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Desensitization of Islet Cells to Bombesin Involves Both Receptor Down-Modulation and Inhibition of Receptor Function

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SUMMARY

The neuropeptide bombesin has a powerful but transient stimulatory effect on insulin secretion in the pancreatic islet cell line HIT-T15. We have previously shown that pretreatment of HIT-T15 cells with a saturating concentration of bombesin (100 nM) for 1.5–2 hr abolishes their secretory response to a second challenge with peptide and decreases [^{125}I-Tyr^4]bombesin binding by over 90%. In this study we examined the mechanisms involved in desensitization to bombesin. To determine whether receptor modulation was responsible, we compared the effect of bombesin pretreatment on [^{125}I-Tyr^4]bombesin binding and on the ability of bombesin to stimulate insulin release. Both effects occurred very rapidly and were maximal by 10 min. Although pretreatment of cells for 90 min with a subsaturating concentration of bombesin did not affect either the ED_{50} for bombesin-stimulated secretion or the apparent K_d for bombesin binding, it decreased both the maximum secretory response to a subsequent challenge with the peptide and bombesin receptor number. However, the extent of desensitization was greater than the extent of receptor down-regulation at all times examined during pretreatment and recovery. Furthermore, bombesin was 3 times more potent at inducing desensitization (ED_{50} = 0.35 ± 0.08 nM) than down-regulation (ED_{50} = 1.1 ± 0.4 nM). These results suggest that desensitization was not due solely to a reduction in receptor number. Because bombesin stimulates diacylglycerol production in HIT-T15 cells, we used the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) to determine whether protein kinase C also played a role in desensitization to the peptide. Pretreatment of cells with TPA did not affect either [^{125}I-Tyr^4]bombesin binding or the dose dependence for bombesin-stimulated hormone release. However, TPA pretreatment did decrease the maximum secretory response to bombesin by 40% and caused a 50% reduction in bombesin-induced accumulation of inositol trisphosphates and elevation of intracellular free calcium. Conversely, bombesin pretreatment reduced the secretory response to TPA by 40%. These studies indicate that the mechanism for desensitization to bombesin is a complex process that involves down-regulation of the bombesin receptor, inhibition of intracellular second messenger production, and reduction of protein kinase C-stimulated secretion.

Although bombesin was originally isolated from the skin of the frog *Bombina bombina* (1), it was subsequently shown to belong to a family of structurally homologous mammalian peptides (2). This group of peptides has a broad spectrum of biological actions affecting the metabolism, proliferation, or differentiated functions of a variety of mammalian cell types (2). Our work has focused on the acute effect of bombesin to regulate hormone secretion in a clonal pancreatic islet cell line called HIT cells. Because in these cells bombesin stimulates insulin secretion very rapidly (by 5 sec) (3, 4), we were able to determine the relationship between this biological activity and the biochemical effects of the peptide on intracellular messengers. Our studies have shown that the effect of bombesin on secretion is mediated by specific plasma membrane receptors that are linked to polyphosphoinositide hydrolysis via a pertussis toxin-insensitive guanine nucleotide-binding protein (4–6).

Interestingly, bombesin-stimulated insulin release in HIT cells returns to the basal rate by 90 min in the continued presence of peptide (3). The time course of this secretory response resembles that observed *in vivo* as well as in perfused pancreas; the stimulatory effect of bombesin on insulin release is always transient (7, 8). The loss of HIT cell responsiveness to bombesin is not due either to peptide inactivation or to depletion of intracellular stores of releasable insulin (3). Rather, bombesin-induced desensitization results from changes

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ABBREVIATIONS: HIT cells, cells of the HIT-T15 cell line; $[Ca^{2+}]_i$, intracellular free calcium ion concentration; IP_3, inositol trisphosphate; quin2, 2-{[2-bis(acetylamino)-5-methylphenyloxy]methyl-6-methoxy-8-bis(acetylamino)}-quinoline; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

specifically associated with activation of the bombesin receptor, because bombesin pretreatment does not inhibit the stimulatory effects of glucagon or high K^+ on insulin release (3).

In this study we examined the mechanism by which HIT cells become desensitized to bombesin. Although we previously found that pretreating cells for 90 min with bombesin decreased the subsequent binding of [^{125}I-Tyr^4]bombesin (5), we show here that desensitization cannot be fully explained by receptor down-modulation. Therefore, we determined whether the second messengers that mediate the secretory effect of bombesin were also involved in the desensitization process. A preliminary report of some these data has been presented (9).

Materials and Methods

Materials were obtained from the sources reported previously (3–5).

Cell culture. The properties of the HIT cell line and the methods of its culture have been described previously (3, 10). [^{125}I-Tyr^4]Bombesin binding and insulin release experiments were carried out with cells in monolayer culture grown in 35-mm wells (3, 5). Measurements of $[Ca^{2+}]_i$ and IP_3 formation were performed with cells in suspension (4). All experiments were carried out at least twice with similar results. The data are expressed as the mean ± range for two experiments or the mean ± standard error for three or more experiments.

Measurement of insulin secretion. Insulin release was determined as previously described (3, 4). Incubations of 30 min or longer were performed at 37° in 5% CO_2/95% air, in a HEPES-buffered salt solution (118 mM NaCl, 4.6 mM KCl, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 5 mM HEPES, and 1 mg of bovine serum albumin/ml, pH 7.2) containing 1 mg/ml sodium bicarbonate. Incubations that were shorter than 30 min were performed at 37° in ambient atmosphere, in the same HEPES-buffered salt solution but without sodium bicarbonate. The concentration of peptide or TPA that elicited a half-maximal stimulation (ED_{50}) of secretion was determined by linear regression analysis of a log-logit transformation of the concentration-response data.

Measurement of [^{125}I-Tyr^4]bombesin binding. [^{125}I-Tyr^4]bombesin was prepared by the glucose oxidase-lactoperoxidase method described previously (5). Binding experiments were performed at 4° for 12 hr in ambient atmosphere in HEPES-buffered salt solution (5). Saturable binding was calculated as the difference between the amount of [^{125}I-Tyr^4]bombesin bound in the absence (total binding) and presence (nonsaturable binding) of 100 nM bombesin. The data shown represent saturable binding unless stated otherwise. The concentration of bombesin that produced half-maximal inhibition (ID_{50}) of [^{125}I-Tyr^4]bombesin binding was calculated by linear regression analysis of the log-logit transformation of competition binding data. Receptor density was calculated from the same data, using our previous observation that [^{125}I-Tyr^4]bombesin and bombesin bind to HIT cell receptors with identical affinities (5). Thus, at each concentration of bombesin, the number of receptors occupied by both labeled and unlabeled ligands was determined, and these data were then analyzed by the method of Scatchard (11).

