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# The biphasic stimulation of insulin secretion by bombesin involves both cytosolic free calcium and protein kinase C

Sheridan L. SWOPE* and Agnes SCHONBRUNN†
Department of Pharmacology, Harvard Medical School, and Laboratory of Toxicology, Harvard School of Public Health, Boston, MA 02115, U.S.A.

Members of the bombesin family of peptides potently stimulate insulin release by HIT-T15 cells, a clonal pancreatic cell line. The response to bombesin consists of a large burst in secretion during the first 30 s, followed by a smaller elevation of the secretory rate, which persists for 90 min. The aim of this study was to identify the intracellular messengers involved in this biphasic secretory response. Addition of 100 nM-bombesin to cells for 20 s increased the cellular accumulation of [$^3$H]diacylglycerol (DAG) by 40% and that of [$^3$H]inositol monophosphate (Ins$P$), bisphosphate (Ins$P_2$) and trisphosphate (Ins$P_3$) by 40%, 300%, and 800%, respectively. In contrast, cyclic AMP concentrations were unaffected. Bombesin stimulation of [$^3$H]Ins$P_3$ formation was detected at 2 s, before the secretory response, which was not measurable until 5 s. Furthermore, the potency of bombesin to stimulate [$^3$H]Ins$P_3$ generation ($ED_{50}$ = 14±9 nM) agreed with its potency to stimulate insulin release ($ED_{50}$ = 6±2 nM). Consistent with its effects on [$^3$H]Ins$P_3$ formation, bombesin raised the intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) from a basal value of 0.28±0.01 μM to a peak of 1.3±0.1 μM by 20 s. Chelation of extracellular $Ca^{2+}$ did not abolish either the secretory response to bombesin or the rise in $[Ca^{2+}]_i$, showing that $Ca^{2+}$ influx was not required. Although the $Ca^{2+}$ ionophore ionomycin (100 nM) mimicked the $[Ca^{2+}]_i$ response to bombesin, it did not stimulate secretion. However, pretreating cells with ionomycin decreased the effects of bombesin on both $[Ca^{2+}]_i$ and insulin release, suggesting that elevation of $[Ca^{2+}]_i$ was instrumental in the secretory response to this peptide. To determine the role of the DAG produced upon bombesin stimulation, we examined the effects of another activator of protein kinase C, the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA). TPA did not affect $[Ca^{2+}]_i$, but it increased insulin secretion after a 2 min lag. However, an immediate increase in secretion was observed when ionomycin was added simultaneously with TPA. These data indicate that the initial secretory burst induced by bombesin results from the synergistic action of the high $[Ca^{2+}]_i$ produced by Ins$P_3$ and DAG-activated protein kinase C. However, activation of protein kinase C alone appears to be sufficient for a sustained secretory response.

## INTRODUCTION

Bombesin, a tetradecapeptide orginally isolated from the skin of the frog *Bombina bombina* [1], is one of a family of structurally homologous amphibian and mammalian peptides. In mammals, bombesin-like peptides affect behaviour, as well as metabolic, exocrine and endocrine processes [2]. In addition, bombesin has been shown to act as a mitogen for Swiss 3T3 fibroblasts [3], normal bronchial epithelial cells [4], and human small-cell lung carcinomas [5,6]. Thus bombesin-like peptides regulate the growth, metabolism and secretory function of diverse mammalian cell types.

Our work has focused on the action of bombesin to regulate insulin secretion by the endocrine pancreas. Using HIT-T15 cells (HIT cells), a clonal insulin-secreting cell line, we have shown that bombesin has a direct, potent and rapid stimulatory effect on the β-cell of the pancreas [7]. The secretory response of HIT cells is mediated by specific plasma-membrane receptors, which appear to be coupled to a guanine nucleotide-binding protein [8,9]. In the present study, we have examined the biochemical mechanisms by which bombesin stimulates insulin secretion in HIT cells.

Early studies in rat exocrine pancreas showed that bombesin-induced amylase secretion was associated with increased phosphatidylinositol hydrolysis [10]. More recently, several studies have shown that the mitogenic effect of bombesin in Swiss 3T3 cells is preceded by the generation of the inositol trisphosphates (Ins$P_3$) inositol 1,3,5-trisphosphate and 1,3,4-trisphosphate, the elevation of intracellular free $Ca^{2+}$ ($[Ca^{2+}]_i$), and the activation of protein kinase C [11–14]. However, the mechanism by which the formation of these intracellular second messengers within seconds of bombesin addition leads to an increase in DNA synthesis hours to days later remains to be elucidated. Furthermore, it has also been suggested that the actions of bombesin in 3T3 fibroblasts result from a tyrosine kinase activity associated with the bombesin receptor in those cells [15]. In the present

Abbreviations used: $[Ca^{2+}]_i$, intracellular free $Ca^{2+}$ concentration; DAG, 1,2-diacylglycerol; $ED_{50}$, concentration that elicits a half-maximal effect; HBSS, Hepes-buffered salt solution; HIT cells, the HIT-T15 cell line; Ins$P$, inositol monophosphate; Ins$P_2$, inositol bisphosphate; Ins$P_3$, inositol trisphosphate; IBMX, 3-isobutyl-1-methylxanthine; quin2, 2-{[2-bis(acetylamino)-5-methylphenyloxy]methyl-6-methoxy-8-bis(acetylamino)}quinoline; RIA, radioimmunoassay; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

* Present address: Department of Biology, University of California, San Diego, La Jolla, CA 92093, U.S.A.
† To whom reprint requests should be addressed. Present address: Department of Pharmacology, University of Texas Medical School, P.O. Box 20708, Houston, TX 77225, U.S.A.

study, we have determined which intracellular messengers are altered by bombesin in an endocrine target, the $\beta$-cell of the pancreas. In addition, we have examined how elevated $[Ca^{2+}]_i$, inositol phosphates and 1,2-diacylglycerol (DAG) interact to mediate the rapid and complex effect of bombesin on insulin release.

## EXPERIMENTAL

### Materials

Media and sera for cell culture were purchased from GIBCO (Grand Island, NY, U.S.A.), and plastic culture dishes were from Falcon. Quin2 acetoxymethyl ester, glucagon, 1-mono-oleoyl-*rac*-glycerol, 1,2-diolein, triolein and phospholipase C (type V) were purchased from Sigma (St. Louis, MO, U.S.A.). Synthetic peptides were from either Peninsula Laboratories (San Carlos, CA, U.S.A.) or Bachem (Torrance, CA, U.S.A.). TPA was purchased from LC Services Corp. (Woburn, MA, U.S.A.). [5,6,8,9,11,12,14,15-$^3$H(n)]Arachidonic acid (60–100 Ci/mmol), [1-$^{14}$C]arachidonic acid (40–60 mCi/mmol) and en$^3$Hance spray were obtained from New England Nuclear (Boston, MA, U.S.A.). *myo*-[2-$^3$H]-Inositol (15 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). Chloroform, methanol, toluene and ScintiVerse II scintillation fluid were purchased from Fisher (Fair Lawn, NJ, U.S.A.). 1,2-Dichloroethane was obtained from Aldrich (Milwaukee, WI, U.S.A.). Silicic acid and Dowex 1-X8 were purchased from Bio-Rad (Richmond, CA, U.S.A.). XAR-5 X-ray film was from Kodak (Rochester, NY, U.S.A.) and disposable 6 cm polyethylene columns were obtained from Evergreen Scientific (Los Angeles, CA, U.S.A.). Merck silica-gel 60 t.l.c. plates were purchased from VWR (Boston, MA, U.S.A.).

