fig. 2D) but not adenosine (Fig. 2C), even at a 2 mM concentration of the latter (not shown). Neither guanosine nor adenosine treatment alone (500 μM for 6 hr) affected basal $[Ca^{2+}]_i$ or the responses to stimuli in the absence of MPA (results not shown).

The increase in $Ca^{2+}$ permeability of the plasma membrane induced by bombesin also was demonstrated in another series of experiments. When extracellular $Ca^{2+}$ (normally 1.5 mM) was chelated by adding 3 mM EGTA (free $Ca^{2+}$ levels in medium were 41 ± 3 nM, N = 3), bombesin only induced a single $[Ca^{2+}]_i$ spike by mobilizing $Ca^{2+}$ from intracellular stores. In control cells, restoration of extracellular free $Ca^{2+}$ brought $[Ca^{2+}]_i$ back to the levels seen prior to the addition of EGTA (Fig. 3A). In contrast to the results seen in normal buffer containing $Ca^{2+}$, there was no enhancement of the bombesin-induced $[Ca^{2+}]_i$ rise in GN-depleted cells when stimulated in the absence of extracellular free $Ca^{2+}$ (Fig. 3B). However, the addition of $Ca^{2+}$ back to the medium resulted in an overshoot of the $Ca^{2+}$ plateau (Fig. 3, B and F), which could be blocked by verapamil (Fig. 3E). Moreover, guanosine (Fig. 3D), but not adenosine (Fig. 3C), prevented these MPA effects. In addition, the elevated basal $[Ca^{2+}]_i$ levels in MPA-treated cells returned to control levels in the absence of extracellular free $Ca^{2+}$ (134 ± 10 nM, compared with 146 ± 15 nM in controls; $P = 0.28$, N = 10).

The enhanced $[Ca^{2+}]_i$ responses to bombesin after MPA treatment, expressed in terms of incremental changes, could be abolished by prior hyperpolarization of the cells with diazoxide, an opener of $K_{ATP}$ channels [22]. Thus, bombesin-induced integrated increments of $[Ca^{2+}]_i$ during 60 sec of stimulation were 9772 ± 621 nM (N = 10; $P <$ 0.001 vs control) in MPA-treated cells, higher than the increments that occurred in control cells (4722 ± 368 nM,