Measurement of intracellular messengers. [^{3}H]Inositol phosphate accumulation was determined as described previously (4). $[Ca^{2+}]_i$ was measured using the fluorescent calcium indicator quin 2, using previously published procedures (4).

Pretreatment and challenge protocols. A similar protocol was used for all the pretreatment experiments. To determine effects on bombesin receptor binding and biological responsiveness, cells in monolayer were preincubated at 37° with either control buffer, bombesin, or TPA at the appropriate concentration. After the desired time, the cells were washed twice with 1 ml of buffer. To study insulin secretion, cells were challenged at 37° with the desired concentration of secretagogue. Following the release incubation, the buffers were collected and the accumulated insulin was measured by radioimmunoassay. For binding studies, HIT cells were shifted to 4° after pretreatment and incubated with [^{125}I-Tyr^4]bombesin (50,000–80,000 cpm/ml) for 12 hr. The cells were collected, and saturable binding was measured as described above. Desensitization is defined as the decrease in the response to bombesin, whereas receptor down-modulation is defined as a reduction in the binding capacity of the cells.

To determine the effect of TPA pretreatment on the ability of bombesin to elevate $[Ca^{2+}]_i$ levels and to stimulate [^{3}H]IP_3 accumulation, cells were incubated in monolayer culture with 300 ng/ml TPA for 60–90 min at 37°. Thereafter, the usual protocols were followed, except that TPA was included in subsequent steps to avoid recovery from desensitization during the required equilibration periods. Thus, for measurement of $[Ca^{2+}]_i$ levels after TPA pretreatment, cells were suspended, incubated with quin2 for 30 min at 37°, and washed two times by centrifugation, all in the continued presence of TPA. A third wash was performed in the absence of TPA before the cells were used to measure effects on $[Ca^{2+}]_i$. TPA did not affect quin2 loading; the intracellular quin2 concentration was 86 ± 3 and 88 ± 7 μM in TPA-pretreated and control cells, respectively (two experiments for each). To determine the ability of bombesin to stimulate [^{3}H]IP_3 accumulation in TPA-pretreated cells, cells were suspended, washed three times, and equilibrated at 37° for 20 min, all in the continued presence of TPA. The final challenge with 100 nM bombesin was also carried out with TPA in the incubation buffer. In both the quin2 and [^{3}H]IP_3 experiments, the total time of pretreatment with TPA was 2 hr.

Results

Effect of bombesin pretreatment on insulin release. Pretreating HIT cells with bombesin altered their subsequent secretory activity in two ways (Table 1). First, the rate of basal secretion was markedly increased following preincubation with 100 nM bombesin, indicating that there was some residual stimulation of insulin release. Second, the pretreatment abolished the secretory response to a new bombesin challenge. This desensitizing effect was very rapid; after 5 min of pretreatment, insulin release during the challenge incubation was the same in the absence and presence of bombesin (Table 1). We have previously demonstrated that the desensitization produced by bombesin pretreatment is specific for bombesin; the secretory response to 40 mM K^+ or 1 μM glucagon is unaffected (3).

Involvement of receptor down-modulation in desensitization. Following binding to its plasma membrane receptor, [^{125}I-Tyr^4]bombesin is rapidly ($t_{1/2} \approx 2$ min) internalized by HIT cells via receptor-mediated endocytosis (5). Furthermore, exposure of cells to 100 nM bombesin for 90 min decreases the subsequent binding of [^{125}I-Tyr^4]bombesin by over 90% (5). To investigate whether desensitization could be due to receptor down-modulation, we compared the effect of bombesin pretreatment on the binding properties of the bombesin receptor and the secretory response to bombesin. Because pretreatment of cells with a saturating concentration of bombesin reduces

TABLE 1

Time course for homologous desensitization to bombesin

HIT cells (5 × 10^6/dish) were incubated with 100 nM bombesin at 37° for the indicated time. Each group of cells was then rinsed rapidly and incubated, in the absence or presence of 100 nM bombesin, for 60 min. The insulin released during the second challenge incubation was measured by radioimmunoassay. The data shown represent the mean ± standard error of triplicate dishes.

Challenge	Insulin released		
	0[a]	5[a]	60[a]
	ng/ml		
Control	2 ± 0.2	13 ± 0.1	8 ± 1.0
Bombesin	31 ± 2.0	13 ± 0.9	9 ± 1.0

[a] Time of pretreatment (min).

[^{125}I-Tyr^4] bombesin binding and bombesin stimulation of insulin release to such an extent that residual effects cannot be characterized, in the following experiments cells were preincubated with submaximal concentrations of peptide. Fig. 1A shows that pretreatment of cells with 1 nM bombesin decreased [^{125}I-Tyr^4] bombesin binding by 55% but did not significantly alter the ED_{50} for unlabeled bombesin. Scatchard analysis (11) of the competition data showed that receptor number was reduced from 11,000 ± 1,000 to 4,500 ± 300 per cell. However, receptor affinity for bombesin was not altered; the apparent equilibrium dissociation constants (K_d) were 0.18 ± 0.04 nM for control cells and 0.17 ± 0.02 nM for pretreated cells. In three experiments like that shown in Fig. 1B, the potency of bombesin to stimulate insulin secretion was unaffected by bombesin pretreatment. Both in control cells (eight experiments) and in cells preincubated with 0.5 nM bombesin (three experiments), the ED_{50} for bombesin stimulation of insulin release was 0.4 ± 0.1 nM. In contrast, stimulation by maximal concentrations of bombesin was reduced by 77 ± 3% following pretreatment with 0.5 nM bombesin.