### Methods

**Cell culture.** The establishment and properties of the HIT cell line have been described previously [7,16]. Cells were grown in monolayer cultures on 35 mm-diam. dishes for cyclic AMP and insulin-release measurements and on 10 cm-diam. dishes for experiments involving the measurement of $[Ca^{2+}]_i$, inositol phosphates and DAG. Cultures were inoculated at a density of approx. $1.5 \times 10^5$ cells/35 mm dish and $1.2 \times 10^6$ cells/10 cm dish and grown as previously described [7]. Cell number was determined at the time of each experiment with a haemocytometer. All experiments were performed at least twice with similar results. Data are presented as mean $\pm$ S.E.M. when $n \geqslant 3$ and as mean $\pm$ range when $n = 2$.

**Measurement of insulin and cyclic AMP release.** Insulin secretion was determined by incubating cells at 37 °C in a Hepes-buffered salt solution (HBSS-I) containing 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 at pH 7.2. The protocol followed has been described in detail previously [7]. To determine effects on cyclic AMP production, HIT cells were pretreated for 30 min in HBSS-I with 100 $\mu$M-3-isobutyl-1-methylxanthine (IBMX) and then incubated with the indicated peptide in the continued presence of IBMX for an additional 30 min at 37 °C. The cyclic AMP accumulated in the medium was measured by RIA as previously described [17].

**Measurement of DAG accumulation.** The incorporation of [$^3$H]arachidonic acid into DAG was assayed as described previously [18]. HIT cell cultures were incubated for 24 h at 37 °C in growth medium containing 1 $\mu$g of [$^3$H]arachidonic acid/ml, at which time > 90 % of the radioactivity was cell-associated. The cells were then suspended in a Hepes-buffered salt solution (HBSS-II: 118 mM-NaCl, 4.6 mM-KCl, 1 mM-$CaCl_2$, 10 mM-glucose and 20 mM-Hepes, pH 7.2). Portions of the cell suspension ($\sim 10^7$ cells/0.5 ml) were incubated with the test compounds at 37 °C for 20 s. The incubations were stopped and the lipids were extracted by adding 2 ml of ice-cold chloroform/methanol (2:1, v/v). DAG (50 $\mu$g) and [$^{14}$C]DAG ($\sim$ 5000 c.p.m., prepared as described below) were added to each tube to increase and to monitor recovery respectively. The lower lipid layer of each extract was evaporated to dryness, dissolved in 1 ml of chloroform and applied to a 2.5 ml preactivated silicic acid column. The neutral lipids were eluted with 5 ml of chloroform, concentrated and analysed by t.l.c. on silica-gel 60 plates with the solvent system dichloroethane/methanol (49:1, v/v). After identification of the lipid spots with iodine vapour, the chromatography plates were sprayed with en$^3$Hance and used to expose XAR-5 X-ray film at −70 °C. Radioactivity was observed in spots with $R_F$ values of $0.94 \pm 0.01$, $0.68 \pm 0.06$ and $0.52 \pm 0.09$, and migrated as triolein, 1,3-diolein and 1,2-diolein standards respectively. The radioactive 1,3-diolein and 1,2-diolein spots were cut out, extracted with 1 ml of toluene, and the $^3$H and $^{14}$C were measured by scintillation counting. All values were corrected for the recovery of the added [$^{14}$C]DAG, which ranged between 55 and 100 % and averaged $80 \pm 1$ % ($n = 60$). Bombesin did not affect the $^3$H associated with 1,3-diolein, triolein, or 1-mono-oleoyl-*rac*-glycerol.

**Preparation of [$^{14}$C]DAG.** The [$^{14}$C]DAG used to measure sample recovery was prepared as previously described for [$^3$H]DAG [18]. Approx. $5 \times 10^7$ HIT cells were incubated in growth medium containing 1 $\mu$Ci of [$^{14}$C]arachidonic acid/ml for 24 h. The lipids were extracted with chloroform/methanol (2:1, v/v) as described above and the upper layer was concentrated and applied to a silicic acid column. The column was washed with 5 ml of chloroform followed by 5 ml of methanol to elute phospholipids. The phospholipids were treated with phospholipase C (type V; 20 $\mu$g/ml) for 60 min at 37 °C, extracted, and applied to a fresh silicic acid column. The [$^{14}$C]DAG was eluted with chloroform. Phospholipase C treatment converted all of the radioactivity in the phospholipid fraction into [$^{14}$C]1,2-diolein, as shown by t.l.c.

**Measurement of inositol phosphate(s) accumulation.** Inositol phospholipid metabolism was examined as previously described [19]. HIT cells were incubated at 37 °C for 48 h in growth medium containing 1 $\mu$Ci of [$^3$H]inositol/ml, at which time 20–45 % of the added radioactivity was cell-associated. The cells were then suspended in HBSS-II, and 0.5 ml samples were incubated at 37 °C with the desired agents for the times indicated. The reactions were stopped with 0.5 ml of ice-cold 20 % (w/v) trichloroacetic acid, the precipitated proteins pelleted (650 *g*, 15 min), and the supernatants extracted with water-saturated diethyl ether. Samples were applied to 2 ml Dowex 1-X8 anion-exchange

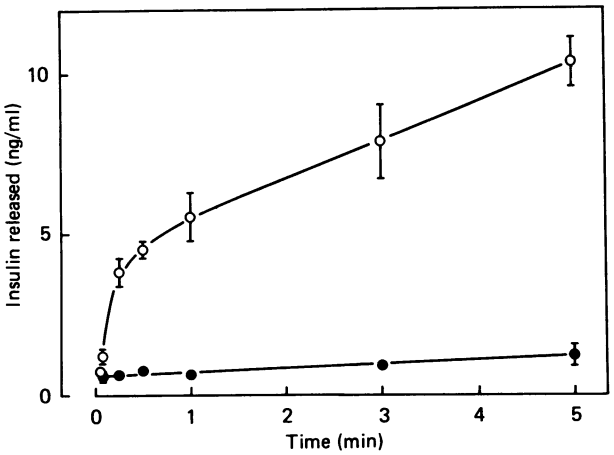


**Fig. 1. Time course for bombesin stimulation of insulin secretion**

HIT cells [4.4(±0.4) × $10^6$ cells/dish] were incubated at 37 °C in the absence (●) or presence (○) of 100 nM-bombesin. At the times shown, the buffers were collected and the released insulin was measured by RIA. The data represent means±S.E.M. for triplicate dishes.

columns ($Cl^-$ form). [$^3$H]Inositol, [$^3$H]Ins*P*, [$^3$H]Ins$P_2$ and [$^3$H]Ins$P_3$ were eluted with 4 × 2 ml of water, 4 × 2 ml of 30 mM-HCl, 4 × 2 ml of 90 mM-HCl and 4 × 1 ml of 500 mM-HCl respectively. The $^3$H in each fraction was measured by scintillation counting and represents the radioactivity incorporated into all isomers of the particular inositol phosphate eluted in that fraction.

**Measurement of $[Ca^{2+}]_i$.** This was done with the fluorescent calcium indicator quin2 [20] as described previously [21]. Each experiment was performed with 0.6 × $10^7$–1.5 × $10^7$ quin2-loaded cells suspended in 2.5 ml of HBSS-II. Quin2 fluorescence was continuously monitored at 37 °C with a Perkin–Elmer 650-10S spectrofluorimeter at an excitation wavelength of 340 nm, an emission wavelength of 492 nm and slit widths of 5 nm each. Calibration of the fluorescent signal in terms of $[Ca^{2+}]_i$ was performed as described previously [21].

The intracellular quin2 concentration was calculated by a published procedure [21] by using an intracellular water volume of 8.0 μl per $10^6$ HIT cells, determined by the method of Rottenberg [22] with [$^3$H]water and [$^{14}$C]sorbitol. The intracellular concentration of quin2 ranged between 60 and 110 μM and averaged 78±3 μM ($n$ = 25). Cells grown and suspended by the protocol used for measuring $[Ca^{2+}]_i$ responded to 100 nM-bombesin with a 2.5-fold increase in insulin secretion during a 20 min incubation. Loading the cells with quin2 did not alter either basal hormone release or the secretory response to bombesin.

