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Unusual Carbachol Responses in RINm5F Cells: Evidence for a "Distal" Site of Action in Stimulus-Secretion Coupling

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SUMMARY

The mechanisms by which carbachol stimulates insulin release were studied in RINm5F cells. Stimulation was associated with mobilization of Ca^{2+} from thapsigargin-sensitive intracellular stores and elevation of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). However, when the elevation of $[Ca^{2+}]_i$ was blocked by prior treatment of the cells with thapsigargin or with the anticalmodulin agents W-7 or W-13, the effect of carbachol to stimulate insulin release was unchanged. Thus, the effect of carbachol to increase $[Ca^{2+}]_i$ was dissociated from the stimulation of release. The role of protein kinase C (PKC) was next investigated. Carbachol-stimulated insulin release was unchanged by phorbol ester-induced down-regulation of PKC, at a time when the stimulation of release by 12-*O*-tetradecanoylphorbol-13-acetate was abolished. Similarly, when the effect of 12-*O*-tetradecanoylphorbol-13-acetate to stimulate release was blocked by each of three separate PKC inhibitors (staurosporine, bisindolylmaleimide, or 1-*O*-hexadecyl-2-*O*-methylglycerol), carbachol stimulated insulin release normally. Thus, the carbachol activation of PKC was also dissociated from the stimulation of insulin release. Finally, the effect of carbachol was examined in PKC-down-regulated cells in the simultaneous presence of thapsigargin. Carbachol still stimulated insulin release normally. It is concluded that carbachol stimulates insulin release in RINm5F cells by a novel mechanism that does not involve the elevation of $[Ca^{2+}]_i$ or the activation of PKC. The action of carbachol appears to be exerted at a "distal site," beyond the point of increased $[Ca^{2+}]_i$, in stimulus-secretion coupling.

Control over the rate of insulin secretion by pancreatic β cells is closely associated with an elevation of $[Ca^{2+}]_i$ (1). Glucose and other nutrient secretagogues raise $[Ca^{2+}]_i$ by stimulating Ca^{2+} influx through voltage-sensitive Ca^{2+} channels in the plasma membrane. A number of hormones and transmitters stimulate insulin secretion by mechanisms that include mobilization of Ca^{2+} from intracellular Ca^{2+} stores (2). For example, muscarinic stimulation of insulin release has been associated with membrane phosphatidylinositol hydrolysis and mobilization of Ca^{2+} from intracellular Ca^{2+} pools. In islet β cells (3, 4) and in insulin-secreting β cell lines, such as RINm5F cells (5, 6), as in many other secretory cells (7), activation of muscarinic receptors stimulates phospholipase C. Phosphatidylinositol-4,5-bisphosphate is hydrolyzed by phospholipase C to $Ins(1,4,5)P_3$, a second messenger that releases Ca^{2+} from intracellular stores (8), and DAG, which is capable of activating PKC isoforms (9). The generation of $Ins(1,4,5)P_3$ due to muscarinic stimulation precedes the increase in $[Ca^{2+}]_i$, and the elevation of $[Ca^{2+}]_i$ precedes the stimulation of insulin secretion (6). The muscarinic effect following increased generation of DAG (10, 11) and the activation of PKC (11, 12) may enhance the stimulation of insulin release by sensitizing the secretory apparatus to Ca^{2+} (13). Inhibition of PKC attenuates or abolishes muscarinic stimulation of insulin secretion in islet β cells (11, 14) and in HIT cells (15). Therefore, it is generally thought that muscarinic receptor activation stimulates insulin secretion by the combined effects of activated phospholipase C to increase DAG and to mobilize Ca^{2+}. The rise in $[Ca^{2+}]_i$ and activation of PKC act in synergy to bring about the full physiological response of the β cells to muscarinic activation. These mechanisms are thought to apply to other secretagogues, such as cholecystokinin, that also increase the turnover of phosphatidylinositol (16). However, in the studies on RINm5F cells reported here, we provide evidence that muscarinic receptor activation can stimulate insulin release by an unusual mechanism that is independent of the elevation of $[Ca^{2+}]_i$ and independent of the activation of PKC. It appears that the stimulation of insulin release by muscarinic activation in these cells is exerted at a late stage in stimulus-secretion coupling, a stage that is beyond the rise in $[Ca^{2+}]_i$ and independent of any rise of $[Ca^{2+}]_i$.

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ABBREVIATIONS: $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; $Ins(1,4,5)P_3$, inositol-1,4,5-trisphosphate; KRB, Krebs-Ringer bicarbonate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DAG, diacylglycerol; PKC, protein kinase C; AMG-C_{16}, 1-*O*-hexadecyl-2-*O*-methylglycerol; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; MPA, mycophenolic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

Experimental Procedures

Cell culture. RINm5F cells were seeded at a density of 3×10^5 cells/ml in 75-cm^2 plastic culture flasks, in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 100 pg/ml streptomycin, and 100 units/ml penicillin. The cell culture was kept at 37° in a 95% air/5% CO_2 atmosphere, and the medium was changed every other day. The maintenance flasks were subcultured every 6 days, when the cells usually reached confluence. Cells of passages 57–64 were used in this study.

Measurement of $[Ca^{2+}]_i$. To load the cells with the fluorescent dye fura-2, cells were detached from the culture flasks by trypsin treatment and were washed twice with a modified KRB buffer containing 128.8 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.0 mM $CaCl_2$, 5.0 mM $NaHCO_3$, 10 mM HEPES, 0.1% bovine serum albumin, and 2.8 mM glucose. Sulfinpyrazone (250 μM) was added to reduce the transport of fura-2 out of the cells. The cell suspension was then incubated for 30 min at 37° in the presence of 1 μM fura-2/acetoxymethyl ester. The cells were washed twice with the modified KRB buffer and resuspended at a concentration of 1×10^6/ml. Fluorescence was measured with excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm, using a Perkin-Elmer LS-5 fluorescence spectrophotometer equipped with four quartz cuvettes. Three milliliters of cell suspension were placed in each cuvette and stirred with a small magnetic bar within the cuvette. The temperature within the cuvettes was maintained at 37° by circulation of warm water through the cuvette holder. The $[Ca^{2+}]_i$ was calculated according to the formula of Grynkiewicz *et al.* (17), $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$, where K_d is the dissociation constant for Ca^{2+} binding to fura-2 (224 nM) (17) and F is the observed fluorescence. At the end of the experiment, 10% Triton X-100 was added to the cuvettes to lyse the cells and saturate the fura-2 with Ca^{2+}. This fluorescence is referred to as F_{max}. Subsequent addition of 13 mM EGTA eliminated all fluorescence due to Ca^{2+} binding to fura-2. This value is referred to as F_{min}. At the beginning and end of each experiment, $MnCl_2$ (5 mM) was added to determine fluorescence due to extracellular fura-2, which allowed the leakage rate of fura-2 during the course of the experiment to be estimated. Because the leakage rate was constant throughout the experiments, the extracellular fura-2 level at any time was determined by extrapolation. Dual excitation at 340 and 380 nm, in turn (by manual adjustment of the wavelength), was used to differentiate the Ca^{2+}-sensitive fluorescence changes from Ca^{2+}-insensitive fluorescence changes.