This, preincubation with bombesin decreased both the number of bombesin receptors per cell and the maximal secretory response to the peptide, without affecting either receptor affinity or the potency of bombesin to stimulate insulin release. Although these observations are consistent with receptor down-modulation being involved in the process of desensitization, a quantitative discrepancy was observed between the magnitude of the two effects. Thus, pretreatment of cells with 1 nM bombesin reduced [^{125}I-Tyr^4]bombesin binding by 42 ± 12%, whereas pretreatment with only 0.5 nM bombesin decreased secretion by 77 ± 3%. This discrepancy was further examined by comparison of the dose dependence and temporal characteristics of desensitization and receptor down-modulation (Figs. 2 and 3). In three experiments similar to that shown in Fig. 2, bombesin was more potent at eliciting desensitization (0.35 ± 0.08 nM) than receptor down-modulation (1.1 ± 0.4 nM). Fur-

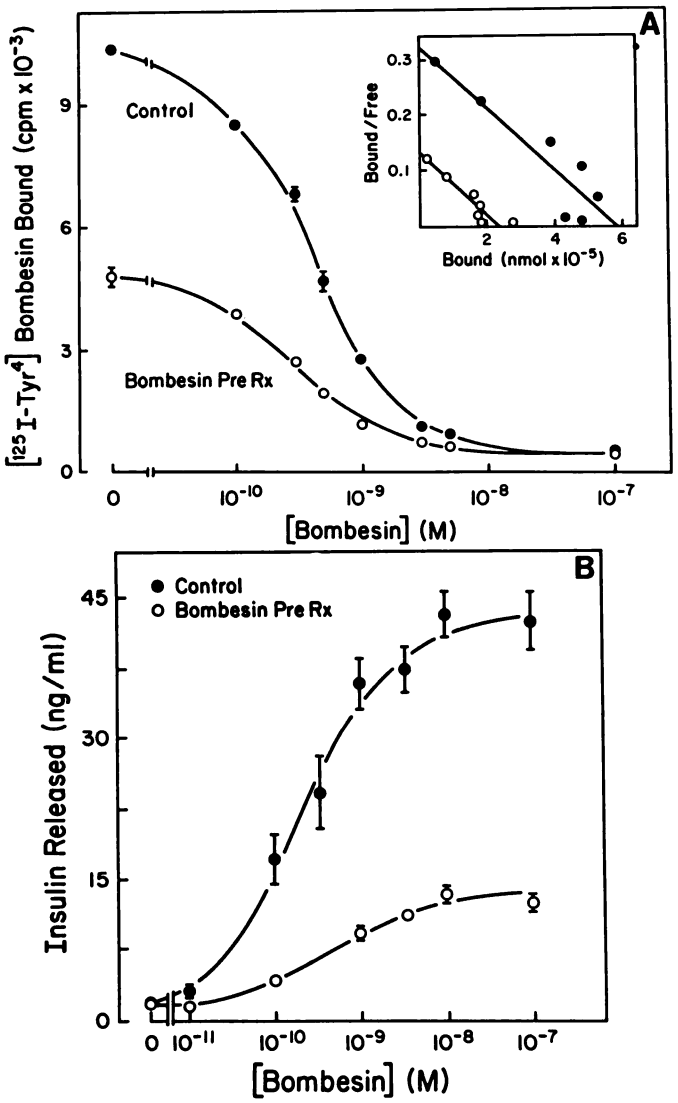

Fig. 1. Effect of bombesin pretreatment on the binding and responsiveness of HIT cells to bombesin. A, HIT cells (3×10^6 cells/dish) were pretreated for 90 min in the presence (○) or absence (●) of 1 nM bombesin. The cells were then rinsed rapidly and incubated at 4° for 12 h with [^{125}I-Tyr^4]bombesin (45,000 cpm/ml) and increasing concentrations of unlabeled bombesin. The amount of [^{125}I-Tyr^4]bombesin bound was determined as described in Materials and Methods. The data represent the mean ± standard error of the total [^{125}I-Tyr^4]bombesin bound in triplicate dishes. The ED_{50} for bombesin inhibition of binding was 0.37 ± 0.02 nM in control cells and 0.51 ± 0.08 nM in pretreated cells. *Inset*, the data plotted by the method of Scatchard (11). B, HIT cells (5×10^6/dish) were pretreated for 90 min in the presence (○) or absence (●) of 0.5 nM bombesin, rinsed, and then incubated for 60 min at 37° with the concentration of bombesin shown. The released insulin was measured by radioimmunoassay. The data represent the mean ± standard error of triplicate dishes.

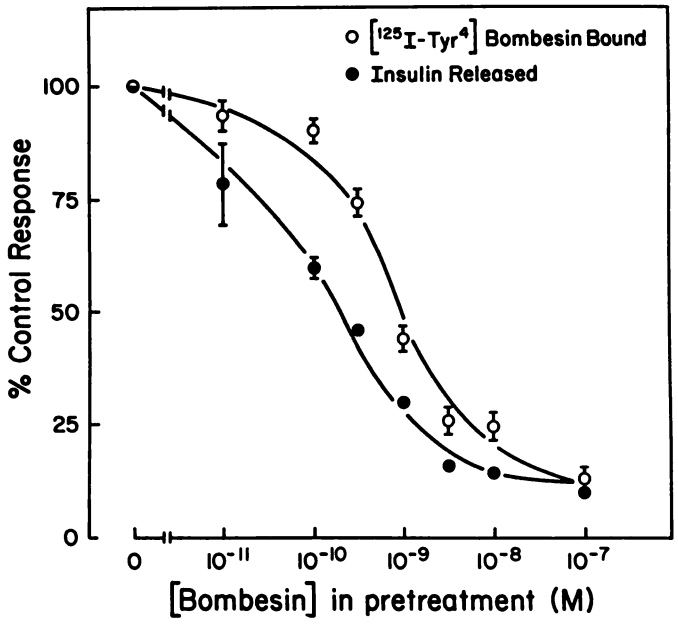

Fig. 2. Potency of bombesin to induce desensitization and receptor down-modulation. HIT cells (5×10^6/dish) were pretreated at 37° for 90 min with the indicated concentration of bombesin, rinsed, and then divided into two groups. One group was challenged with 100 nM bombesin and the insulin secreted during the following 60-min incubation was measured by radioimmunoassay (●). The second group was incubated with [^{125}I-Tyr^4]bombesin (50,000 cpm/ml) for 12 hr at 4° and saturable binding was determined as described in Materials and Methods (○). Cells that were preincubated in the absence of bombesin (control) bound 1840 ± 70 cpm of [^{125}I-Tyr^4]bombesin/dish and released 4.2 ± 0.3 ng of insulin in response to bombesin stimulation. The data are expressed as a percentage of the response observed in these control cultures and represent the means ± standard error of either quadruplicate (○) or triplicate (●) dishes. A 50% reduction of secretory response and [^{125}I-Tyr^4]bombesin binding was produced by pretreatment with 0.19 ± 0.04 nM and 0.62 ± 0.09 nM bombesin, respectively.