## RESULTS

### Effect of bombesin on insulin secretion

The secretory response to 100 nM-bombesin was biphasic, consisting of a rapid burst of secretion followed by a smaller stimulation (Fig. 1). In six experiments, increased secretion was measurable at 5 s (2.4±0.9-fold over basal), but was not detected at 3 s. The initial burst phase of secretion was linear for 15–30 s and was 180±20 times the basal rate ($n$ = 6). The second phase of secretion was 13±3 times the basal rate ($n$ = 6) and was linear for up to 5 min. We have previously shown that the second phase of secretion continues for 90 min [7].

**Table 1. Effect of bombesin on the concentrations of DAG and cyclic AMP**

DAG was measured in HIT cells prelabelled with [$^3$H] arachidonic acid as described under 'Methods'. Samples (0.5 ml) of the labelled cell suspension were added to tubes containing 10 μl of a 50-fold-concentrated stock solution of each of the peptides shown. After 20 s at 37 °C, the incubations were stopped and the radioactivity incorporated into DAG was determined. Each incubation contained 9.2(±0.2) × $10^7$ cells. To measure changes in cyclic AMP concentrations, HIT cells [3.5(±0.3) × $10^6$ cells/dish] were incubated with 100 μM-IBMX and the indicated peptide for 30 min at 37 °C as described under 'Methods'. The cyclic AMP released into the buffer was measured by RIA. In each case, the data represent means±S.E.M. for triplicate incubations: ND, not determined.

| Treatment | [$^3$H]DAG (c.p.m.) | Cyclic AMP (pmol/ml) |
|---|---|---|
| Control | 1240±30 | 4±1 |
| Bombesin (100 nM) | 1820±20 | 4±1 |
| Glucagon (1 μM) | 1240±30 | 92±3 |
| Somatostatin (100 nM) | 1240±80 | ND |

### Generation of specific intracellular messengers in response to bombesin

The results in Table 1 and Fig. 2 show the effect of bombesin on a number of second messengers which are known to regulate secretory processes. Bombesin did not affect cyclic AMP concentrations, whereas it stimulated [$^3$H]DAG accumulation (Table 1). In the same experiment glucagon and somatostatin, two peptides which also regulate insulin secretion by HIT cells [7], did not alter [$^3$H]DAG accumulation, although glucagon produced a 23-fold increase in cyclic AMP (Table 1). In five experiments, bombesin increased [$^3$H]DAG by 40±3% after 20 s. Bombesin also rapidly stimulated inositol mono- and poly-phosphate accumulation (Fig. 2), whereas glucagon and somatostatin had no effect after a 20 s incubation (results not shown). Both [$^3$H]Ins$P_2$ and [$^3$H]Ins$P_3$ were significantly elevated above basal values within 2 s of bombesin addition (Fig. 2). By comparison, the initial burst of bombesin-stimulated insulin secretion was not detected until 5 s (Fig. 1). In three experiments, [$^3$H]Ins$P_2$ and [$^3$H]Ins$P_3$ accumulation reached a peak between 5 s and 15 s. In contrast, stimulation of [$^3$H]Ins*P* accumulation was not detected until 15 s, and reached a peak at 1 min. Thereafter, all the inositol phosphates returned to near-basal values (Fig. 2). These data demonstrate the specificity of bombesin's effect on different intracellular messengers in HIT cells. In addition, they show that increases in cellular [$^3$H]Ins$P_2$ and [$^3$H]Ins$P_3$, but not [$^3$H]Ins*P*, occurred before the initial burst of insulin release produced by bombesin.

To determine whether Ins$P_2$ or Ins$P_3$ could function as intracellular mediators of bombesin-induced insulin release, we also examined the concentration-dependence for the increased [$^3$H]Ins$P_2$ and [$^3$H]Ins$P_3$ accumulation

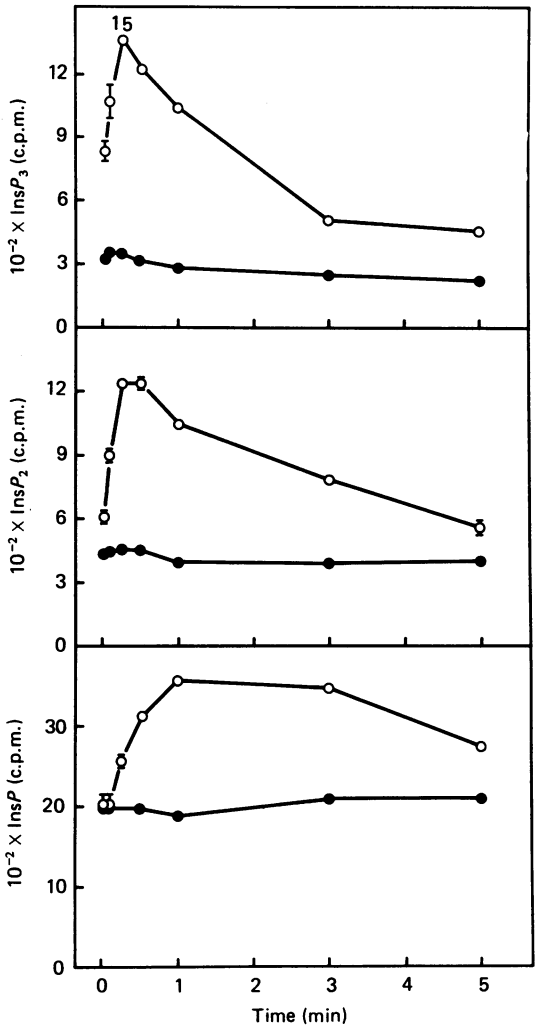


**Fig. 2. Time course for bombesin stimulation of inositol phosphate formation**

HIT cells were prelabelled with [$^3$H]inositol as described under 'Methods'. Portions of the prelabelled cell suspension [10(±2) × $10^7$ cells/0.5 ml] were added to 10 $\mu$l of vehicle (●) or 5 $\mu$M-bombesin (○), producing a final bombesin concentration of 100 nM. After incubation at 37 °C for the times shown, the cells were precipitated and the radioactivity associated with Ins$P$, Ins$P_2$ and Ins$P_3$ was measured as described under 'Methods'. The data represent means ± S.E.M. for triplicate samples.