Measurement of membrane potential. Membrane potential was measured using the fluorescent dye bis-oxonol. The cells were detached from culture flasks using trypsin and washed twice with a modified KRB-gelatin buffer (0.05% gelatin in place of 0.1% bovine serum albumin). The cell suspension (1.5×10^6/ml) was then incubated with 100 nM bis-oxonol at 37° for 10 min, and 3 ml of cell suspension were added to each quartz cuvette. Fluorescence was monitored with a Perkin-Elmer LS-5 spectrophotometer, using an excitation wavelength of 540 nm and an emission wavelength of 580 nm.

Insulin secretion. Insulin release was monitored using both cell perifusion and static incubation conditions. For perifusion experiments, cells (10^6 cells/2 ml) were seeded in plastic Petri dishes (35-mm diameter) 4 or 5 days before the experiment. On the day of the experiments, cells were detached from the dishes with trypsin and washed twice with KRB buffer. Then, $0.5–1 \times 10^6$ cells were placed in each of four parallel chambers (700 μl each) of a perifusion apparatus and perifused with the KRB buffer at a rate of 1 ml/min, at 37°. Cells were perifused for 45 min, to equilibrate and stabilize the cells and secretion rates, before the experiments were begun. Samples of the perifusate (1 ml each) were collected and kept at −20° before being assayed for insulin content. For static incubation experiments, cells (4×10^5/ml) were seeded in each well of 24-well polycarbonate culture plates 4 or 5 days before the experiments. On the day of the experiments, the culture medium was aspirated and the cells were washed with KRB buffer. Cells were then incubated with KRB buffer at 37° for 30 min for equilibration and stabilization of insulin release. After stimulation for 8–10 min, samples were collected and centrifuged to remove any cells that may have detached from the wells. The supernatants were kept at −20° until they were assayed for insulin content by radioimmunoassay.

[^{3}H]Arachidonic acid release. RINm5F cell monolayers were incubated with 0.25 μCi/ml [^{3}H]arachidonic acid under tissue culture conditions for 24 hr. On the day of the experiment, the culture medium was aspirated and the cells were washed twice with KRB buffer before incubation in KRB buffer at 37° for 40 min. The buffer was then aspirated, agonists in KRB buffer were added, and the incubation was continued for 8 min. Release of [^{3}H]arachidonic acid into the medium was measured by withdrawal of an aliquot of 200 μl of the KRB buffer (followed by centrifugation to remove any detached cells) for scintillation counting.

Reagents. RPMI 1640 medium, fetal calf serum, streptomycin, and penicillin were purchased from GIBCO. ^{125}I-Insulin (for radioimmunoassay) was purchased from DuPont-New England Nuclear. Bis-oxonol was from Molecular Probes. Thapsigargin was from Research Biochemicals (Natick, MA). Bisindolylmaleimide, forskolin, and AMG-C_{16} were from Calbiochem. Carbachol, fura-2/acetoxymethyl ester, TPA, sulfinpyrazone, staurosporine, nitrendipine, and mellitin were from Sigma. W-compounds (W-5, W-7, W-12, and W-13) were from Seikagaku America (St. Petersburg, FL).

Statistical analysis. Results are presented as means ± standard errors. Differences between means were analyzed by Student's *t* test or by analysis of variance as appropriate.

Results

Effects of carbachol on $[Ca^{2+}]_i$ and on membrane potential. In Fig. 1 are shown the effects of carbachol on $[Ca^{2+}]_i$ in RINm5F cells in suspension. Carbachol (200 μM) caused a prompt but transient rise in $[Ca^{2+}]_i$ to almost double the resting level of 75 nM. The elevated $[Ca^{2+}]_i$ returned to the resting level within 2 min. This short period of elevated $[Ca^{2+}]_i$ is in contrast to the prolonged stimulatory effect of carbachol on insulin release (see below). Subsequent addition of TPA (50 nM) also elevated $[Ca^{2+}]_i$ (Fig. 1A). The effect of carbachol was not affected either by 1 μM nitrendipine, a voltage-dependent Ca^{2+} channel antagonist (Fig. 1B), or by removal of extracellular Ca^{2+} by chelation with 5 mM EGTA (Fig. 1C). In contrast, the effect of TPA was abolished by these treatments (Fig. 1, B and C). When membrane potential was monitored with the fluorescent dye bis-oxonol, it was found that carbachol did not change the membrane potential, whereas TPA, used as a positive control, caused prompt depolarization of the membrane. Another control, the sodium ionophore gramicidin (2 μM), also resulted in the expected cell depolarization (Fig. 1D). These results are comparable to other reports demonstrating that carbachol releases Ca^{2+} from intracellular stores (6, 18), whereas TPA elevates $[Ca^{2+}]_i$ by depolarization and increased activity of voltage-dependent Ca^{2+} channels (18, 19). Atropine (100 μM) completely blocked the effect of carbachol (data not shown).