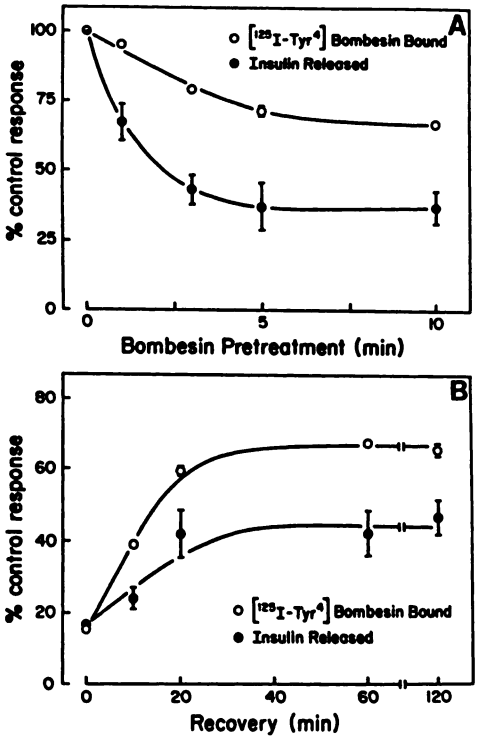

Fig. 3. Time course for induction and recovery from desensitization and receptor down-modulation. A, HIT cells (6×10^6/dish) were pretreated with 1 nM bombesin at 37° for the times shown, rinsed, and then divided into two groups. One group was challenged with 100 nM bombesin for 60 min at 37° and insulin secretion was measured by radioimmunoassay (●). The second group was incubated with [^{125}I-Tyr^4]bombesin (30,000 cpm/ml) for 12 hr at 4° and saturable binding was determined as described in Materials and Methods (○). Cells that were preincubated in the absence of bombesin (control) bound 5800 ± 85 cpm of [^{125}I-Tyr^4] bombesin/dish and released 16 ± 2 ng of insulin in response to bombesin stimulation. The data are presented as a percentage of the control response measured in nonpretreated cells and represent the mean ± standard error of triplicate dishes. B, HIT cells (3.5×10^6/dish) were preincubated for 90 min at 37° in the presence or absence of 100 nM bombesin, rinsed, and then incubated again at 37° in the absence of peptide. After the indicated time, the cells were rinsed again and divided into two groups. One group was challenged with 100 nM bombesin at 37° for 60 min and insulin secretion was measured by radioimmunoassay (●). The second group was incubated with [^{125}I-Tyr^4]bombesin (50,000 cpm/ml) for 12 hr at 4° and saturable binding was determined as described in Materials and Methods (○). The data are presented as a percentage of the response given by nonpretreated cells. The control secretory response was 14 ± 1 ng of insulin/ml and the control [^{125}I-Tyr^4]bombesin binding was 7580 ± 120 cpm/dish. The data represent the mean ± standard error of triplicate dishes.

thermore, pretreatment of cells with 1 nM bombesin reduced bombesin stimulation of insulin release to a greater extent than [^{125}I-Tyr^4]bombesin binding at all times examined (Fig. 3A). Finally, when cells were allowed to recover after pretreatment with a maximal concentration of bombesin, [^{125}I-Tyr^4]bombesin binding was greater than the secretory response at all times (Fig. 3B). The quantitative differences observed between bombesin-induced desensitization and receptor down-modulation suggest that the desensitization process is not due solely to a loss of receptors.

Involvement of protein kinase C in bombesin-induced desensitization. Bombesin stimulates the accumulation of diacylglycerol (4), which is known to activate protein kinase C (12). Because phorbol esters mimic this effect of diacylglycerol (12, 13), we used TPA to examine the role of protein kinase C in desensitization to bombesin. Fig. 4 shows that heterologous cross-desensitization between bombesin and TPA occurs. In four similar experiments, pretreatment with a maximal concentration of TPA (300 ng/ml) abolished the secretory response to TPA in a challenge incubation and reduced the response to bombesin by 42 ± 4%. In contrast, the stimulatory effect of 40 mM K^+ was not inhibited. Thus, TPA is able to selectively desensitize HIT cells to bombesin.

The effect of bombesin pretreatment on the ability of TPA to stimulate insulin release was also examined in Fig. 4. As in previous experiments, pretreatment of HIT cells with bombesin markedly inhibited the secretory response to bombesin in a challenge incubation, whereas the response to high K^+ was unaffected. Most interestingly, the response to TPA was reduced. In six experiments, pretreatment of HIT cells with 100 nM bombesin for 2 hr decreased the response to TPA by 43 ± 4%. These data show that bombesin causes specific desensitization within the protein kinase C-mediated pathway.

To determine whether the process by which bombesin elicits desensitization to TPA could be the same as that involved in the homologous desensitization to bombesin, we compared the time course and dose dependence for these two bombesin-induced effects. In three experiments, maximal desensitization to TPA was observed after 5 min of pretreatment with 100 nM bombesin. Therefore, bombesin elicited heterologous desensitization to TPA with a time course similar to that for homologous desensitization to bombesin. In addition, the potency of bombesin to elicit the two effects was similar. In two experiments like that shown in Fig. 5, bombesin induced heterologous desensitization to TPA with an ID_{50} of 0.25 ± 0.08 nM. This value agreed well with the ID_{50} for bombesin-induced homologous desensitization (0.35 ± 0.08 nM; three experiments).