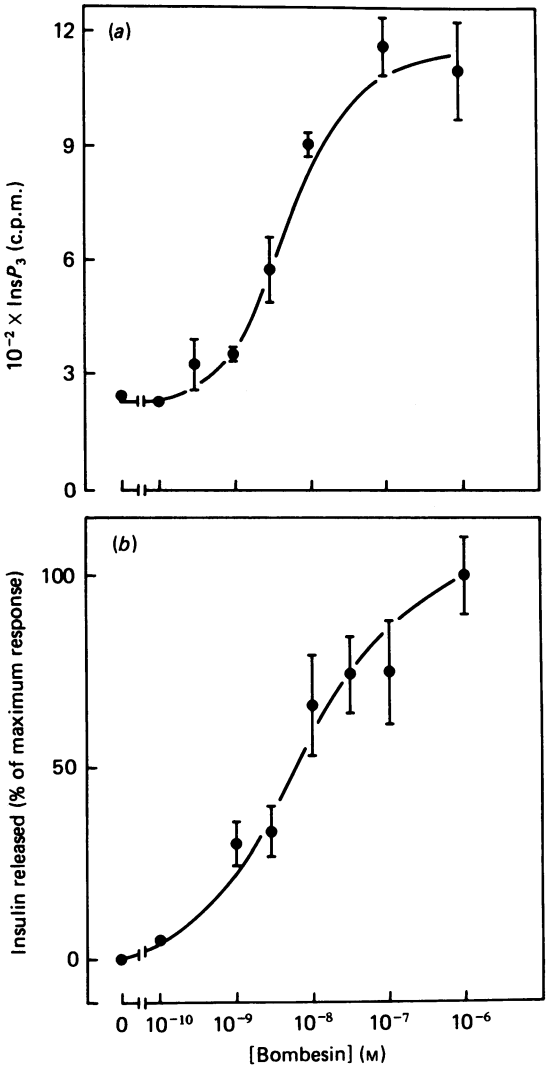


**Fig. 3. Concentration-dependence for bombesin stimulation of inositol phosphate formation and insulin secretion**

(*a*) HIT cells were prelabelled with [$^3$H]inositol as described under 'Methods'. Samples of the labelled cell suspension [8.1(±0.1) × $10^7$ cells/0.5 ml] were added to 10 $\mu$l of a 50-fold stock solution of bombesin to produce the final concentrations indicated. After incubation at 37 °C for 20 s, the cells were precipitated and the radioactivity associated with Ins$P_3$ was measured as described under 'Methods'. The data represent means ± S.E.M. for triplicate aliquots. (*b*) HIT cells [3.3(±0.5) × $10^6$/dish] were incubated at 37 °C with the indicated concentrations of bombesin. After 20 s, the buffers were collected and the released insulin was measured by RIA. The data represent means ± S.E.M. for six dishes from two independent experiments.

in response to bombesin. In the experiment shown in Fig. 3(*a*), the concentration of bombesin which elicited a half-maximal stimulation ($ED_{50}$) of [$^3$H]Ins$P_3$ generation was 4.8±0.1 nM. In a second experiment, the $ED_{50}$ for bombesin-stimulated [$^3$H]Ins$P_3$ accumulation was 22.5±0.1 nM. Bombesin also stimulated [$^3$H]Ins$P_2$ generation in a dose-dependent manner, with an $ED_{50}$ of 15±9 nM ($n = 2$). These concentrations are higher than the previously reported $ED_{50}$ of 0.5 nM for bombesin-stimulated insulin release [7]. However, the previous value for secretion was obtained during a 60 min incubation with bombesin. Therefore we determined the concentration-dependence for the effect of bombesin on insulin release at 20 s (Fig. 3*b*). At this early time point, 6±2 nM-bombesin elicited a half-maximal effect, in much better agreement with the $ED_{50}$ value for [$^3$H]Ins$P_3$ and [$^3$H]Ins$P_2$ generation measured after the same incubation period. Thus both the time course and the concentration-dependence of bombesin action support the involvement of Ins$P_3$ and/or Ins$P_2$ accumulation in mediating the secretory response to bombesin.

### Mobilization of an intracellular $Ca^{2+}$ pool by bombesin

Ins(1,4,5)$P_3$ has been shown to release $Ca^{2+}$ from microsomal fractions of a rat insulinoma [23]. To determine whether the bombesin-induced generation of

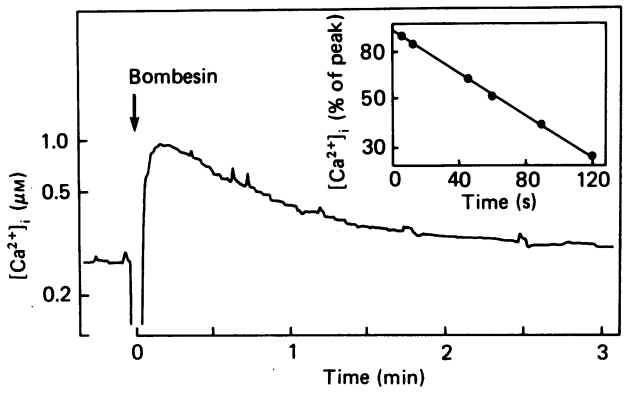


**Fig. 4. Time course of the bombesin-induced changes in $[Ca^{2+}]_i$**

$[Ca^{2+}]_i$ was measured in HIT cells [1.21(±0.04) × $10^7$ cells/2.5 ml] by monitoring the fluorescence of intracellular quin2 at 37 °C. The $Ca^{2+}$ concentrations shown were calculated from the intensity of the fluorescence signal as described under 'Methods'. The arrow indicates the time of addition of a 200-fold-concentrated stock solution of bombesin, giving a final peptide concentration of 100 nM. The inset shows a semi-logarithmic plot of the decrease in $[Ca^{2+}]_i$ from a peak value attained after bombesin addition.

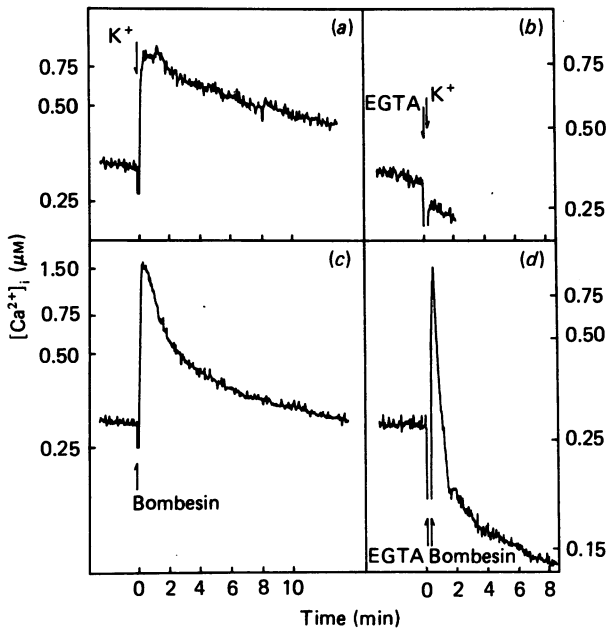


**Fig. 5. Effect of EGTA on bombesin- and $K^+$-stimulated increases in $[Ca^{2+}]_i$**

$[Ca^{2+}]_i$ was measured in HIT cells [1.1(±0.2) × $10^7$ cells/2.5 ml] by monitoring the fluorescence of intracellular quin2 at 37 °C. The $Ca^{2+}$ concentrations shown were calculated from the intensity of the fluorescence signal as described under 'Methods'. The arrows indicate the time of addition of 200-fold-concentrated stock solutions of $K^+$, bombesin or EGTA, which gave final concentrations of 40 mM, 100 nM and 1.1 mM respectively.

[$^3$H]Ins$P_3$ was associated with a rise in $[Ca^{2+}]_i$, we directly examined the ability of bombesin to elevate the $[Ca^{2+}]_i$, using the fluorescent $Ca^{2+}$ indicator quin2. As shown in Fig. 4, 100 nM-bombesin had a rapid and powerful effect on $[Ca^{2+}]_i$, eliciting a rise from a basal value of 255 nM to a peak of 920 nM by 30 s. A change in $[Ca^{2+}]_i$ was detected 3 s after addition of bombesin. In replicate experiments, the resting $[Ca^{2+}]_i$ was 0.28±0.01 μM ($n$ = 30), and bombesin caused a rise to a peak value of 1.3±0.1 μM at 20±2 s ($n$ = 16). Thus the maximal change in $[Ca^{2+}]_i$ caused by bombesin treatment was 1.0±0.1 μM, corresponding to a 4.5±0.3-fold stimulation. In the continued presence of bombesin, the $[Ca^{2+}]_i$ returned toward the basal value with a half-time of 46±6 s ($n$ = 13; Fig. 4 inset).