Effect of thapsigargin on the carbachol-induced increase in $[Ca^{2+}]_i$. After the addition of 200 μM carbachol, the administration of 2 μM thapsigargin, a microsomal Ca^{2+}-ATPase inhibitor (20), also elevated $[Ca^{2+}]_i$. It did so in the presence of extracellular Ca^{2+} (Fig. 2A) or in the absence of extracellular Ca^{2+} (Fig. 2C). In the presence of extracellular Ca^{2+}, thapsigargin-induced elevation of $[Ca^{2+}]_i$ lasted about 6 min. In the absence of extracellular Ca^{2+}, the elevated

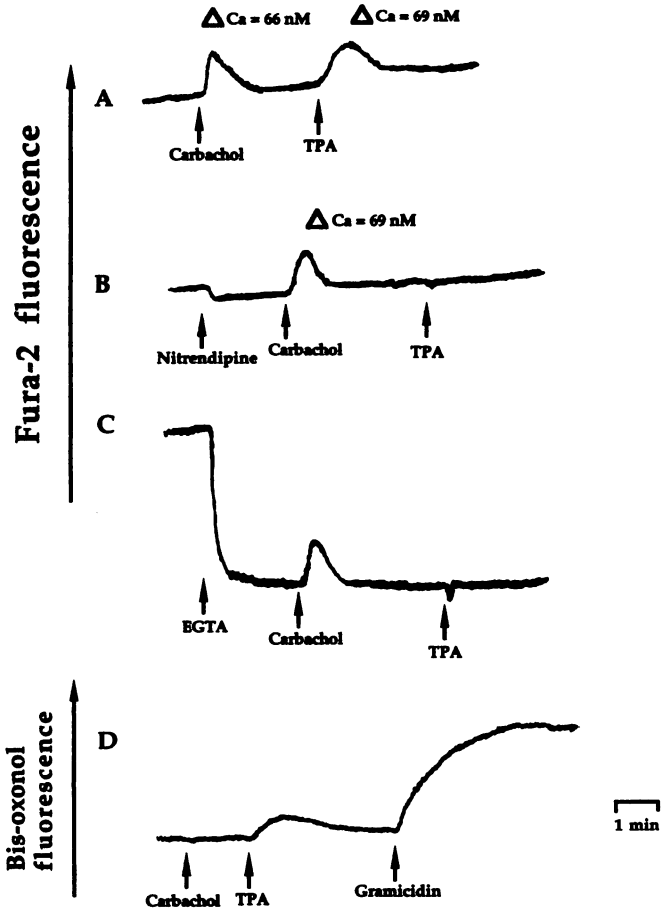

Fig. 1. Effects of 200 μM carbachol and 50 nM TPA on $[Ca^{2+}]_i$ in RINm5F cells, in the presence of 1.0 mM $CaCl_2$ in the medium (A), in the presence of 1 μM nitrendipine (an L-type Ca^{2+} channel blocker) (B), or after removal of extracellular Ca^{2+} by addition of 5 mM EGTA (C), and the effect of carbachol on the membrane potential of the cells (D). Resting levels of $[Ca^{2+}]_i$ were 75 nM (A), 74 nM (B), or 80 nM (C). $\Delta[Ca^{2+}]_i$ values represent the peak rise in $[Ca^{2+}]_i$ above the resting levels in these representative tracings. The experiments have been repeated five times, with similar results.

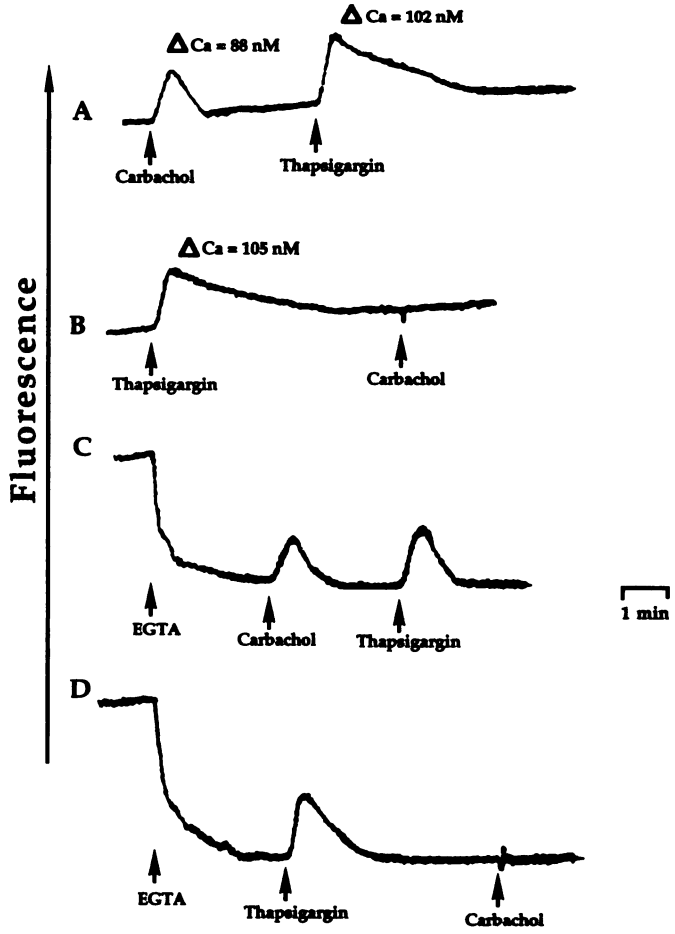

Fig. 2. Effects of 200 μM carbachol and 2 μM thapsigargin on $[Ca^{2+}]_i$ in RINm5F cells, in the presence of 1.0 mM $CaCl_2$ (A and B) or after removal of extracellular Ca^{2+} by addition of 5 mM EGTA (C and D). Resting levels of $[Ca^{2+}]_i$ were 82 nM (A), 84 nM (B), 79 nM (C), or 89 nM (D); $\Delta[Ca^{2+}]_i$ values represent the peak rise in $[Ca^{2+}]_i$ above the resting levels in these representative tracings. The experiments have been repeated three times, with similar results.

phase of $[Ca^{2+}]_i$ was shorter, indicating the contribution of Ca^{2+} influx to the late phase of the $[Ca^{2+}]_i$ rise. The effect of thapsigargin was not influenced by prior addition of nitrendipine (data not shown). When thapsigargin was applied before carbachol, subsequent addition of carbachol no longer raised $[Ca^{2+}]_i$ (Fig. 2, B and D). Because carbachol releases Ca^{2+} from intracellular thapsigargin-sensitive Ca^{2+} stores, this result confirms the effectiveness of the action of thapsigargin to unload the stores.

Effect of the W-compounds, Ca^{2+}/calmodulin inhibitors, on the elevation of $[Ca^{2+}]_i$ caused by carbachol. The W-compounds are naphthalenesulfonamide anticalmodulin agents (21), which have been shown to inhibit the KCl-stimulated rise in $[Ca^{2+}]_i$ (due to blockade of Ca^{2+} influx) and insulin secretion in RINm5F cells (22). Because we found also that they affected the mobilization of intracellular Ca^{2+} produced by carbachol,[2] we used these compounds to block the increase in $[Ca^{2+}]_i$ and confirm, if possible, the data obtained with thapsigargin. As shown in Fig. 3, the prior addition of W-7 at a concentration of 30 μM (the same concentration that inhibited the effects of KCl on $[Ca^{2+}]_i$ and insulin release in these cells) (22) completely blocked the elevation of $[Ca^{2+}]_i$ produced by carbachol. The nonchlorinated analog W-5 (30 μM), which has a lower affinity for calmodulin than does W-7, was inactive. The effect of carbachol on $[Ca^{2+}]_i$ was also completely blocked by another active analog, W-13 (50 μM), whereas its paired, less active, analog W-12 was without effect (data not shown).