The effect of bombesin pretreatment on the dose dependence for TPA stimulation of insulin release is shown in Fig. 6. In two experiments, pretreatment of HIT cells for 60 min with

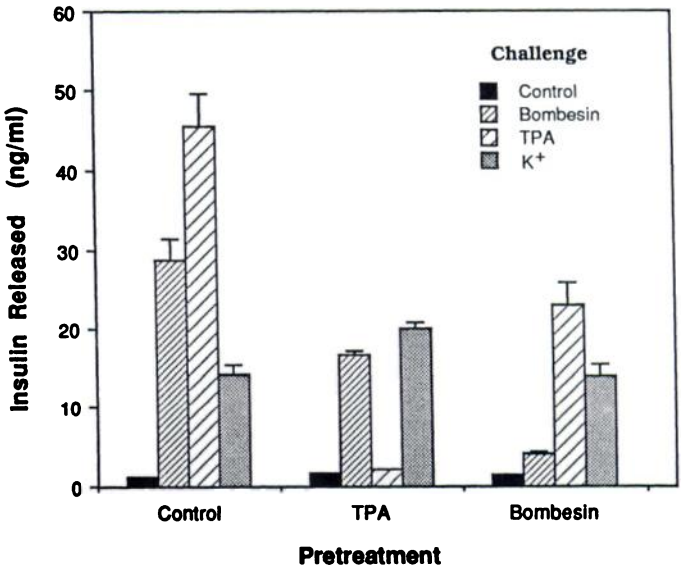

Fig. 4. Heterologous desensitization between bombesin and TPA. HIT cells (6×10^6/dish) were pretreated for 2 hr at 37° with either control buffer, 300 ng/ml TPA, or 100 nM bombesin. The cells were then rinsed and challenged with control buffer, 100 nM bombesin, 300 ng/ml TPA, or 40 mM K^+. After 60 min, the buffers were collected and the released insulin was measured by radioimmunoassay. The data represent the mean ± standard error of triplicate dishes.

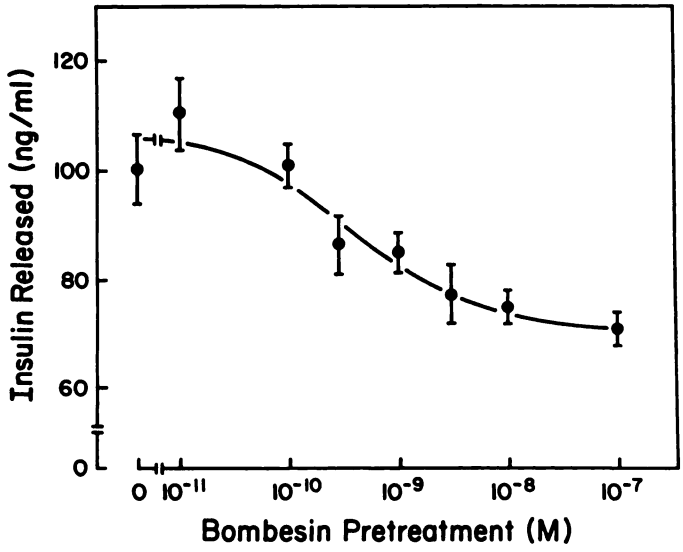

Fig. 5. Potency of bombesin to induce desensitization to TPA. HIT cells (3×10^6/dish) were pretreated for 60 min at 37° with the indicated concentration of bombesin. The cells were then rinsed and incubated with TPA (300 ng/ml) for an additional 60 min. The data represent the mean ± standard error of the insulin released in triplicate dishes.

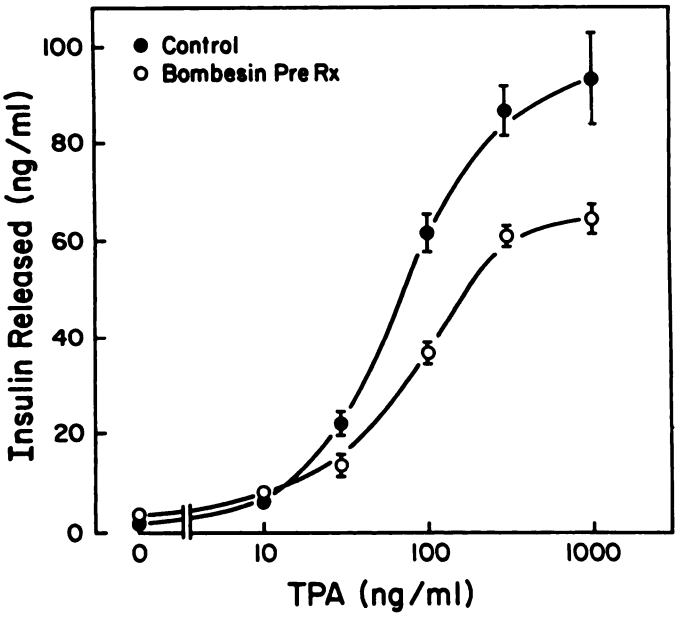

Fig. 6. Effect of bombesin pretreatment on the concentration dependence for TPA stimulation of insulin secretion. HIT cells (5×10^6/dish) were pretreated for 60 min at 37° in the absence (●) or presence (○) of 100 nM bombesin. The cells were then rinsed and incubated with the indicated concentration of TPA for 60 min. The data represent the mean ± standard error of the insulin released in triplicate dishes.

100 nM bombesin reduced the maximal secretory response to TPA by 46 ± 13%. However, the ED_{50} values for TPA were the same in control cells (67 ± 5 ng/ml; three experiments) and in pretreated cells (72 ± 14 ng/ml; two experiments). Thus, bombesin pretreatment decreased the secretory response to maximal concentrations of both TPA and bombesin without altering the potency of either secretagogue.

Mechanisms involved in phorbol ester desensitization of the response to bombesin. To further compare TPA- and bombesin-induced desensitization, we determined how TPA pretreatment affected the dose dependence for bombesin stimulation of insulin release (Fig. 7). Preincubation of HIT cells with 300 ng/ml TPA for 2 hr decreased the maximal secretory response to bombesin by 46 ± 2% (two experiments). In contrast, the dose dependence for bombesin stimulation of insulin secretion was not altered by TPA pretreatment; the ED_{50} for bombesin was 0.37 ± 0.09 nM in control cells (eight experiments) and 0.34 ± 0.02 nM in pretreated cells (two experiments).

The effect of TPA pretreatment on bombesin receptor binding and on the ability of bombesin to stimulate the production of intracellular messengers was examined in order to elucidate the mechanisms involved in the TPA-induced desensitization. The binding of trace concentrations of [^{125}I-Tyr^4]bombesin was not altered in HIT cells pretreated with 300 ng/ml TPA for 2 hr. Specific [^{125}I-Tyr^4]bombesin binding was 4170 ± 150 cpm/dish in control cells and 3970 ± 410 cpm/dish in TPA-pretreated cells (triplicate dishes). Therefore, the effect of TPA to desensitize HIT cells to bombesin appeared to provide a useful paradigm for examining those mechanisms of bombesin desensitization that do not involve a decrease in receptor number.