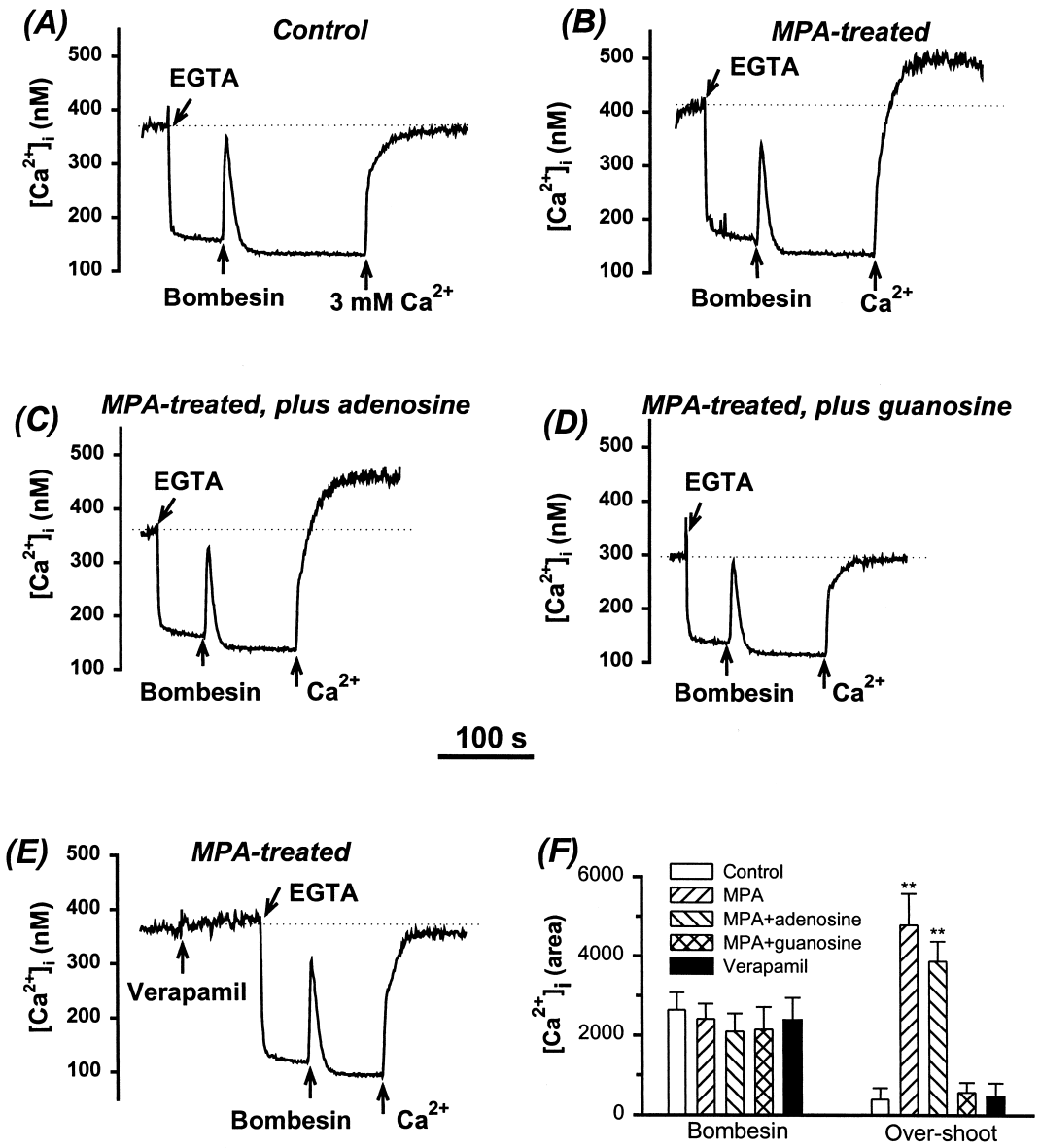


**FIG. 3. Augmentation of extracellular $Ca^{2+}$ entry into MPA-treated cells. HIT cells were treated with 1 μg/mL of MPA for 6 hr. In this figure, calculations of $[Ca^{2+}]_i$ were not corrected through the use of the $Mn^{2+}$-quench technique for contamination by extracellular indo-1. Therefore, the reported $[Ca^{2+}]_i$ values of these traces, before adding EGTA and after restoring extracellular free $Ca^{2+}$, were higher than actual levels (due to contaminating signal from extracellular indo-1). Absolute values for $[Ca^{2+}]_i$ are only completely accurate for the periods when extracellular $Ca^{2+}$ was chelated by EGTA, since, under this condition, no contaminating signal from extracellular indo-1 will be generated (because no free $Ca^{2+}$ is available). However, assessments of relative changes are valid throughout. Final concentrations of additions: EGTA, 3 mM; bombesin, 100 nM; and verapamil, 20 μM. The concentration of both adenosine and guanosine during cell treatment was 500 μM, when present. Traces are representative of at least four observations in each case. The $[Ca^{2+}]_i$ area in panel F was calculated by integrating the increment during 30 sec after adding bombesin and over 60 sec following restoration of extracellular $Ca^{2+}$. Values are means ± SEM of 4–6 observations. Key: (**) $P < 0.01$ vs control.**

N = 9). In the presence of diazoxide (100 μM), the enhanced $[Ca^{2+}]_i$ response to bombesin was prevented: 5348 ± 853 nM (N = 5; $P = 0.4$ vs control). In addition, diazoxide abrogated the enhancement of $[Ca^{2+}]_i$ responses induced by submaximal concentrations (10 and 15 mM) of $K^+$, but had no effect on $[Ca^{2+}]_i$ increase evoked by $K^+$ itself (Fig. 4). These data again suggest that the enhanced $[Ca^{2+}]_i$ responses induced by MPA may be attributable to the closure of $K_{ATP}$ channels, especially since repolarization induced by diazoxide had no apparent effect on $Ca^{2+}$ currents stimulated by $K^+$. The elevated basal $[Ca^{2+}]_i$ levels in GN-depleted cells also were prevented by diazoxide; the decrement of resting $[Ca^{2+}]_i$ induced by 100 μM diazoxide was 3 ± 1 nM in control and 28 ± 5 nM in treated cells (N = 6 each, $P < 0.001$). This suggests that a voltage-gated, but non-L-type $Ca^{2+}$ channel (because of lack of response to verapamil; cf. Fig. 3E), contributed to the basal $[Ca^{2+}]_i$ elevation. Non-L-type (T- or N-type) $Ca^{2+}$ chan-