To investigate the source of the $Ca^{2+}$ mobilized by bombesin, we determined whether extracellular $Ca^{2+}$ was required for bombesin to elicit a rise in $[Ca^{2+}]_i$ (Fig. 5). When the extracellular $Ca^{2+}$ was chelated with 1.1 mM-EGTA, bombesin still increased $[Ca^{2+}]_i$, from 240 nM to 1.1 μM (Fig. 5*d*). By comparison, in the same experiment, bombesin raised $[Ca^{2+}]_i$ from 300 nM to 1.7 μM in the absence of EGTA (Fig. 5*c*). Thus the absolute change in $[Ca^{2+}]_i$ in response to bombesin remained 60% intact and the fold stimulation remained 80% intact when the extracellular $Ca^{2+}$ was chelated. In contrast, addition of 1.1 mM-EGTA completely blocked the increase in $[Ca^{2+}]_i$ in response to 40 mM-$K^+$ (Figs. 5*a* and 5*b*). Since increasing extracellular $K^+$ causes depolarization of the plasma membrane and, as a consequence, increased $Ca^{2+}$ influx through voltage-sensitive $Ca^{2+}$ channels, this result demonstrates that $Ca^{2+}$ influx was effectively prevented by chelating the extracellular $Ca^{2+}$. In replicate experiments with EGTA, the absolute change in $[Ca^{2+}]_i$ in response to bombesin was 40±7% intact ($n$ = 5) and the fold stimulation was 55±7% intact ($n$ = 5), whereas the effect of 40 mM-$K^+$ was abolished ($n$ = 4). These data indicate that the effect of bombesin to elevate $[Ca^{2+}]_i$ was due, at least in part, to mobilization of an intracellular $Ca^{2+}$ pool.

The partial inhibition of the $[Ca^{2+}]_i$ response to bombesin by EGTA could have been due either to blockage of $Ca^{2+}$ influx or to an effect on the intracellular $Ca^{2+}$ pool. After treatment of HIT cells with bombesin in the presence of EGTA, the $[Ca^{2+}]_i$ decayed to 160±20 nM ($n$ = 5), which was less ($P < 0.01$) than the untreated basal concentration of 280±10 nM ($n$ = 30; Figs. 5*c* and 5*d*). In addition, EGTA increased the rate of decay of the elevated $[Ca^{2+}]_i$ produced by bombesin treatment to a half-time of 23±5 s ($n$ = 5), which was less ($P < 0.05$) than the half-time of 46±6 s ($n$ = 30) observed after the addition of bombesin in the absence of EGTA (Figs. 5*c* and 5*d*). Finally, when HIT cells were preincubated with EGTA for longer times, basal $[Ca^{2+}]_i$ continuously declined and the subsequent response to bombesin was diminished (results not shown). Together, these observations suggest that prolonged treatment with EGTA induced a net loss of cellular $Ca^{2+}$ by decreasing the driving force for $Ca^{2+}$ influx. Thus the ability of EGTA to decrease the $[Ca^{2+}]_i$ response to bombesin (Figs. 5*c* and 5*d*) is likely to be due to a diminution of the intracellular $Ca^{2+}$ pool.

We also investigated the role of extracellular $Ca^{2+}$ in the secretory response to bombesin. The presence of 1.1 mM-EGTA did not affect stimulation of insulin secretion by bombesin, but significantly blocked the effect of 40 mM-$K^+$ (Table 2). In three experiments the secretory response to $K^+$ was decreased by 71±5%, whereas the response to bombesin was unaffected. In summary, our data indicate that the effects of bombesin both on $[Ca^{2+}]_i$ and on insulin secretion do not require

**Table 2. Effect of EGTA on bombesin- and $K^+$-stimulated insulin secretion**

HIT cells [4.3(±0.4) × $10^6$/dish] were incubated at 37 °C with the indicated secretagogues in the absence or presence of 1.1 mM-EGTA. After 15 s, the buffers were collected and the released insulin was measured by RIA. The extracellular $Ca^{2+}$ concentration was 0.5 mM in the absence of EGTA and approx. 1 μM in the presence of 1.1 mM-EGTA [24]. The data represent means ± S.E.M. for triplicate dishes.

| | Insulin released (ng/ml) | |
|---|---|---|
| Treatment | Control | EGTA |
| Control | 0.56±0.03 | 0.7±0.1 |
| $K^+$ (40 mM) | 2.1±0.2 | 1.0±0.2 |
| Bombesin (100 nM) | 3.4±0.3 | 3.1±0.4 |

high extracellular $Ca^{2+}$, suggesting that mobilization of intracellular $Ca^{2+}$ stores rather than $Ca^{2+}$ influx is involved in these responses to the peptide.

### Effect of ionomycin on the bombesin-induced elevation of $[Ca^{2+}]_i$

We used the $Ca^{2+}$-selective ionophore ionomycin [25] to examine the role of intracellular $Ca^{2+}$ mobilization in bombesin action. At 100 nM, ionomycin increased $[Ca^{2+}]_i$ in a manner similar to bombesin (Fig. 6*a*). The peak $[Ca^{2+}]_i$ response was 1.5±0.2 μM and occurred 31±3 s ($n = 10$) after the addition of ionomycin. This was somewhat later ($P < 0.05$) than the peak response to bombesin (20±2 s; $n = 16$). However, the maximum $[Ca^{2+}]_i$ produced by ionomycin was similar to that elicited by bombesin (1.3±0.1 μM; $n = 16$); both agents resulted in almost complete saturation of the quin2 dye.

To test whether the ionomycin effect resulted from increased $Ca^{2+}$ influx, HIT cells were treated with 1.15 mM-EGTA before ionomycin addition (Fig. 6*b*). Although this concentration of EGTA blocked the effect of 40 mM-$K^+$ on $[Ca^{2+}]_i$ (Figs. 5*a* and 5*b*), it did not abolish the response to ionomycin (Fig. 6*b*). In three experiments, the peak $[Ca^{2+}]_i$ response elicited by 100 nM-ionomycin in the presence of 1.15 mM-EGTA was 0.57±0.02 μM. Thus the increase in $[Ca^{2+}]_i$ produced by ionomycin in the presence of EGTA was 27±1% of that observed in control cells: the fold stimulation produced by ionomycin in the presence of EGTA was 41±4% intact. These data suggest that, as for bombesin, a part of the action of ionomycin to elevate $[Ca^{2+}]_i$ was independent of extracellular $Ca^{2+}$, and therefore must result from the release of an intracellular $Ca^{2+}$ pool.

To investigate whether ionomycin and bombesin released the same intracellular store of $Ca^{2+}$, we examined the effect of ionomycin on bombesin action. As shown in Fig. 7(*a*), pretreatment of HIT cells with 100 nM-ionomycin decreased the effect of 100 nM-bombesin on $[Ca^{2+}]_i$. After ionomycin pretreatment, bombesin increased $[Ca^{2+}]_i$ by only 0.1±0.1 μM ($n = 2$). Thus ionomycin decreased the response to bombesin by 86±1%, calculated from the absolute rise in $[Ca^{2+}]_i$, or by 94±1%, calculated from the fold stimulation above the $[Ca^{2+}]_i$ present just before bombesin addition.