Dissociation of the carbachol-stimulated elevation of $[Ca^{2+}]_i$ from carbachol-stimulated insulin secretion. Because a rise in $[Ca^{2+}]_i$ is thought to be critical for stimulus-secretion coupling in pancreatic islet β cells, the correlation between the effect of carbachol on $[Ca^{2+}]_i$ and the effect of carbachol on insulin secretion was examined under conditions in which the elevation of $[Ca^{2+}]_i$ produced by carbachol was blocked. This was achieved by prior addition of either thapsigargin or W-7 at the concentrations (2 μM and 30 μM, respectively) that completely blocked the carbachol-induced rise in $[Ca^{2+}]_i$. In perifusion experiments, 200 μM carbachol caused a rapid increase in the rate of insulin release from the RINm5F cells, which reached a peak within 2–5 min and returned to the basal level after 20–30 min (Fig. 4A). Interestingly, prior perifusion of the cells with 2 μM thapsigargin

[2] S. H. Tang, G. C. Yaney, and G. W. G. Sharp, unpublished observations.

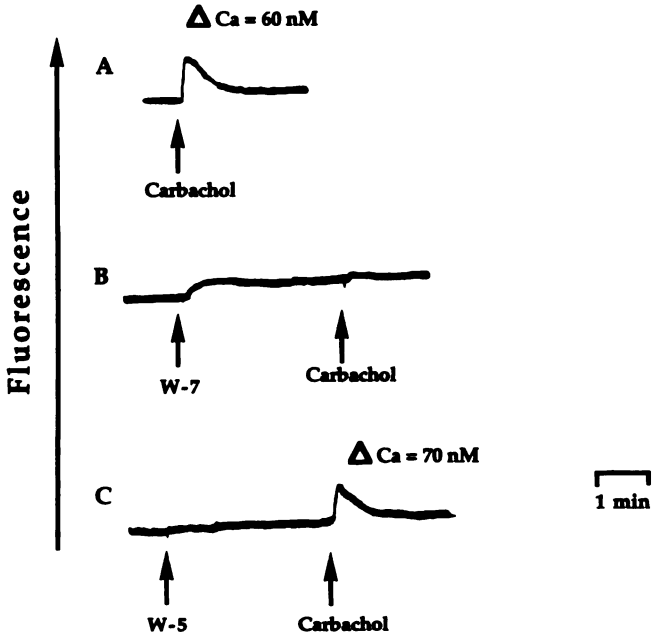

Fig. 3. Effects of 200 μM carbachol on the elevation of $[Ca^{2+}]_i$, when given alone (A) or in the presence of 30 μM W-7 (B) or 30 μM W-5 (C). The resting $[Ca^{2+}]_i$ was 95 nM in each case. $\Delta[Ca^{2+}]_i$ values represent the peak rise in $[Ca^{2+}]_i$ above the resting levels in these representative tracings from three different experiments.

6 min before the addition of carbachol (to block the carbachol-induced rise in $[Ca^{2+}]_i$) did not block the effect of carbachol on insulin release (Fig. 4). Thapsigargin, which transiently increases $[Ca^{2+}]_i$, slightly lowered the basal level of insulin release, rather than stimulating it (Fig. 4).

Under static incubation conditions, 30 μM W-7, which completely blocked the elevation of $[Ca^{2+}]_i$ produced by carbachol, also failed to block carbachol-stimulated insulin release. The basal rate of insulin release and the carbachol-stimulated insulin release were 9.7 ± 0.03 and 14.7 ± 0.05 pmol/10^7 cells/min (mean ± standard error, three experiments), respectively ($p < 0.01$). In the presence of 30 μM W-7, carbachol-stimulated insulin release was 15.3 ± 0.06 pmol/10^7 cells/min (mean ± standard error, three experiments), which was not significantly different from the value for carbachol stimulation alone (i.e., 9.7 ± 0.03 pmol/10^7 cells/min) ($p > 0.05$). Similar results were obtained with W-13.

Effects of PKC inhibitors on the stimulation of insulin release by carbachol. Muscarinic activation of RINm5F cells increases the generation of DAG, which activates PKC (11, 12). Because we found that the elevation of $[Ca^{2+}]_i$ and insulin release during carbachol stimulation could be dissociated, we naturally assumed that the activation of PKC mediated the insulin release caused by carbachol. Remarkably, we found that neither down-regulation of PKC nor inhibition of PKC affected carbachol-induced insulin release. As illustrated in Fig. 5A, down-regulation of PKC by pretreatment of the cells with 300 nM TPA for 24 hr decreased the basal level of insulin release but not the stimulation of insulin release by carbachol. As a control for the efficiency and extent of PKC down-regulation, the TPA-induced response was examined and found to be completely abolished (Fig. 5B). Furthermore, carbachol-induced insulin release was not inhibited by the PKC inhibitor bisindolylmaleimide (Figs. 6A and 7C), at a concentration that eliminated the