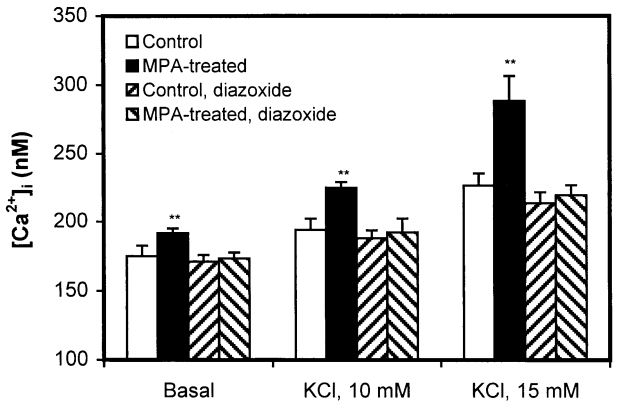


**FIG. 4. Effect of diazoxide on $K^+$-evoked $[Ca^{2+}]_i$ rises in control and MPA-treated HIT cells. Cells were treated with 1 μg/mL of MPA for 6 hr. When present, 100 μM diazoxide was added 5 min before stimulation by increasing KCl to the indicated concentrations. Values are means ± SEM from 3–6 observations. Key: (**) $P < 0.01$ vs control.**

nels have been identified previously in pancreatic β-cells [23–25], and they may be involved because of their different sensitivities to membrane potential.

### *Effect of MPA Treatment on Membrane Potential*

The above observations from studies on $[Ca^{2+}]_i$ suggested that MPA-treated cells might be more depolarized. To test this hypothesis, we used a fluorescent probe (bisoxonol) to monitor membrane potential [16].

Treatment with 1 μg/mL of MPA for 3 hr slightly, but significantly, depolarized the resting membrane potential of cells (69.5 ± 1.1% in control and 73.8 ± 1.0% in treated cells; N = 10 each, $P = 0.01$). The resting membrane potential of MPA-treated cells (1 μg/mL for 6 hr) was depolarized further (Fig. 5) compared with control (73.5 ± 1.3% in control and 80.6 ± 1.1% in treated cells; N = 13 each, $P < 0.001$). Non-saturating concentrations of $K^+$ (10 and 15 mM) brought membrane potential to higher levels in treated cells (Fig. 5, A–C), reminiscent of the enhanced $[Ca^{2+}]_i$ rises induced by these concentrations of $K^+$. The greater depolarization by 10–15 mM $K^+$ in MPA-treated cells was due to the elevations of resting membrane potential because the increments evoked by $K^+$ were not increased. No difference was found upon further addition of $K^+$ (23 mM), a concentration at which $[Ca^{2+}]_i$ enhancement by MPA also was not observed. The MPA-induced alteration in membrane potential was sensitive to the $K_{ATP}$ channel opener diazoxide (100 μM), which hyperpolarized resting membrane potential in treated cells to a level similar to that achieved in control cells (Fig. 5, D–F). After diazoxide, $K^+$ additions evoked depolarization of both cell cultures to the same extent. The peak depolarization due to bombesin was increased (84.4 ± 2.6 vs 78.8 ± 3.0 in control; N = 4 each; $P < 0.01$) in treated cells, but the