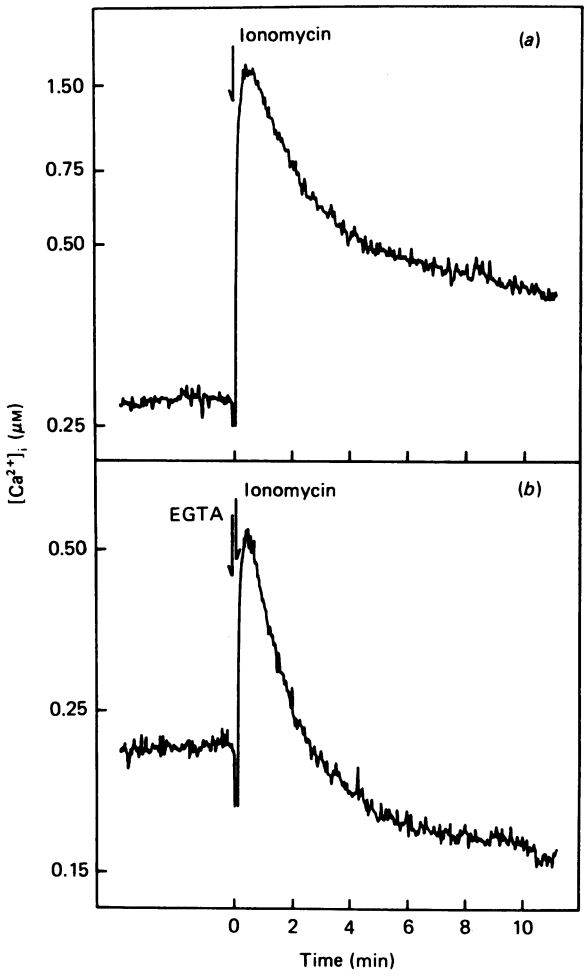


**Fig. 6. Effect of ionomycin on $[Ca^{2+}]_i$ in the presence and absence of EGTA**

$[Ca^{2+}]_i$ was measured in HIT cells [1.3(±0.2) × $10^7$ cells/2.5 ml] by monitoring the fluorescence of intracellular quin2 at 37 °C. The $Ca^{2+}$ concentrations shown were calculated from the intensity of the fluorescence signal as described under 'Methods'. The arrows indicate the time of addition of 200-fold-concentrated stock solutions of ionomycin or EGTA, giving final concentrations of 100 nM and 1.15 mM respectively.

Furthermore, pretreating cells with 100 nM-bombesin also decreased the effect of ionomycin (Fig. 7*b*). After bombesin treatment, ionomycin elevated $[Ca^{2+}]_i$ by only 0.25±0.07 μM ($n = 3$), a decrease of 79±6% in the absolute rise in $[Ca^{2+}]_i$ and of 71±5% in the fold stimulation. These data are consistent with ionomycin and bombesin acting to mobilize the same intracellular $Ca^{2+}$ pool.

### Role of $[Ca^{2+}]_i$ in bombesin stimulation of insulin secretion

To determine whether an increase in $[Ca^{2+}]_i$ was important in the action of bombesin to stimulate insulin release, we examined the effect of ionomycin on the time course of peptide-induced secretion (Fig. 8). Ionomycin alone did not stimulate insulin release by HIT cells. However, pretreatment with 100 nM-ionomycin blunted the secretory response to bombesin. Inhibition of the response to bombesin was detected by 15 s, that is during

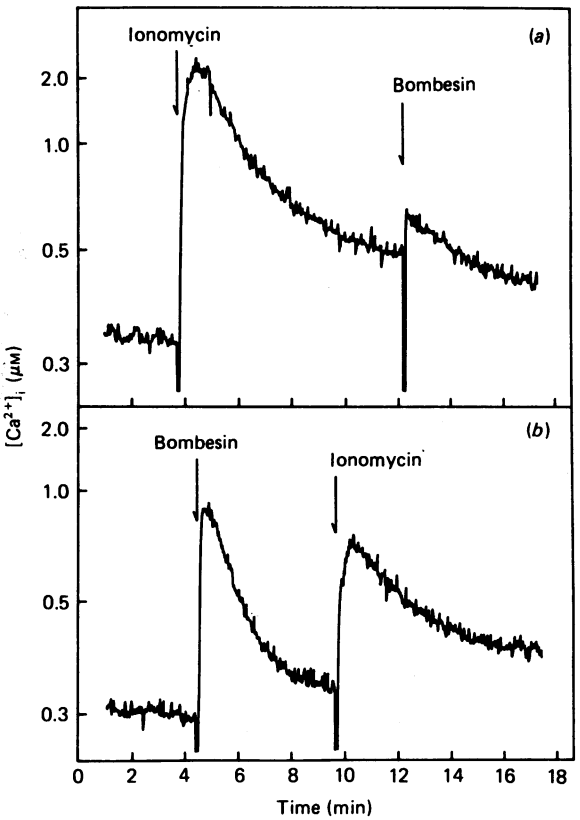


**Fig. 7. Effect of a prior challenge with the heterologous agent on bombesin- and ionomycin-induced increases in $[Ca^{2+}]_i$**

$[Ca^{2+}]_i$ was measured in HIT cells [1.3(±0.2) × $10^7$ cells/2.5 ml] by monitoring the fluorescence of intracellular quin2 at 37 °C. The $Ca^{2+}$ concentrations shown were calculated from the intensity of the fluorescence signal as described under 'Methods'. Cells were treated first with ionomycin and then challenged with bombesin (*a*) or first with bombesin and then with ionomycin (*b*). At the times shown by the arrows, either bombesin or ionomycin, as indicated, was added from 200-fold-concentrated stock solutions to give a final concentration of 100 nM.

the initial phase of secretion. In the experiment shown in Fig. 8, the secretory rate during the second phase also appeared to be decreased. However, in similar experiments the second phase was not consistently affected. Thus an elevation of $[Ca^{2+}]_i$ may be important for the early rapid phase of bombesin-stimulated insulin release. However, mobilization of the intracellular $Ca^{2+}$ pool, as produced by ionomycin, was not sufficient to stimulate secretion.

### Role of protein kinase C activation in bombesin stimulation of insulin release

The inability of ionomycin to stimulate secretion suggested that elevation of $[Ca^{2+}]_i$ by mobilization of an intracellular store may act in concert with another intracellular messenger(s) to mediate the effect of bombesin. As shown in Table 1, treatment of HIT cells with bombesin stimulated [$^3$H]DAG accumulation. This second messenger has been shown to activate protein kinase C [26,27], an effect which is mimicked by phorbol esters [28]. We therefore used the phorbol ester TPA to examine the role of protein kinase C in bombesin action.

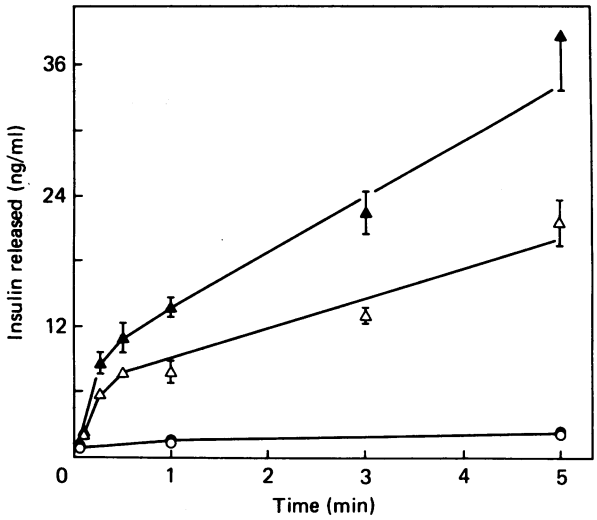


**Fig. 8. Effect of ionomycin pretreatment on the time course for bombesin stimulation of insulin secretion**

HIT cells [4.1(±0.5) × $10^7$ per dish] were incubated at 37 °C for 30 min in the absence (●, ▲) or presence (○, △) of 100 nM-ionomycin. Fresh buffer was then added and the cells were further incubated at 37 °C in the continued absence or presence of ionomycin, either without (●, ○) or with (▲, △) 100 nM-bombesin. At the indicated times, the buffers were collected and the released insulin was measured by RIA. The data represent means ± S.E.M. for triplicate dishes.