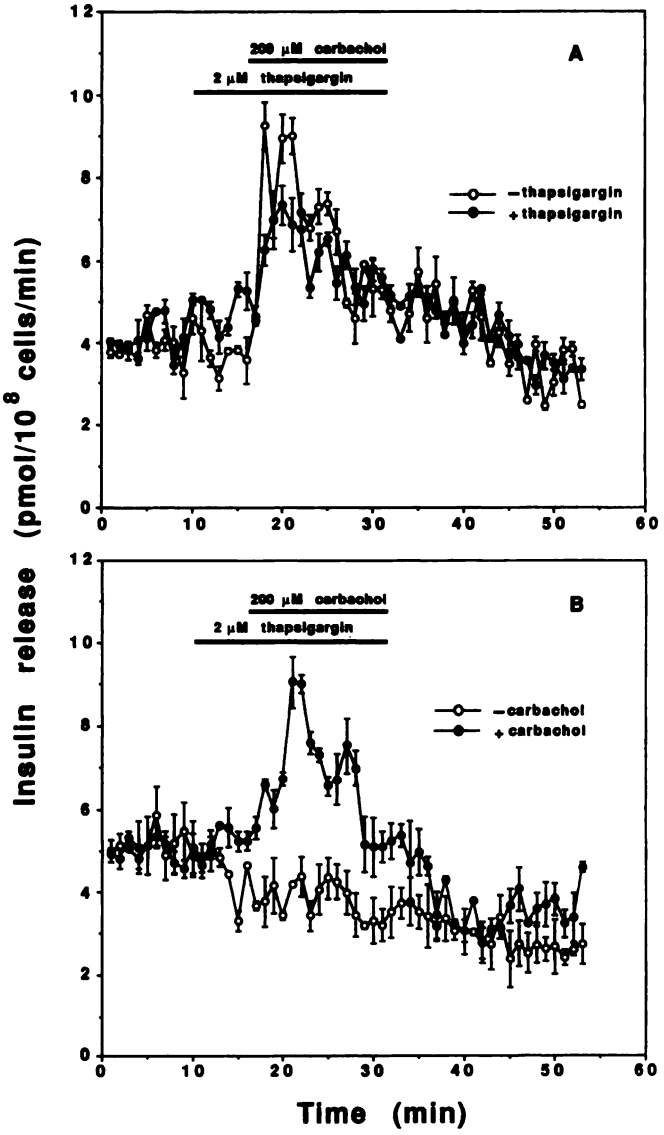

Fig. 4. Effects of thapsigargin on insulin release stimulated by carbachol. RINm5F cells were perifused at 37° for 45 min before time 0 on the trace. A, Carbachol-stimulated insulin release in the absence of thapsigargin (○) or after addition of thapsigargin 6 min before administration of carbachol (●). B, Effect of thapsigargin alone on insulin release (○) and the effect of carbachol 6 min after the addition of thapsigargin (●). Values are presented as the mean ± standard deviation from duplicate samples in one representative experiment. The experiments were repeated three times, with similar results.

effect of TPA to stimulate insulin release (Fig. 6B). Two other PKC inhibitors, staurosporine (100 nM) and AMG-C_{16} (120 μM), were also without effect on carbachol-induced insulin release, whereas they too suppressed the effect of TPA (Fig. 7, A and B). Because neither down-regulation or inhibition of PKC nor blockade of the elevation of $[Ca^{2+}]_i$ (by thapsigargin or the W-compounds) abolished carbachol-induced insulin release individually, we took an additional step to determine whether simultaneous suppression of the activation of PKC and the elevation of $[Ca^{2+}]_i$ would have any effect on carbachol-induced insulin release. As shown in Fig. 8, carbachol elicited a nearly normal response of insulin release after thapsigargin application to block the $[Ca^{2+}]_i$ rise in PKC-down-regulated cells. In contrast, the effect of TPA was

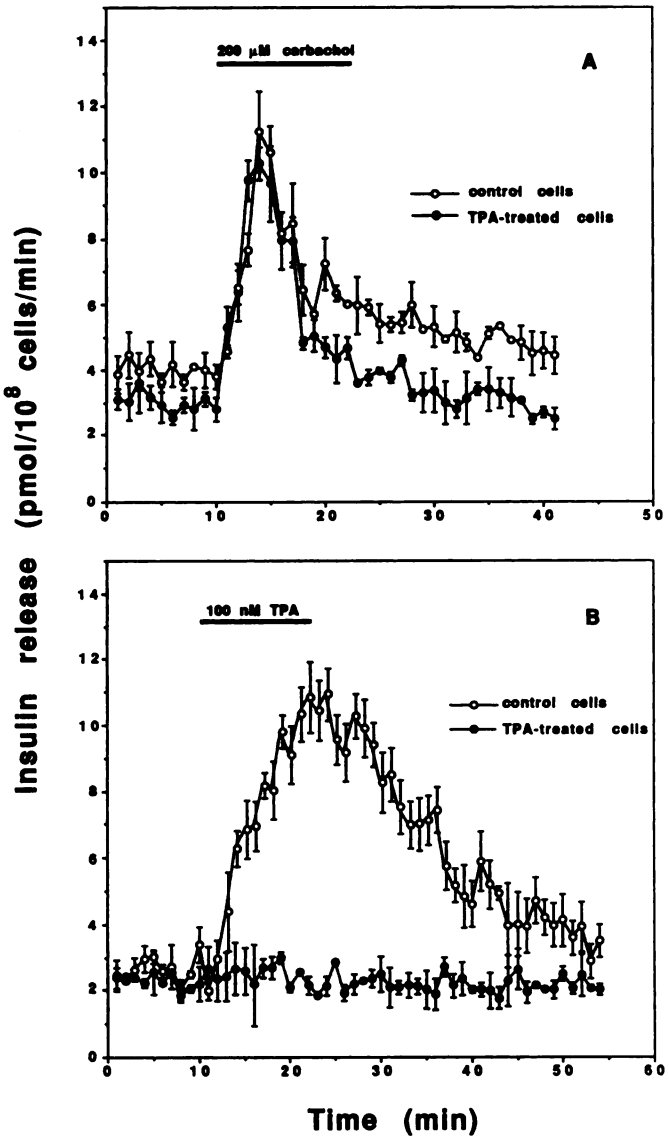

Fig. 5. Effects of down-regulation of PKC on carbachol-stimulated insulin release. RINm5F cells were treated with 300 nM TPA or, for the control cells, with vehicle (0.1% dimethylsulfoxide) for 24 hr under culture conditions. The cells were then perifused at 37° for 45 min before time 0. Basal insulin secretion was monitored for 10 min before the addition of carbachol or TPA. A, Effects of carbachol on insulin release in 24-hr TPA-treated cells (●) and in control cells (○). B, Effects of TPA on insulin release in 24-hr TPA-treated cells (●) and in control cells (○). Values are presented as the mean ± standard deviation from duplicate samples in one representative experiment. The experiments were repeated three times, with similar results.