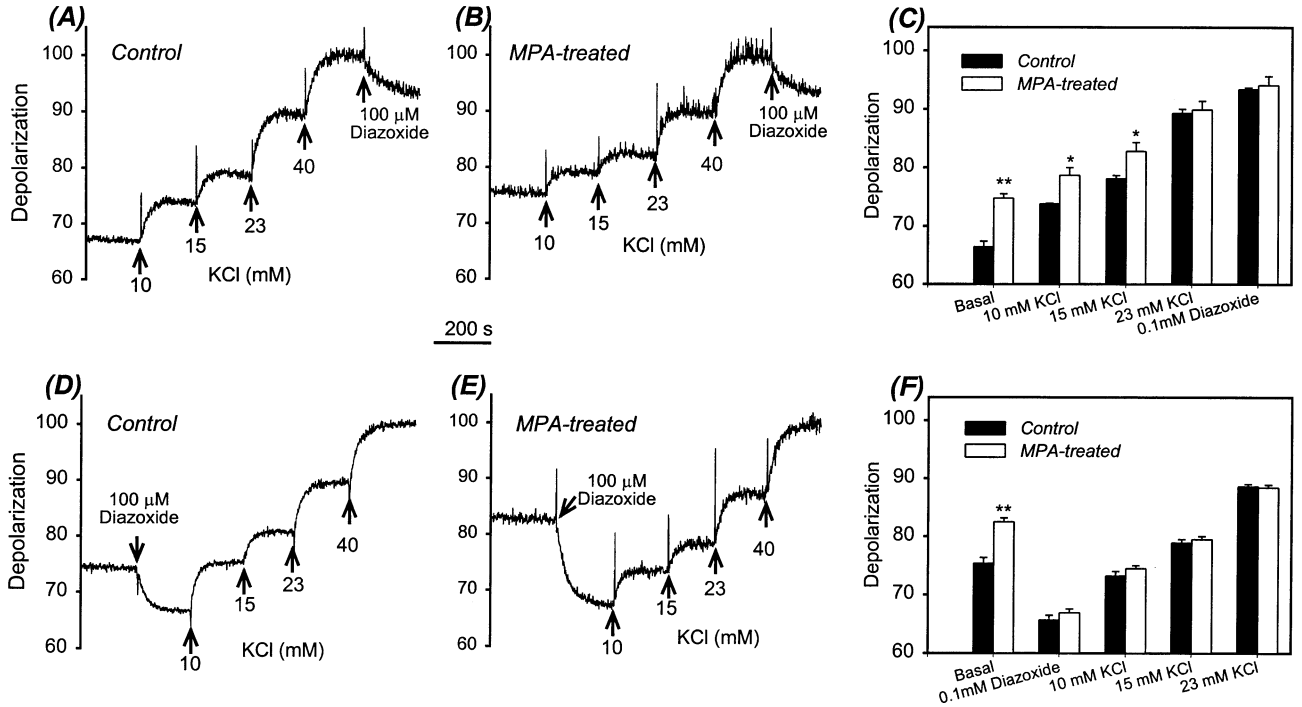


**FIG. 5. Effect of MPA treatment on membrane potential in HIT cells. After cells were pretreated with 1 μg/mL of MPA for 6 hr, membrane potential was monitored with the voltage-sensitive fluorescent probe bisoxonol. Increase of fluorescence (upward) indicates depolarization, whereas decrease of fluorescence (downward) indicates hyperpolarization. The results were expressed as a percentage of the depolarizing signal achieved with a saturating concentration of KCl (40 mM) for each trace. Traces are representative of at least four observations in each case. The KCl additions indicated in the figure denote the final concentrations reached in the medium. The bars in panel C associated with diazoxide were achieved following the addition of 40 mM KCl. Values in panels C and F are means ± SEM of 4–8 observations. Key: (*) $P < 0.05$, and (**) $P < 0.01$ compared with controls.**