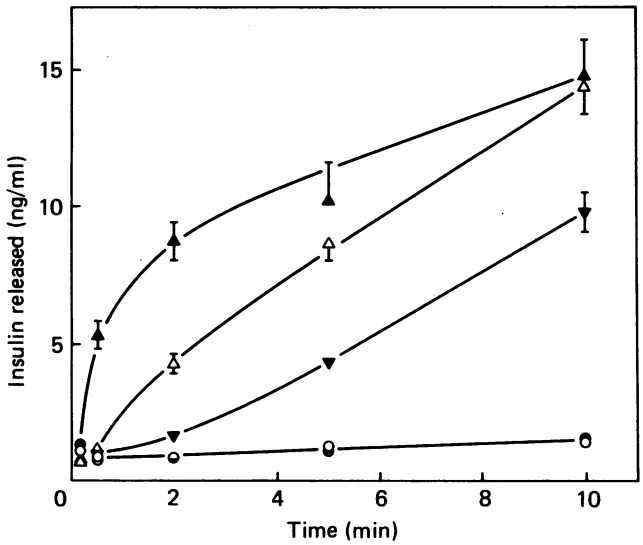


**Fig. 9. Effect of ionomycin on TPA-stimulated insulin release**

HIT cells [1.7(±0.1) × $10^6$ per dish] were incubated at 37 °C in buffer alone (●), or in buffer containing 100 nM-ionomycin (○), 60 ng of TPA/ml (▼), 100 nM-ionomycin plus 60 ng of TPA/ml (△), or 100 nM-bombesin (▲). The amount of insulin released after the indicated times was measured by RIA. The data represent means ± S.E.M. for triplicate dishes.

In contrast with the biphasic response to bombesin, TPA (300 ng/ml) elicited a linear rate of insulin release which was 65 ± 4 times ($n$ = 2) the basal rate. Interestingly, insulin secretion returned to the basal rate after 60 min in the continued presence of TPA, consistent with

a desensitization to the phorbol ester. During 60 min incubations, TPA elicited a half-maximal effect on secretion at a concentration of $67 \pm 5$ ng/ml ($n = 3$).

The effect of TPA on insulin secretion by HIT cells was not dependent on an elevation of $[Ca^{2+}]_i$. In four experiments, exposure of cells to TPA (300 ng/ml) did not alter the $[Ca^{2+}]_i$ as measured with quin2 (results not shown). These results show that in HIT cells activation of protein kinase C and elevation of $[Ca^{2+}]_i$ can occur independently. Furthermore, TPA alone, presumably via activation of protein kinase C, was sufficient for stimulation of insulin release, but did not mimic the biphasic secretory response to bombesin.

### Ionomycin potentiates the effects of TPA on insulin release

Our results showed that ionomycin mimicked the effect of bombesin on $[Ca^{2+}]_i$, but did not stimulate insulin secretion. In contrast, TPA induced a linear rate of secretion without altering $[Ca^{2+}]_i$. In an attempt to mimic the biphasic secretory response elicited by bombesin, we examined the combined effects of ionomycin and TPA (Fig. 9). As shown above, the stimulatory effect of bombesin was biphasic, with an initial burst of release (4.6 ng/ml per min per $10^6$ cells), being followed by a smaller second phase (0.42 ng/ml per min per $10^6$ cells). This first phase of secretion was 265 times the basal rate of 0.17 ng/ml per min per $10^6$ cells, whereas the second phase was 25 times basal. As previously, ionomycin did not stimulate secretion, whereas, after a lag of about 90 s, TPA elicited a linear secretory rate of 0.62 ng/ml per min per $10^6$ cells. However, in the presence of both ionomycin and TPA, there was a rapid secretory response of 1.3 ng/ml per min per $10^6$ cells, which was 75 times the basal rate. After the first few minutes, the rate of secretion in the presence of TPA plus ionomycin decreased to that observed with TPA alone. At 2 min, the amount of insulin released in the presence of TPA alone was twice the control value, whereas in the presence of TPA plus ionomycin stimulation was 5.2-fold. In two other experiments, TPA did not elicit insulin release by 2 min, whereas in the presence of TPA plus ionomycin stimulation was detected. These data demonstrate that an elevation of $[Ca^{2+}]_i$ and activation of protein kinase C work synergistically to elicit a rapid secretory response. In addition, the results are consistent with activation of protein kinase C alone mediating the second, slower, phase of secretion in response to bombesin.

## DISCUSSION

We have shown that bombesin acts on the insulin-secreting pancreatic HIT cell line to elicit a biphasic secretory response. This response was associated with the cellular accumulation of [$^3$H]DAG, [$^3$H]Ins$P_2$ and [$^3$H]Ins$P_3$, and a rise in $[Ca^{2+}]_i$. Our results support the involvement of these second messengers in the stimulation of insulin release by bombesin. Thus insulin secretion was increased by pharmacological agents which mimic the action of DAG to activate protein kinase C and that of Ins$P_3$ to increase the $[Ca^{2+}]_i$. Further, the concentrations of these messengers changed appropriately in response to the peptide. Accordingly, generation of [$^3$H]Ins$P_2$ and [$^3$H]Ins$P_3$ and elevation of $[Ca^{2+}]_i$ occurred before insulin secretion. Furthermore, the dose-dependence for bombesin stimulation of [$^3$H]Ins$P_2$ and [$^3$H]Ins$P_3$ accumulation and insulin release agreed. Although our data do not differentiate between Ins$P_2$ and Ins$P_3$ as possible intracellular messengers, results from other workers indicate that Ins$P_3$ acts as a signal to mobilize intracellular $Ca^{2+}$ stores in insulin-secreting cells, whereas Ins$P_2$ does not [23,29].

Bombesin induced the release of $Ca^{2+}$ from an intracellular pool, as shown by its ability to elevate $[Ca^{2+}]_i$ when influx was inhibited by chelating extracellular $Ca^{2+}$. Our data are consistent with the hypothesis that the second messenger responsible for mobilizing intracellular $Ca^{2+}$ is Ins$P_3$: Ins$P_3$ accumulation and $[Ca^{2+}]_i$ elevation were both detected by 2–3 s, attained a peak by 15–30 s, and returned to near-basal values by 3–5 min. These changes are also sufficiently rapid to implicate Ins$P_3$ and $[Ca^{2+}]_i$ in the initial burst phase of insulin release, which began at 5 s and lasted for 1–2 min. In fact, the importance of the rise in $[Ca^{2+}]_i$ for the initial secretory response to bombesin is supported by the observation that pretreating cells with ionomycin blunted bombesin stimulation of both the $[Ca^{2+}]_i$ and insulin release. Similar results have been reported in pancreatic acinar cells, where the ability of bombesin to elevate $[Ca^{2+}]_i$ was also found to be independent of extracellular $Ca^{2+}$ [30]. In addition, pretreating acinar cells with carbachol, which appears to mobilize the same intracellular $Ca^{2+}$ store as bombesin, blunted bombesin's biological actions, suggesting that elevation of $[Ca^{2+}]_i$ was important for bombesin-induced amylase secretion [30]. Thus a rise in $[Ca^{2+}]_i$ owing to the release of $Ca^{2+}$ from cellular stores appears to be involved in bombesin stimulation of secretion by both the exocrine and endocrine pancreas.