We previously showed that bombesin increases the production of IP_3 and causes an elevation of $[Ca^{2+}]_i$ in HIT cells (4). The ability of bombesin to elicit [^{3}H]IP_3 accumulation during a 20-sec incubation was decreased by 51 ± 8% (triplicate samples) after pretreatment of cells with 300 ng/ml TPA for 2 hr. Furthermore, bombesin stimulation of [^{3}H]inositol bisphosphate and [^{3}H]inositol monophosphate formation were reduced in parallel. Similarly, pretreatment with TPA decreased bombesin stimulation of $[Ca^{2+}]_i$ (Fig. 8). The peak $[Ca^{2+}]_i$ response to bombesin was reduced by 50 ± 6% (three experiments), although basal $[Ca^{2+}]_i$ levels were the same in TPA-pretreated cells (270 ± 4 nM; four experiments) and control cells (280 ± 10 nM; 88 experiments). In addition, the rate of decay of the $[Ca^{2+}]_i$ from its peak value was enhanced by TPA pretreatment;

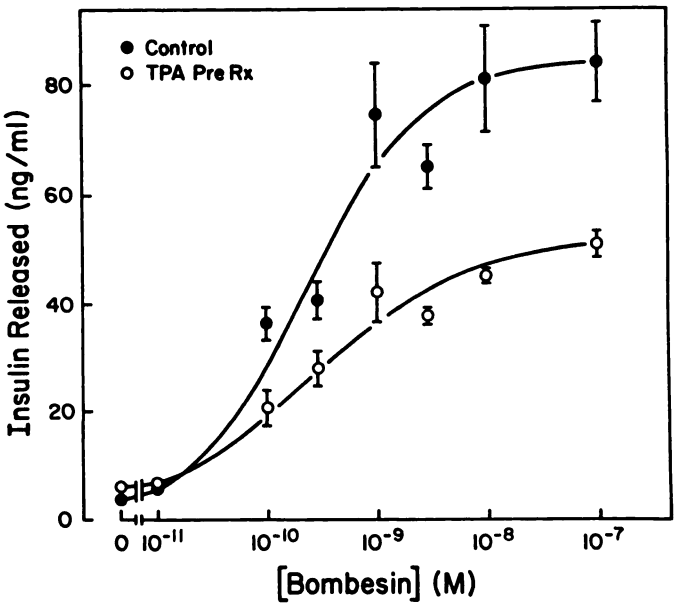

Fig. 7. Effect of TPA pretreatment on bombesin stimulation of insulin secretion. HIT cells (2×10^6/dish) were preincubated for 2 hr at 37° in the absence (●) or presence (○) of 300 ng/ml TPA. The cells were then rinsed and incubated at 37° with the indicated concentration of bombesin. After 60 min, the buffers were collected and the released insulin was measured by radioimmunoassay. The data represent the mean ± standard error of triplicate dishes.

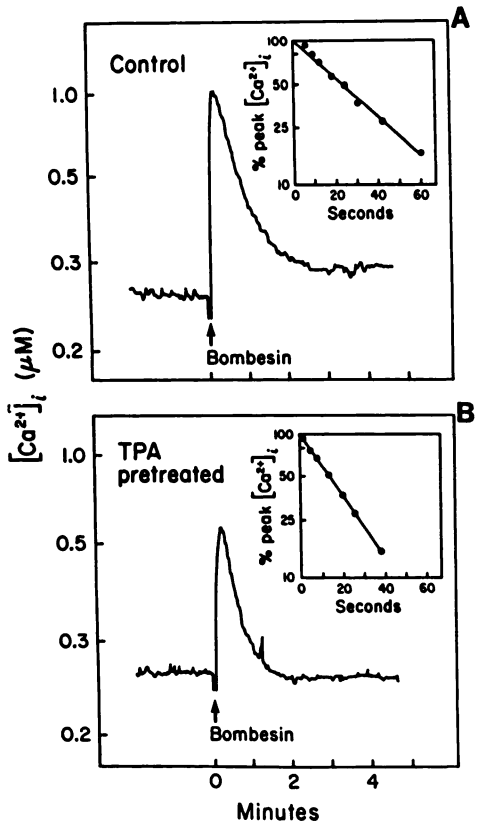

Fig. 8. Effect of TPA pretreatment on bombesin stimulation of the $[Ca^{2+}]_i$. HIT cells were rinsed and preincubated at 37° in the absence (A) or presence (B) of 300 ng/ml TPA. After 60 min, the cells were suspended in the continued absence or presence of TPA, as described in Materials and Methods. Aliquots of the suspension (10^7 cells) were loaded with quin2 and the $[Ca^{2+}]_i$ was continuously monitored at 37°, as described in Materials and Methods. At $t = 0$, bombesin was added to a final concentration of 100 nM. *Inset*, semilogarithmic plot of the decay of $[Ca^{2+}]_i$ from the peak value.

the half-time for the decrease in $[Ca^{2+}]_i$ was 46 ± 6 sec (16 experiments) in control cells and 15 ± 2 (six experiments) in TPA-pretreated cells. Thus, TPA pretreatment markedly inhibited the accumulation of two intracellular messengers in response to bombesin stimulation.

HIT cells respond to bombesin with a rapid initial burst of secretion, followed by a slower sustained phase (4). Our previous studies indicated that $[Ca^{2+}]_i$ and diacylglycerol play distinct roles in the two phases of bombesin-stimulated insulin release (4). The concerted action of increased $[Ca^{2+}]_i$ and activated protein kinase C is necessary to produce the rapid burst of insulin secretion in response to bombesin (4). However, bombesin-induced activation of protein kinase C appears to be sufficient to elicit the smaller second phase of the secretory response (4). Therefore, we determined how pretreatment with TPA affected the time course of bombesin-stimulated insulin release (Fig. 9). Preincubation of cells with TPA resulted in a small residual stimulation of basal secretion following removal of the phorbol ester. However, the effect of TPA on basal secretion was negligible compared with its inhibition of the rapid burst of secretion produced by bombesin (Fig. 9). In three experiments, the amount of insulin secreted during the first minute of bombesin challenge was decreased by 50 ± 10% after preincubation with TPA. In contrast, residual stimulation of basal secretion after TPA pretreatment confounded the effect of TPA on the sustained phase of the bombesin response (Fig. 9). Thus, the rates of insulin release during the second phase of the bombesin response (between 2 and 45 min) were indistinguishable in control and TPA-pretreated cells and were similar to the basal secretion observed after TPA pretreatment. In a separate experiment, the rates of insulin release during the second phase of the bombesin response were again the same in control and pretreated cells but were both somewhat higher than the residual basal secretion observed after TPA pretreatment. These results indicate that bombesin is less effective at stimulating the second phase of secretion after pretreatment with phorbol ester.