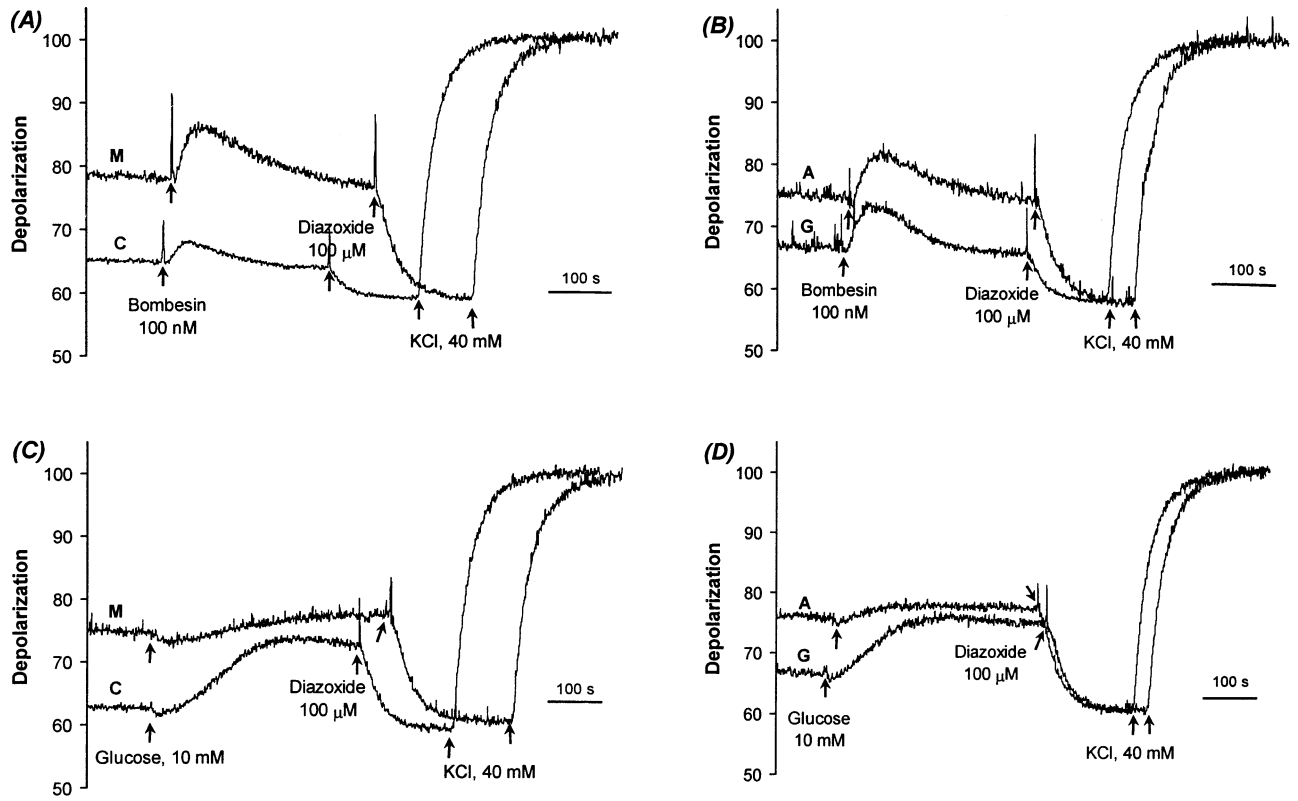


**FIG. 6. Effect of MPA treatment on membrane depolarization induced by bombesin and glucose. Membrane potential was monitored by using bisoxonol (see the legend to Fig. 5 for details). Conditions: C, control; M, treated with MPA (1 μg/mL) for 6 hr; A, adenosine (500 μM) plus MPA for a 6-hr pretreatment; and G, guanosine (500 μM) plus MPA for a 6-hr pretreatment. The traces are representative of at least three experiments in each case.**

incremental change in membrane potential was not decreased (Fig. 6A). In contrast, glucose depolarized membrane potential to identical levels; however, the increment over basal was reduced significantly by 49% (5.8 ± 1.2 vs 11.5 ± 1.8 in control; N = 4 each, $P < 0.01$) in treated cells (Fig. 6C). Mirroring its effect on membrane potential, glucose also raised $[Ca^{2+}]_i$ to a similar final level in both control and MPA-treated cells (see above), despite a slight reduction of the incremental change. This might be explained by the fact that nutrient metabolism not only causes depolarization but also might enhance the opening activity of voltage-gated $Ca^{2+}$ channels in insulin-secreting cells [26, 27]. As with effects on $[Ca^{2+}]_i$, the changes in membrane potential after MPA treatment could be prevented by simultaneous addition of guanosine, but not adenosine (Fig. 6, B and D).

### *Effect of MPA Treatment on Nutrient Metabolism and Insulin Secretion*

Overall glucose metabolism was assessed by measurement of the reduction of a tetrazolium salt (MTS) in intact HIT cells. Glucose-stimulated insulin secretion and glucose-elicited MTS reduction were correlated closely as a function of the sugar concentrations (Fig. 7). Glucose-induced MTS reduction was not affected by up to 3-hr MPA treatment. Six-hour exposure modestly but significantly reduced the glucose-produced signal by ~25% (Table 2). This effect was prevented by guanosine but not adenosine. There was no change of cell viability following a 6-hr MPA treatment, as determined by the trypan-blue exclusion test (not shown).

Basal insulin secretion was not altered by MPA treatment for up to 6 hr. The insulin secretion stimulated by 10 mM glucose alone, or combined with bombesin, as well as that stimulated by 40 mM $K^+$, was inhibited by 50–80% after MPA treatment for either 3 or 6 hr (Fig. 8). Glucose-induced release was most sensitive to GN depletion, whereas that promoted by $K^+$ was less affected. The inhibitory effects of MPA on insulin secretion could be abrogated by co-provision with guanosine but not by adenosine (data not shown).

## DISCUSSION

This study focuses on the effects of endogenous GNs on membrane potential and $[Ca^{2+}]_i$ in intact insulin-secreting cells by using two chemically dissimilar agents that deplete cellular GNs selectively. Several interesting observations were obtained. The data suggested novel roles of GNs in the maintenance of resting (and also the stimulation of)


**FIG. 7. Relationship between glucose-stimulated insulin secretion and nutrient metabolism reflected by MTS reduction. HIT cells were seeded on multi-well plates. After 30 min of preincubation in glucose-free medium, cells were stimulated by different concentrations of glucose in the presence of MTS (for determination of its reduction product formazan) or the absence of MTS (for measurement of insulin secretion), in parallel experiments. Values are means ± SEM of three independent experiments.**

membrane potential, putatively by an action on $K_{ATP}$ channels, thereby indirectly modulating voltage-gated $Ca^{2+}$ channels. In contrast, thapsigargin-sensitive intracellular $Ca^{2+}$ pools appeared unaffected by GN depletion. Finally, GNs modulated exocytosis in insulin-secreting cells at sites beyond the rise in $[Ca^{2+}]_i$, since MPA inhibited insulin release without affecting (or while even enhancing) $[Ca^{2+}]_i$ responses to secretagogues. This formulation is in accord with our earlier data [28], which were obtained by a different approach.