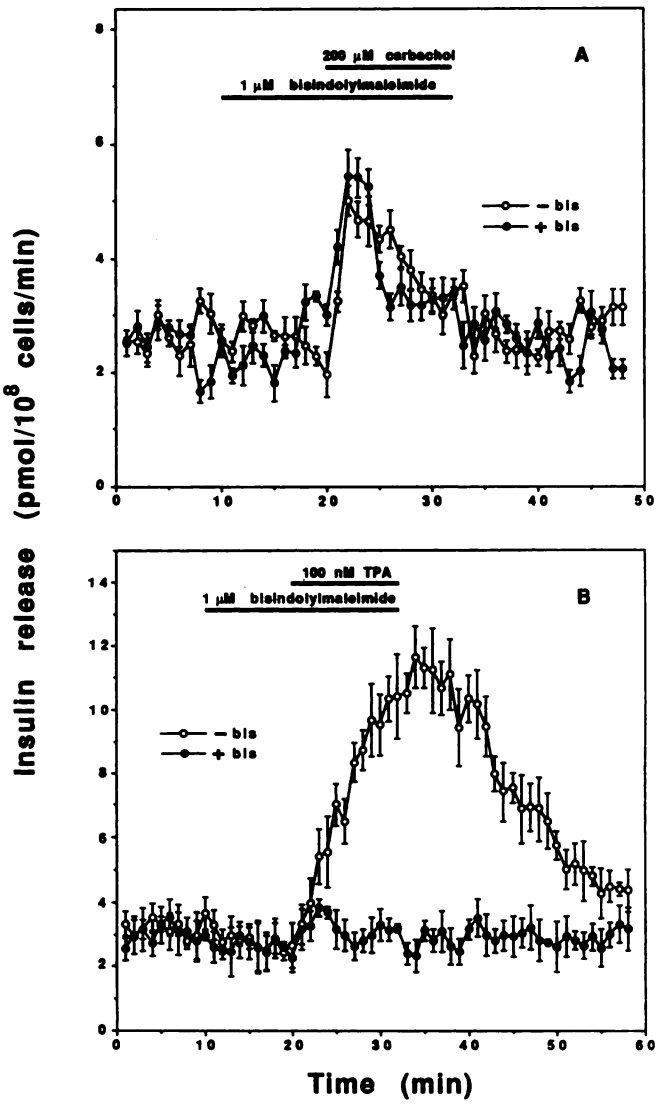

Fig. 6. Effects of bisindolylmaleimide on carbachol-stimulated insulin release. RINm5F cells were perifused at 37° for 45 min before time 0. Basal insulin secretion was monitored for 10 min before the addition of bisindolylmaleimide. A, Effects of carbachol on insulin release in the absence of bisindolylmaleimide (○) or 10 min after the addition of bisindolylmaleimide (●). B, Effects of TPA on insulin release in the absence of bisindolylmaleimide (○) or 10 min after addition of bisindolylmaleimide (●). Values are presented as the mean ± standard deviation from duplicate samples in one representative experiment. The experiments were repeated three times, with similar results.

blocked in PKC-down-regulated cells. The physiological inhibitor norepinephrine (1 μM) completely inhibited this carbachol-induced insulin secretion (data not shown).

Effects of carbachol on insulin release and [^{3}H]arachidonic acid release. It was reported that glucose-stimulated insulin release was partially mediated by the accumulation of arachidonic acid and its metabolites in cells through the action of phospholipase A_2 (23). Because the major phospholipase A_2 form in islet β cells is a Ca^{2+}-independent phospholipase A_2 (24) and because carbachol is reported to cause arachidonic acid accumulation in islet β cells (25), we explored the possibility that arachidonic acid might mediate the effect of carbachol on insulin release in the RINm5F cells. As shown in Table 1, carbachol, at concentrations up to 500 μM, did not increase [^{3}H]arachidonic acid release from RINm5F cells, whereas it increased insulin release. As a positive control, the bee venom component mellitin, which can activate phospholipase A_2 (26), caused a concentration-dependent increase in both [^{3}H]arachidonic acid release and insulin release.

Discussion

The results presented here demonstrate that in RINm5F cells the muscarinic receptor agonist carbachol caused an elevation of $[Ca^{2+}]_i$ without causing cell depolarization. Consequently, the effect of carbachol on $[Ca^{2+}]_i$ was not affected