In summary, our results are consistent with a decrease in protein kinase C activation leading to a desensitization of the second phase of the secretory response to bombesin, although this conclusion is based on a comparison of small effects. More strikingly, our experiments clearly show that protein kinase C activation markedly inhibits the burst phase of bombesin-stimulated insulin release and support the conclusion that inhibition of bombesin-stimulated IP_3 and $[Ca^{2+}]_i$ production is involved in this desensitization.

Discussion

Bombesin has been shown to elicit a large number of specific responses from different target cells, including stimulation of endocrine and exocrine secretion in the pancreas, stimulation of cell proliferation in 3T3 fibroblasts and small cell lung carcinoma cells, and neuromodulation in the central nervous system (2, 3, 14–16). As a result, intense attention has recently been focused on the mechanism of action of this neuropeptide/growth factor. In all target cells examined to date, bombesin produces a rapid increase in IP_3 and diacylglycerol accumulation and a rise in cytosolic calcium. Thus, these intracellular messengers have been proposed to mediate the biological actions of bombesin. Nonetheless, very little is known about how cellular responsiveness to bombesin is regulated. In this study, we show that pretreatment of HIT cells with bombesin for as little as 5 min blocked subsequent stimulation of insulin secretion by the peptide. This desensitization is due to a reduction in the maximum cellular response to bombesin; peptide potency is unaffected. Although a similar, albeit somewhat slower ($t_{1/2} \approx$ 60 min), desensitization to bombesin has been observed previously in pancreatic acinar cells (17), homologous desensitization of the proliferative effects of bombesin has not been examined. Furthermore, the mechanisms involved in desensitization to bombesin are not well understood.

The results presented here show that pretreatment of HIT cells with bombesin rapidly ($t_{1/2} \approx 2$ min) decreased receptor number without affecting the apparent affinity of the bombesin receptor. The loss of cell surface receptors was only partially reversed during a subsequent 2-hr incubation at 37° in the absence of peptide, suggesting that at least some of the internalized receptors were degraded. The rapid partial recovery of cell surface receptors could result either from receptor recycling or from the utilization of an intracellular pool of receptors. Although our results do not distinguish between these mechanisms, they clearly show that the density of bombesin receptors on the cell surface is decreased sufficiently rapidly to be involved in desensitization to the peptide.

In contrast to the down-regulation of bombesin receptors observed in HIT cells, pretreatment of Swiss 3T3 fibroblasts

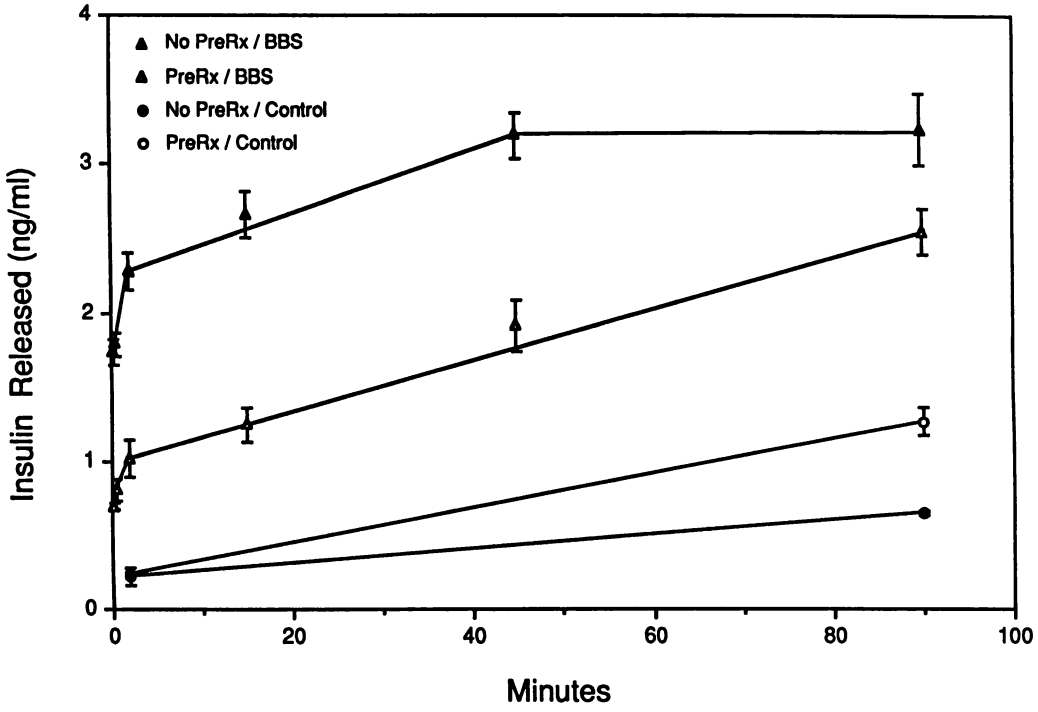

Fig. 9. Effect of TPA pretreatment on the time course for bombesin stimulation of insulin secretion. HIT cells (4×10^6/dish) were incubated for 2 hr at 37° in the absence (●, ▲) or presence (○, △) of 300 ng/ml TPA. The cells were then rinsed and incubated with control buffer (●, ○) or 100 nM bombesin (▲, △) for the indicated times. The data represent the mean ± standard error of the insulin released in triplicate dishes.

with bombesin does not appear to affect bombesin receptor density (18, 19). The inability of Swiss 3T3 cells to down-regulate bombesin receptors is clearly not due to a general cellular defect in receptor-processing pathways, because these cells do exhibit normal homologous down-regulation of their epidermal growth factor receptors (18). Furthermore, both [^{125}I-Tyr^4]bombesin and the mammalian homolog ^{125}I-gastrin-releasing peptide are rapidly internalized subsequent to receptor binding in Swiss 3T3 cells (18, 19), as in HIT cells (5). There are two possible explanations for such a cell-specific difference in homologous regulation of bombesin receptors. First, the bombesin receptor may not be internalized with its bound ligand in Swiss 3T3 cells, whereas it is internalized in HIT cells. Alternatively, the receptor could be rapidly recycled to the plasma membrane after internalization in Swiss 3T3 cells, whereas in HIT cells the receptor is degraded. In fact, because ligand binding was measured at 37° in both studies examining bombesin receptor down-regulation in 3T3 cells (18, 19), it is possible that receptor levels recovered during the binding assay that followed pretreatment with unlabeled peptide. Interestingly, pancreatic acinar cells behave in a manner that is intermediate between HIT cells and 3T3 cells. In this cell type, homologous bombesin receptor down-regulation is observed, but the rate of this process, as well as the rate of receptor recovery, is much slower than in HIT cells (20). These results indicate that it will be important to determine whether structural differences exist between bombesin receptors in different cell types and whether they can explain the observed variability in receptor regulation.