The modification of membrane potential could be detected following a 3-hr exposure to the GN-depleting agent MPA and was magnified further 6 hr later. Although there was a tendency for $[Ca^{2+}]_i$ to change by 3 hr, clearer alterations were observed only after a 6-hr treatment with MPA. In contrast, the cellular GN content already was reduced markedly (by ~80%) after a 3-hr treatment and only decreased slightly further after a 6-hr exposure [15]. The reason for the delay in $[Ca^{2+}]_i$ changes is unclear. It is possible that an earlier alteration was too small to be detected or that the change in membrane potential did not reach a level sufficient to affect $Ca^{2+}$ channels at 3 hr. Similar alterations were seen in assay medium either with or without glucose, arguing against a role for changes in glucose metabolism. Moreover, inclusion of methylene blue (2 μg/mL) or 6-aminonicotinamide (35 μg/mL), both of which deplete cellular NAD(P)H [29], failed either to mimic the MPA effect (by themselves) or to potentiate the action of MPA on membrane potential and $[Ca^{2+}]_i$ after 3 hr of treatment (data not shown). Another possibility may relate to compartmentation of GNs [30, 31]. The GN "pools" near the target region may have to be reduced below a critical level before the changes of ionic events can be detected [32]. Whatever the exact mechanism, the alterations of membrane potential, $[Ca^{2+}]_i$, and insulin secretion seen in MPA-treated cells all appeared to be specifically attributable to depletion of GNs, since they could be prevented by co-provision of guanosine (which restores GNs and ANs) but not by a wide concentration range (100–2000 μM) of adenosine (which preserves only ANs) [11, 15]. It is unlikely that the reversing effect of guanosine is mediated by actions on an exofacial purine receptor on the plasma membrane [33], since co-exposure of cells to 500 μM GTP or GDP (acting as purinergic agonists) for 6 hr failed to prevent the MPA-induced alterations of $[Ca^{2+}]_i$ (data not shown). Furthermore, a second GN-depleting compound, mizoribine, caused changes similar to those seen with MPA. A slight increase in the ATP/ADP ratio was seen at ~1 μg/mL of MPA, which might influence $K_{ATP}$ channels directly; this cannot explain the ionic changes found in this study, since the latter also were observed at 25 μg/mL of MPA (data not shown), a condition at which no increase of ANs occurred (cf. Table 1). In accord with this formulation, mizoribine

**TABLE 2. Effect of GN depletion on MTS reduction**

| Treatment | Control | MPA | MPA + guanosine | MPA + adenosine |
|---|---|---|---|---|
| 1 hr | 0.85 ± 0.02 | 0.82 ± 0.02 | 0.85 ± 0.02 | 0.83 ± 0.02 |
| 3 hr | 0.82 ± 0.03 | 0.80 ± 0.05 | 0.84 ± 0.02 | 0.81 ± 0.02 |
| 6 hr | 0.87 ± 0.07 | 0.68 ± 0.04* | 0.84 ± 0.03 | 0.64 ± 0.03* |

HIT-cells were seeded on multi-well plates and treated with 1 μg/mL of MPA alone or plus 500 μM adenosine or guanosine for various times. After a 30-min preincubation in glucose-free medium, cells were incubated with 10 mM glucose in the presence of MTS. A colored formazan was formed by reduction of MTS, and its optical density was determined with a microplate reader at 490 nm. The values (in arbitrary units) are means ± SEM of 6–10 observations.

*$P < 0.01$ vs controls.