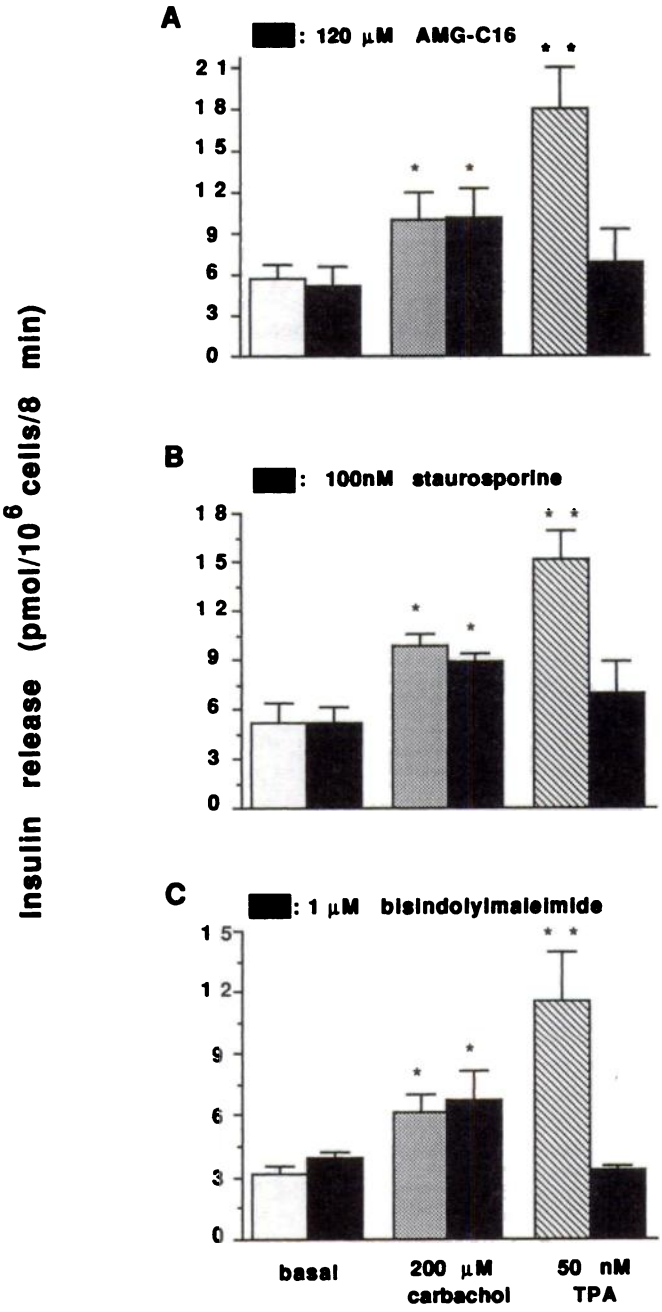

Fig. 7. Effects of the PKC inhibitors AMG-C_{16} (120 μM) (A), staurosporine (100 nM) (B), and bisindolylmaleimide (1 μM) (C) on carbachol-stimulated insulin release in static incubation studies. RINm5F cells were preincubated at 37° for 30 min in modified KRB buffer containing 2.8 mM glucose. The PKC inhibitors were added, as appropriate, at 30 min and the incubation was continued for 8 min. Then, the media were removed and replaced with KRB buffer containing stimulators (200 μM carbachol or 50 nM TPA) or stimulators plus the PKC inhibitors, and the incubation was continued for an additional 8 min. The data are the mean and standard error for three experiments. The level of significance by unpaired *t* tests was as follows: *, $p < 0.05$; **, $p < 0.01$, versus the basal values without inhibitors.

by the voltage-dependent Ca^{2+} channel blocker nitrendipine, nor was it affected by the short term removal of extracellular Ca^{2+} with EGTA. These data agree with previous studies (6, 18) and confirm the conclusion that muscarinic stimulation raises $[Ca^{2+}]_i$ primarily by mobilizing intracellular Ca^{2+} stores. Thapsigargin is a microsomal Ca^{2+}-ATPase inhibitor

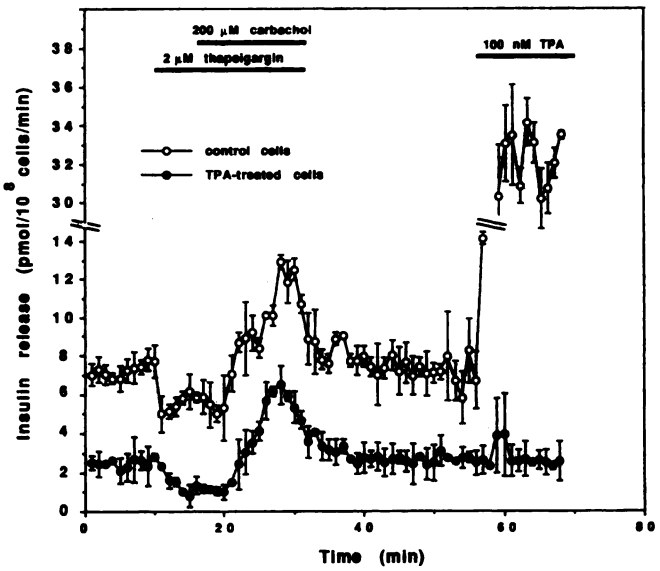

Fig. 8. Effects of carbachol to stimulate insulin release in the presence of thapsigargin, in PKC-down-regulated RINm5F cells (●) or in control cells (○). RINm5F cells were treated with 300 nM TPA for 24 hr in culture, and the control cells were treated with vehicle (0.1% dimethylsulfoxide). The cells were perifused at 37° for 45 min before time 0. Basal insulin secretion was monitored for 10 min before the addition of thapsigargin to both sets of cells. Carbachol was added 6 min after thapsigargin. Values are presented as the mean ± standard deviation from duplicate samples in one representative experiment. The experiments were repeated three times, with similar results.

TABLE 1

Effects of carbachol on [^{3}H]arachidonic acid release and insulin release

RINm5F cells that had been loaded with [^{3}H]arachidonic acid were incubated with modified KRB buffer at 37° for 40 min. The buffer was then aspirated and replaced with KRB buffer containing test agents (carbachol, 20–500 μM; mellitin, 0.5–2 μg/ml). The incubation was continued for 8 min, and the media were sampled and assayed for radioactivity. For insulin release studies, the cells were incubated with modified KRB buffer at 37° for 40 min and then the buffer was replaced with KRB buffer containing carbachol or mellitin. The incubation was continued for 8 min. Values are presented as the mean ± standard error from three separate experiments.

	[^{3}H]Arachidonic acid	Insulin release
	cpm/10^3 cells/8 min	*pmol/10^6 cells/8 min*
Basal	316.0 ± 37	4.0 ± 0.36
Carbachol		
20 μM	316.3 ± 43.9	5.6 ± 0.70[a]
200 μM	375.3 ± 21.5	7.9 ± 1.43[b]
500 μM	382.6 ± 35.7	8.2 ± 0.87[b]
Mellitin		
0.5 μg/ml	786.3 ± 61.4[b]	5.5 ± 0.35[a]
1 μg/ml	1052.6 ± 182.4[b]	9.7 ± 0.55[b]
2 μg/ml	1202.0 ± 24.5[b]	12.0 ± 0.15[b]

[a] $p < 0.05$, versus respective basal values (analysis of variance).
[b] $p < 0.01$.

that causes depletion of certain intracellular Ca^{2+} stores (20). Because carbachol no longer raised $[Ca^{2+}]_i$ after addition of thapsigargin (Fig. 2), it is concluded that carbachol releases Ca^{2+} from intracellular thapsigargin-sensitive stores and that thapsigargin can be used as a tool in the study of carbachol responses.

An unexpected and remarkable phenomenon in the present study was that, under conditions in which the carbachol-induced elevation of $[Ca^{2+}]_i$ was abolished, e.g., with the sulfonamide-derived W-compounds or thapsigargin, car-