The observation that in HIT cells bombesin-induced receptor down-regulation and desensitization were produced and reversed in parallel supports the conclusion that the decrease in receptor number was at least partially responsible for the desensitization process. However, bombesin was more potent at inducing desensitization than receptor down-regulation. Therefore, at submaximal concentrations of bombesin, the magnitude of the desensitization was always greater than the decrease in receptor binding. These results indicated that mechanisms other than a reduction in receptor number also contributed to bombesin-induced desensitization. In contrast, pretreatment of pancreatic acinar cells with a submaximal concentration of bombesin (1 nM) produced a somewhat greater decrease in receptor binding (70–75%) than in bombesin-stimulated amylase secretion (55%) (20). Similarly, full responsiveness to bombesin returned before complete recovery of binding (20). The larger effects of bombesin pretreatment on receptor binding than on biological responsiveness were consistent with the presence of spare bombesin receptors in acinar cells (20). These results, together with the observation that desensitization of acinar cells to bombesin had no effect on their responsiveness to other agonists that stimulate polyphosphatidylinositol hydrolysis, led these investigators to propose that desensitization involved receptor inactivation (17, 20).

In an effort to identify the mechanisms other than receptor down-regulation that desensitize HIT cells to bombesin, we examined the role of diacylglycerol, one of the intracellular messengers produced upon bombesin stimulation (4). These studies were based on precedents from other systems showing that activation of protein kinases by second messengers and phorbol esters can produce desensitization by inhibiting either receptor function or later steps in message transduction (12, 21–23). As in the case of homologous desensitization to bombesin, pretreatment of HIT cells with TPA decreased the maximal secretory response to the peptide, without altering its ED_{50}. However, in contrast to the effect of bombesin, TPA pretreatment did not reduce the binding of trace concentrations

of [^{125}I-Tyr^4] bombesin Thus, the ability of TPA to elicit heterologous desensitization was not due to an effect on either the number or the affinity of bombesin receptors. These data contrast with the effect of TPA to regulate plasma membrane receptors for many other ligands, including epidermal growth factor and α_1-adrenergic agonists (12, 21).

To investigate the mechanisms responsible for the desensitization of HIT cells by TPA, we determined whether either second messenger production or second messenger activity was inhibited in pretreated cells. Preincubation with phorbol ester decreased bombesin-induced accumulation of inositol phosphates and elevation of $[Ca^{2+}]_i$. In addition, TPA pretreatment completely blocked the ability of TPA to stimulate insulin secretion during a subsequent challenge incubation. Consistent with these effects, TPA pretreatment also reduced the secretory response to bombesin. However, the relative importance of effects on second messenger formation and protein kinase C activation in the homologous desensitization to bombesin remains unresolved. Bombesin pretreatment did reduce the responsiveness of HIT cells to a subsequent TPA challenge, indicating that some inhibition of protein kinase C function did occur. Nonetheless, because TPA pretreatment inhibited stimulation of insulin secretion by bombesin to a smaller extent than restimulation by TPA, inactivation of protein kinase C cannot by itself account for the desensitization to bombesin. Thus, further biochemical studies will be needed to determine precisely the relative importance of effects on second messenger production and protein kinase C function in the mechanism by which bombesin causes desensitization.

Although homologous desensitization to bombesin has not been demonstrated in Swiss 3T3 fibroblasts, a number of studies have examined the effects of TPA pretreatment on different aspects of bombesin action in this cell line. Incubation of 3T3 cells with phorbol ester for several days has been shown to decrease both phorbol ester binding sites in intact cells (24) and protein kinase C activity in cell extracts (25, 26). This down-regulation of protein kinase C results in the complete desensitization of 3T3 cells to phorbol esters and the partial inhibition of some of the actions of bombesin, including stimulation of protein phosphorylation (27), induction of c-*fos* and c-*myc* mRNA accumulation (26), and stimulation of DNA synthesis (28). However, chronic TPA pretreatment does not inhibit the effects of bombesin on either $^{45}Ca^{2+}$ efflux or $[Ca^{2+}]_i$ (29) and, in fact, potentiates bombesin stimulation of IP_3 formation (30). Thus, chronic pretreatment with TPA does not cause desensitization to bombesin at the level of second messenger formation. In contrast, pretreatment of 3T3 cells with TPA for short periods (minutes) does not decrease phorbol ester binding (24), does not block bombesin stimulation of protein kinase C-mediated protein phosphorylation (27), but does inhibit the effect of bombesin to increase IP_3 production (30), $^{45}Ca^{2+}$ efflux (30, 31), and $[Ca^{2+}]_i$ (31, 32). Thus, acute TPA pretreatment appears to inhibit the production of some, but apparently not all, intracellular messengers in response to bombesin. These studies have been interpreted to suggest that the diacylglycerol that is produced following bombesin stimulation acts via a feed-back mechanism involving protein kinase C to inhibit bombesin receptor function (30). Given the complex effects of TPA on bombesin action in 3T3 cells, it will be of interest to determine the extent to which they pertain to the homologous regulation of cellular sensitivity to bombesin itself. Our studies in HIT cells show for the first time that bombesin pretreatment inhibits the effects of protein kinase C activation. Furthermore, aspects of bombesin-induced homologous desensitization can be mimicked by preincubation of cells with TPA.

In conclusion, the results presented here indicate that desensitization of bombesin-stimulated insulin secretion in HIT cells is a multistep process involving 1) receptor down-regulation as a consequence of ligand-induced receptor internalization, 2) inhibition of bombesin-stimulated second messenger accumulation, and 3) inhibition of the protein kinase C-mediated pathway. It will now be of importance to identify the biochemical targets involved in each of these regulatory mechanisms.

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