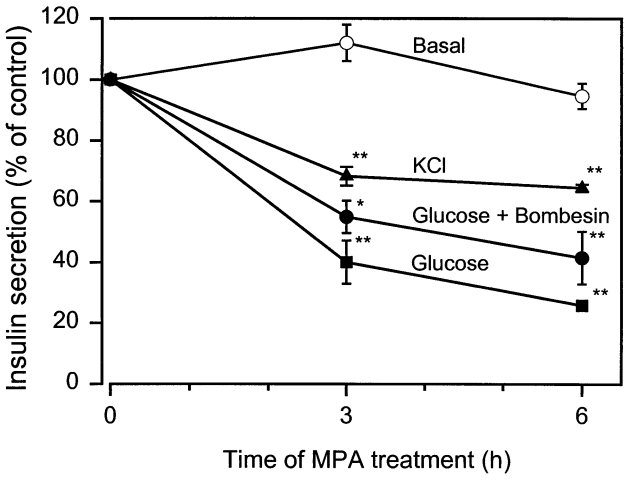


**FIG. 8. Effect of MPA treatment on stimulated insulin secretion from HIT cells. HIT cells were treated with 1 μg/mL of MPA for various time periods. When present, the concentrations of stimuli were: 40 mM KCl, 10 mM glucose, and 100 nM bombesin. Values are means ± SEM of 8–10 observations. The original data are expressed as percent of content (in control cells, these values were 1.22 ± 0.04 [basal], 5.64 ± 0.45 [at 10 mM glucose], 19.54 ± 4.10 [at 10 mM glucose plus 100 nM bombesin], and 6.25 ± 0.65 [at 40 mM KCl]). Key: (*) $P < 0.05$, and (**) $P < 0.01$ compared with controls.**

induced similar alterations of $[Ca^{2+}]_i$, but without affecting the ATP/ADP ratio [15].

The changes of membrane potential and $[Ca^{2+}]_i$ seen in GN-depleted cells might be explained by closure of $K_{ATP}$ channels in the resting state, in turn opening voltage-gated $Ca^{2+}$ channels, since diazoxide, an opener of $K_{ATP}$ channels [22, 34], was able to abolish these alterations of ion fluxes. An effect of GN depletion on some other types of current (e.g. opening a cation channel or closing an ATP-insensitive $K^+$ channel), rather than on $K_{ATP}$ channels, to induce depolarization cannot be excluded completely. However, the fact that the action of GN depletion on depolarization and $[Ca^{2+}]_i$ could be prevented almost completely by diazoxide, which had no apparent effect on L-type $Ca^{2+}$ currents at submaximal or maximal concentrations of $K^+$, argues against a major involvement of other channels. Direct evidence for an effect of GN depletion on $K_{ATP}$ channels was not provided in the current studies; however, GN effects on $K_{ATP}$ channels have been demonstrated in membrane patches by the patch-clamp technique [4, 5, 34]. GN-depleted cells displayed a greater degree of depolarization of resting membrane potential, achieving levels reached by 10 mM $K^+$ in control cells. Membrane potential after adding a subsaturating concentration of $K^+$ (10 and 15 mM) or bombesin, a weak depolarizing receptor agonist [16], also was depolarized to more positive levels in MPA-treated cells. Thus, GN depletion increases the sensitivity of β-cells to stimulation by submaximal depolarizing conditions, resulting in an enhanced $[Ca^{2+}]_i$ increment, which in turn, seems to explain the elevated basal $[Ca^{2+}]_i$ and the enhanced $[Ca^{2+}]_i$ rises under the same conditions. However, this augmentation would not be evident in the $[Ca^{2+}]_i$ responses to already maximal depolarization and $Ca^{2+}$ influx, such as those seen with 23 or 40 mM $K^+$. The enhanced $[Ca^{2+}]_i$ responses to low $K^+$ and bombesin were attributable to the opening of L-type $Ca^{2+}$ channels, since both verapamil and nifedipine (results not shown) abolished them.

It is well known that receptor agonists can change membrane potential by action on $K_{ATP}$ channels, an effect mediated via heterotrimeric G-proteins [6, 35, 36]. In insulin-secreting cells, there is evidence for both the closing and the opening of $K_{ATP}$ channels by receptor agonists, e.g. vasopressin, galanin, and glucagon [6, 7, 16]. This effect of the G-protein can be mediated via either its α-subunit or βγ-dimers [36]. We cannot exclude the possibility that GN depletion leads to the inhibition of a trimeric or small G-protein that activates $K_{ATP}$ channels. Similar G-proteins are involved both in inhibition of insulin secretion and in the activation of $K_{ATP}$ channels by inhibitory hormones [6, 37]. However, the trimeric G-proteins ($G_i/G_o$ class) coupled to inhibitory agonists such as epinephrine appear to be intact after MPA treatment, since the ability of epinephrine to inhibit insulin secretion remains unaffected [11]. The phospholipase C signaling pathway, elicited by receptor-mediated agonists using the $G_q/G_{11}$ family of trimeric G-proteins, also appeared unaffected by MPA treatment, since a normal or even increased $Ca^{2+}$ mobilization was elicited by bombesin in treated cells. These findings might indicate that modulation of $K_{ATP}$ channels by at least certain G-proteins remains intact.