bachol still stimulated insulin release. In a previous paper we reported that W-7 and W-13 (at the same concentrations as used here, i.e., 30 and 50 μM, respectively) inhibited both KCl-induced elevation of $[Ca^{2+}]_i$ and KCl-stimulated insulin release in RINm5F cells (22). However, W-7 and W-13 inhibited the carbachol-induced elevation of $[Ca^{2+}]_i$ without affecting carbachol-induced insulin release. Also, depletion of intracellular Ca^{2+} stores with thapsigargin abolished the effect of carbachol on $[Ca^{2+}]_i$ but not the effect of carbachol on insulin release. Clearly, carbachol-induced insulin secretion involves a mechanism that is independent of the elevation of $[Ca^{2+}]_i$ in these cells. Because muscarinic activation stimulates phosphatidylinositol hydrolysis, with the generation of Ins(1,4,5)P_3 and DAG, resulting in both elevation of $[Ca^{2+}]_i$ and activation of PKC (6, 11, 12, 27), it was natural to think that the effect of carbachol on insulin release might be due to activation of PKC. In islet β cells (11, 14) and HIT cells (15), down-regulation of PKC with TPA or application of PKC inhibitors attenuated or abolished the stimulating effects of muscarinic receptor agonists on insulin release. Because we demonstrated that carbachol can induce insulin release without elevation of $[Ca^{2+}]_i$, it seemed very likely that the effect of carbachol on insulin release was mediated by PKC activation. However, down-regulation of PKC or application of a number of PKC inhibitors did not affect the stimulation of insulin release by carbachol under conditions in which the stimulating effect of TPA on insulin release was abolished. Although we concluded that carbachol can induce insulin release without activation of PKC, one might argue that RINm5F cells may have a unique isoform of PKC that is not sensitive to TPA treatment and is not sensitive to the several PKC inhibitors that we used. To date, 10 different PKC isozymes, encoded by eight different genes, have been identified and cloned from a variety of tissues. These PKC isozymes are differentially expressed in cells and tissues and have distinct biochemical properties (28). PKC isozymes show distinct patterns of responsiveness to different phorbol esters, which themselves have individual functional profiles in biological systems (29). For example, in rat adipocytes TPA selectively down-regulated PKC isozymes α, β, γ, δ, and ζ but not ε, whereas in BC_3H4 myocytes TPA down-regulated PKC-α but did not affect PKC-β (30). Different PKC isozymes also have different levels of responsiveness to the PKC inhibitor staurosporine (31). Therefore, it is conceivable that RINm5F cells contain a PKC isozyme that is not sensitive to TPA pretreatment and not sensitive to some PKC inhibitors. Islet β cells have been reported to have α and β forms of PKC (32, 33), and recently Biden and co-workers (34) reported that in RINm5F cells a novel PKC isozyme (τ form) was present. Although the responsiveness of these PKC isoforms to phorbol esters and PKC inhibitors is not clear at present, there would have to be very stringent requirements for the PKC isozymes in RINm5F cells to escape being suppressed in our study. We have used both TPA pretreatment (to down-regulate PKC) and a variety of PKC inhibitors, of which bisindolylmaleimide and staurosporine interact with the ATP binding sites on PKC (35) and AMG-C_{16} blocks the DAG binding sites (36). Furthermore, in all cases, the down-regulation or application of inhibitors blocked the effect of TPA on insulin secretion. Nevertheless, we cannot exclude the possibility that a unique PKC isoform, which escapes down-regulation by TPA and inhibition by the inhibitors we have used, is the mediator of the effects described.

In searching for the mechanism of action of carbachol, we have assumed that the release mechanism is exocytotic. There is no evidence of nonspecific leakage or damage, because this would have been revealed by an easily detectable leak of fura-2 in these experiments. Also, norepinephrine inhibits the carbachol-stimulated release of insulin (data not shown). We have measured [^{3}H]arachidonic acid release from the cells. Arachidonic acid and its metabolites were reported to be responsible for glucose-stimulated insulin release through the action of phospholipase A_2 (23), and carbachol caused arachidonic acid accumulation in islet β cells (25). In our study, carbachol did not increase [^{3}H]arachidonic acid release from RINm5F cells, whereas mellitin, a bee venom component that activates phospholipase A_2 (26), caused a concentration-dependent increase in both [^{3}H]arachidonic acid release and insulin release (Table 1). Therefore, the effect of carbachol on insulin release appears not to be mediated through the action of phospholipase A_2. Carbachol also did not affect the cellular level of cAMP (data not shown).

The data show that neither elimination of the carbachol-induced rise in $[Ca^{2+}]_i$ nor down-regulation or inhibition of PKC blocks the stimulation of insulin release. Furthermore, simultaneous elimination of the increases in $[Ca^{2+}]_i$ and PKC activity also fails to block the stimulation of insulin release (Fig. 8). Clearly then, there must be some mechanism other than the elevation of $[Ca^{2+}]_i$ and the activation of PKC involved in carbachol-induced insulin secretion. According to the current understanding of stimulus-secretion coupling, a rise in $[Ca^{2+}]_i$ is a critical event in the sequence of reactions that lead to the stimulation of insulin release. Stimulus-secretion coupling has been defined in terms of proximal and distal events, relative to the elevation of $[Ca^{2+}]_i$, and it is the proximal events about which we know most. Little is known about the distal events. However, from the data presented here it seems clear that the effect of carbachol on RINm5F cells is exerted at a site that is distal to the elevation of $[Ca^{2+}]_i$ in stimulus-secretion coupling. At present we do not know the mechanism by which the stimulation is brought about. However, we do know that all of the muscarinic effects of carbachol analyzed thus far are mediated by heterotrimeric G proteins. Therefore, the possibility exists for a stimulatory effect of one or more heterotrimeric G proteins at a late stage in stimulus-secretion coupling, in addition to the presumed interaction of low molecular weight G proteins. In 1992, Metz *et al.* (37) reported that, using MPA to inhibit *de novo* GTP synthesis in rat islets, glucose-induced insulin release was blocked. Guanine circumvented this block via the nucleotide "salvage" pathway. MPA also reduced the cellular ATP content and raised the cellular UTP content, but these changes were not responsible for the defective insulin secretion, because adenine, which restored ATP and UTP levels to nearly normal values, did not alter the reduction of GTP content and insulin secretion. By applying the same strategy to RINm5F cells, we found that, in cells that had been pretreated with MPA (25 μg/ml), carbachol-induced insulin release was completely blocked and this block was prevented by the addition of guanine (100 μM) during the pretreatment period. Also, the addition of adenine (150 μM) during the pretreatment period did not alter the effect of MPA (data not shown). This study suggests that GTP is required for the

action of carbachol on insulin secretion and supports the idea that G proteins are involved in the action. It is also interesting to note that mastoparan, a tetradecapeptide purified from wasp venom (38), also acts at a late stage in stimulus-secretion coupling to increase the rate of insulin secretion (39), that mastoparan activates heterotrimeric G proteins (40), and that the stimulatory effect of mastoparan on insulin release appears to require GTP (41). Although these findings suggest a G protein-controlled mechanism for exocytosis, other possibilities, such as activation of tyrosine kinase or some unknown action of inositol phosphates, are also conceivable. The unusual response of RINm5F cells to carbachol may be unique to this cell type. Nevertheless, it seems likely that investigation of the mechanisms underlying this effect in the RINm5F cell line will provide valuable information on the late stages of stimulus-secretion coupling and/or exocytosis.

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