$Ca^{2+}$ has long been believed to be a necessary and sufficient link in stimulus–secretion coupling [31]. Thus micromolar concentrations of $Ca^{2+}$ stimulate insulin secretion by permeabilized islets [32,33]. In addition, the $Ca^{2+}$ ionophores A23187 and ionomycin at 2–10 $\mu$M have been shown to stimulate insulin release by isolated islets, dispersed pancreatic cells and the RIN clonal insulin-secreting cell line [25,34,35]. These last studies contrast with the results presented here, which showed that treatment of HIT cells with 100 nM-ionomycin increased $[Ca^{2+}]_i$ to micromolar without stimulating insulin secretion. However, the ability of the high concentrations of ionophores used in previous studies to increase insulin release may be due to an action to permeabilize the plasma membrane, resulting in $Ca^{2+}$ influx. The observation that stimulation of secretion by high concentrations of ionophore is dependent on extracellular $Ca^{2+}$ in some systems [25,35] supports an effect at the plasma membrane. Thus the intracellular location of the rise in $Ca^{2+}$ may determine whether secretion is stimulated. Influx of $Ca^{2+}$ could generate a very high local $[Ca^{2+}]_i$ just inside the plasma membrane and thereby regulate membrane-bound enzymes that are important for secretion. In fact, we and others [36] have found that addition of 40 mM-$K^+$ to HIT cells results both in an increase in $[Ca^{2+}]_i$ as a consequence of stimulated $Ca^{2+}$ influx and in increased insulin release. Since ionomycin did not affect the secretory response to 40 mM-$K^+$ (S. L. Swope & A. Schonbrunn, unpublished work), the inability of ionomycin to stimulate secretion was not due to an effect of the ionophore to blunt $Ca^{2+}$ action. Therefore, our data indicate that, although $Ca^{2+}$ influx can stimulate

insulin release, elevation of $[Ca^{2+}]_i$ via mobilization of intracellular $Ca^{2+}$ is not sufficient to induce secretion.

An elevation of $[Ca^{2+}]_i$ is also not necessary for stimulation of insulin secretion, since TPA elicited a secretory response by HIT cells without altering $[Ca^{2+}]_i$. However, pretreatment with ionomycin blunted bombesin's effects on $[Ca^{2+}]_i$ and the burst phase of stimulated secretion. Therefore, the increase in $[Ca^{2+}]_i$ appears to play a modulatory role in the secretory response to bombesin.

Our results did not provide a value for $[Ca^{2+}]_i$ which could support a rapid secretory response to bombesin. Pretreatment with ionomycin blocked the effect of bombesin on $[Ca^{2+}]_i$ by 90% and blunted the initial rapid phase of secretion. By comparison, when extracellular $Ca^{2+}$ was chelated with EGTA, the $Ca^{2+}$ response to bombesin was inhibited by 40% and the secretory response was unaffected. Thus ionomycin appeared to be more effective than EGTA at inhibiting bombesin-stimulated elevation of $[Ca^{2+}]_i$ and insulin release. However, the percentage inhibition of bombesin action was calculated from the increase in $[Ca^{2+}]_i$ above the concentration present just before peptide addition. The absolute $[Ca^{2+}]_i$ attained in response to bombesin was similar ($P > 0.1$) after ionomycin ($0.58 \pm 0.06\,\mu M$) and EGTA ($0.79 \pm 0.09\,\mu M$) pretreatment. The fact that only ionomycin blunted the secretory response to bombesin suggests that the $[Ca^{2+}]_i$ present before the bombesin challenge may affect the subsequent response. Perhaps those intracellular targets which are regulated by $Ca^{2+}$ became desensitized by the ionomycin-induced elevation of $[Ca^{2+}]_i$. Conversely, after pretreatment with EGTA, $Ca^{2+}$-regulated proteins may become supersensitive to a subsequent elevation of $[Ca^{2+}]_i$. Thus HIT cells may respond differently to the same absolute $[Ca^{2+}]_i$, depending on prior conditions.

When cells were treated with bombesin, both $InsP_3$ and $[Ca^{2+}]_i$ returned to near-basal values by 5 min, although bombesin-induced insulin secretion continued for 90 min [7]. Furthermore, mimicking bombesin's effect on $[Ca^{2+}]_i$ with ionomycin did not increase the rate of insulin release. These results suggested that additional second messengers may be important in the secretory response to this peptide. Since [$^3$H]DAG was rapidly increased by bombesin, some of bombesin's actions are likely to involve activation of protein kinase C. The fact that TPA also stimulated insulin release by HIT cells demonstrated that activation of protein kinase C could induce secretion. A role for protein kinase C in bombesin action is supported in other target cells as well. In Swiss 3T3 fibroblasts, bombesin also increased DAG [12], and TPA and bombesin stimulated the phosphorylation of some of the same proteins [13].

Our results support an interaction between protein kinase C activation and an elevation of $[Ca^{2+}]_i$ in the secretory response of HIT cells to bombesin. Pharmacological activation of protein kinase C with TPA elicited a linear secretory response which resembled the second phase of bombesin-stimulated secretion. The response to TPA showed a lag of 90 s and was not accompanied by a rise in $[Ca^{2+}]_i$. Ionomycin elevated $[Ca^{2+}]_i$, did not by itself stimulate secretion, but potentiated the effect of TPA, resulting in a rapid secretory response. The ability of high $[Ca^{2+}]_i$ and protein kinase C activation to act synergistically has been demonstrated in diverse cell types, including platelets [27,37], pancreatic acini [38], a pituitary cell line [39], as well as pancreatic islets [35]. In these studies, neither elevation of $[Ca^{2+}]_i$ by a $Ca^{2+}$ ionophore nor pharmacological activation of protein kinase C can independently elicit the maximal cellular response observed with a physiological agonist. In most systems examined, TPA does not stimulate a rapid early response, but elicits a slow response [35,39,40] which does not require a rise in $[Ca^{2+}]_i$ [37,40]. In addition, elevation of $[Ca^{2+}]_i$ with ionophore often dramatically increases the response to TPA at early times [37,39,40]. Thus the ability of TPA plus ionomycin to elicit a rapid early secretory response in HIT cells agrees with the action of this combination of drugs in a number of other systems and supports the involvement of both DAG and elevated $[Ca^{2+}]_i$ in bombesin action.

Our conclusion that an elevation of $[Ca^{2+}]_i$ and activation of protein kinase C work in concert to mediate the secretory response to bombesin is in conflict with the proposed mechanism of bombesin action in some other systems. While the experiments presented here were in progress, bombesin treatment of Swiss 3T3 cells was shown to cause polyphosphoinositide breakdown as well as generation of inositol polyphosphate and DAG and elevation of $[Ca^{2+}]_i$ [11,12]. One group has proposed that the action of bombesin to increase binding of $^{125}$I-labelled epidermal growth factor in Swiss 3T3 cells is mediated by an elevation of $[Ca^{2+}]_i$ [41], whereas others have concluded that it is due entirely to activation of protein kinase C [13]. Even less is known about the mechanism by which the rapid generation of different intracellular messengers upon bombesin stimulation is linked to the mitogenic response which is measured hours to days later. For Swiss 3T3 cells the cellular increase in inositol polyphosphates and $[Ca^{2+}]_i$ in response to bombesin was transient. However, DAG concentrations remained elevated for 60 min, the longest time examined. In addition, TPA mimicked the mitogenic effect of bombesin and, like bombesin, was synergistic with insulin in stimulating DNA synthesis [12]. These results suggest that a prolonged activation of protein kinase C rather than a transient elevation of $[Ca^{2+}]_i$ may mediate the mitogenic effect of bombesin in 3T3 cells: however, this remains to be critically tested.

In summary, the data presented here support the involvement of polyphosphoinositide metabolism in the action of bombesin to stimulate insulin secretion by a pancreatic $\beta$-cell line. Our results are consistent with the increased $InsP_3$ formed in response to bombesin acting to mobilize an intracellular pool of $Ca^{2+}$. The resulting elevation of $[Ca^{2+}]_i$ is important for the initial rapid phase of insulin release. However, release of this intracellular pool is not sufficient for stimulation of secretion, but acts together with activation of protein kinase C to elicit a rapid secretory response. Further, whereas an elevation of $[Ca^{2+}]_i$ was necessary for the initial burst of secretion, the bombesin-induced activation of protein kinase C via the generation of DAG appears sufficient to mediate the second, slower, secretory response.

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