It is more likely that the alterations of membrane potential and $[Ca^{2+}]_i$ in GN-depleted cells are due to direct action of GNs on $K_{ATP}$ channels. In the presence of $Mg^{2+}$ (i.e. the physiological condition), GDP activates $K_{ATP}$ channels with a potency almost equal to ADP, whereas GTP is less effective [4, 38]. Both Mg-GTP and Mg-GDP act on the same site on the regulatory subunit of the channel as Mg-ADP [34, 39]. Thus, the results from the patch-clamp and molecular cloning studies indicate that, under physiological conditions, the dominant effect of ambient endogenous GNs would be the activation of $K_{ATP}$ channels. Since MPA reduces [GDP] and [GTP], the net effect would be predicted to be removal of a tonic activation of $K_{ATP}$ channels, which could explain the partial depolarization occurring in the GN-depleted cells observed in this study. It is interesting to note that $K_{ATP}$ channels also have been identified in mitochondria. These channels also are opened by both GTP ($K_{1/2}$ = 7 μM) and GDP ($K_{1/2}$ = 140 μM), and appear to play an important role in modulating the sensitivity of the channels to the physiological, ambient concentrations of ATP [40]. Taken together, these findings suggest that $K_{ATP}$ channels may be modulated also by GNs in intact β-cells, in addition to the direct regulation by ANs. Changes in GNs are unlikely to exert a quantitatively important role in the direct regulation of $K_{ATP}$ channels and $[Ca^{2+}]_i$ under physiological conditions, in view of the dramatic reduction (80%) of GNs required for MPA to affect these variables. However,

an important role for GNs on ion fluxes may occur in pathophysiological states such as exposure to interleukin-1β [41], in which GTP content and GTP/GDP ratio are decreased dramatically, or conditions wherein high energy stores are greatly reduced, such as hypoxia or mitochondrial dysfunction. Under the latter conditions, the opening of $K_{ATP}$ channels induced by the decline in the ATP/ADP ratio might be modulated by the concurrent declines in the GTP/GDP ratio [42]. Further studies are required to examine these possibilities.

Insulin release induced by various secretagogues is impeded in GN-depleted β-cells [11, 12, 15, 43]. Reduction of insulin secretion occurred after a 3-hr exposure to MPA and was accentuated further by 6 hr. In contrast, no clear change in $[Ca^{2+}]_i$ homeostasis occurred at 3 hr, and $[Ca^{2+}]_i$ profiles were actually higher at 6 hr following GN depletion. Thus, the inhibition of secretion cannot be explained by decreased $[Ca^{2+}]_i$ upon stimulation of the cells. Nor is it likely to be caused by impaired nutrient metabolism as assessed by the MTS test, since an inhibitory effect on the latter was seen only at 6 hr but not at 3 hr, a time when insulin secretion was inhibited. It should be noticed that Komatsu *et al*. [43] did not find an inhibitory effect of MPA (20 hr) on glucose oxidation or utilization in rat islets. In addition, glucose was excluded from the medium when cells were stimulated by high $K^+$, but insulin secretion was still inhibited. Therefore, the fundamental action of GN depletion on insulin secretion does not relate to alterations of fuel metabolism, based on the MTS assay, although the modestly reduced metabolism seen after a 6-hr MPA treatment might explain the additional inhibition of glucose-induced insulin secretion, in particular to glucose stimulation (−75% at 6 hr vs −60% at 3 hr). Thus, at least one site(s) of the action of GNs on secretion appears to include a distal step beyond $[Ca^{2+}]_i$ rises [28]. It has been supposed for a long time that a G-protein ($G_E$) may control exocytosis directly [44]. Inhibition of insulin secretion in GTP-depleted cells could suggest that the small G-proteins, which possess lower affinity for GTP [45], may be the best candidates to comprise the $G_E$. Indeed, there is evidence for a role of the low molecular weight G-proteins Rab3 and Cdc42 in regulation of insulin release [14, 46–49]; the GTP-dependent carboxyl methylation of Cdc42 was inhibited in MPA-treated cells [14]. In addition, MPA impeded the loading of Ras by GTP in neonatal islet cells [50]. These studies suggest that the function of certain small G-proteins that act as positive effectors of secretion may be impeded upon GTP depletion.

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