Early Events Elicited by Bombesin and Structurally Related Peptides in Quiescent Swiss 3T3 Cells. II. Changes in Na^+ and Ca^{2+} Fluxes, Na^+/K^+ Pump Activity, and Intracellular pH

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Abstract. The amphibian tetradecapeptide, bombesin, and structurally related peptides caused a marked increase in ouabain-sensitive $^{86}Rb^+$ uptake (a measure of Na^+/K^+ pump activity) in quiescent Swiss 3T3 cells. This effect occurred within seconds after the addition of the peptide and appeared to be mediated by an increase in Na^+ entry into the cells. The effect of bombesin on Na^+ entry and Na^+/K^+ pump activity was concentration dependent with half-maximal stimulation occurring at 0.3–0.4 nM. The structurally related peptides litorin, gastrin-releasing peptide, and neuromedin B also stimulated ouabain-sensitive $^{86}Rb^+$ uptake; the relative potencies of these peptides in stimulating the Na^+/K^+ pump were comparable to their potencies in increasing DNA synthesis (Zachary, I., and E. Rozengurt, 1985, *Proc. Natl. Acad. Sci. USA.*, 82:7616–7620). Bombesin increased Na^+ influx, at least in part, through an Na^+/H^+ antiport. The peptide augmented intracellular pH and this effect was abolished in the absence of extracellular Na^+. In addition to monovalent ion transport, bombesin and the structurally related peptides rapidly increased the efflux of $^{45}Ca^{2+}$ from quiescent Swiss 3T3 cells. This Ca^{2+} came from an intracellular pool and the efflux was associated with a 50% decrease in total intracellular Ca^{2+}. The peptides also caused a rapid increase in cytosolic free calcium concentration.

Prolonged pretreatment of Swiss 3T3 cells with phorbol dibutyrate, which causes a loss of protein kinase C activity (Rodriguez-Pena, A., and E. Rozengurt, 1984, *Biochem. Biophys. Res. Commun.*, 120:1053–1059), greatly decreased the stimulation of $^{86}Rb^+$ uptake and Na^+ entry by bombesin implicating this phosphotransferase system in the mediation of part of these responses to bombesin. Since some activation of monovalent ion transport by bombesin was seen in phorbol dibutyrate–pretreated cells, it is likely that the peptide also stimulates monovalent ion transport by a second mechanism.

THE amphibian tetradecapeptide, bombesin, is a potent mitogen for quiescent Swiss 3T3 cells. The peptide stimulates DNA synthesis at nanomolar concentrations in the absence of other exogenous growth factors (52). In addition, peptides which have close homology to the carboxyl-terminal heptapeptide of bombesin, including gastrin-releasing peptide (GRP[1–27]),[1] neuromedin B, and litorin (see reference 69 for structures), are also mitogenic for Swiss 3T3 cells. These peptides bind to a novel class of specific, high-affinity receptors (70), and they stimulate a variety of biochemical events which precede the onset of DNA synthesis. The preceding paper (69) reports that peptides of the bombesin family stimulate the phosphorylation of an M_r 80,000 cellular protein (designated 80k) which reflects the activation of the Ca^{2+}-sensitive, phospholipid-dependent protein kinase (protein kinase C) (54, 55) and decreases the affinity of the epidermal growth factor receptor for its ligand (69).

Rapid changes in the fluxes and intracellular concentration of monovalent and divalent cations may play a role in the mitogenic signalling of many growth factors (for reviews see references 49, 51). The mitogens stimulate Na^+ entry into Swiss 3T3 cells (8, 13, 24, 28, 29, 56, 57, 65) by an amiloride-

1. *Abbreviations used in this paper:* ^{14}C-DMO, 5,5-dimethyl [2-^{14}C] oxazolidine-2,4-dione; $[Ca^{2+}]_i$, cytoplasmic free calcium concentration; DME, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GRP(1–16), NH_2-terminal fragment of gastrin-releasing peptide; GRP(1–27), gastrin-releasing peptide; PBt_2, phorbol 12,13-dibutyrate; PDGF, platelet-derived growth factor; pH_i, intracellular pH; SP, substance P.

sensitive Na^+/H^+ antiport. This increases intracellular Na^+ and causes cytoplasmic alkalinization (7–9, 24, 56, 65). Since the activity of the Na^+/K^+ pump is regulated by intracellular Na^+ (28, 57), there is a secondary stimulation of Na^+/K^+ pump activity, increasing intracellular K^+ and restoring the electrochemical gradient for Na^+ (8, 13, 23, 24, 28, 29, 50, 56, 57, 65). Rapid changes of Na^+, K^+, and H^+ fluxes occur in a variety of other cell types following a mitogenic signal (2, 15, 19, 20, 32, 33, 35, 36, 39, 41–43). In addition, treatment of quiescent Swiss 3T3 cells with various mitogens causes a rapid efflux of $^{45}Ca^{2+}$ from cells preloaded with the isotope (21, 22). This results in a marked decrease (~50%) in total intracellular Ca^{2+}. Mitogenic stimulation of a variety of cell types causes an increase in Ca^{2+} efflux measured isotopically (4, 40) or in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) measured with the fluorescent Ca^{2+} indicator quin 2 (31, 34, 37, 60). In the present paper, we report that the peptides of the bombesin family, a novel class of mitogens (52, 70), induce marked changes in Na^+ and Ca^{2+} fluxes, Na^+/K^+ pump activity, and intracellular pH. The effects occurred within seconds, and were also elicited by structurally related peptides including litorin, GRP(1–27), and neuromedin B, and were partially inhibited by the bombesin antagonist (D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]) substance P (SP).

Materials and Methods

Cell Culture

Cultures of Swiss 3T3 cells (61) were grown at 37°C in humidified 10% CO_2/90% air in Dulbecco's modified Eagle's medium (DME) containing 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml). For experiments, 10^5 cells were subcultured into 33-mm Nunc Petri dishes with DME containing 10% FBS. These cultures were used 6–8 d later at a time when the cells were confluent and quiescent as judged by cytofluorometric and autoradiographic analysis (12).

Measurements of $[Ca^{2+}]_i$ were made on quiescent Swiss 3T3 cells grown on Cytodex 2 beads. For these experiments, Cytodex 2 beads were allowed to swell in phosphate-buffered saline (PBS) for at least 4 h, rinsed twice with PBS, and autoclaved in siliconized glass bottles. 2.5×10^7 cells were seeded into a flask containing 750 mg of Cytodex 2 beads and 160 ml of DME supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% FBS. The mixture of cells, beads, and medium was gassed with 10% CO_2/90% air, maintained at 37°C, and stirred intermittently (2 min on, 30 min off). On the following day, the medium volume was increased to 500 ml and the mixture was re-gassed with 10% CO_2/90% air and stirred continuously at the slowest rate which prevented the beads from settling (~25 rpm). 5–7 d later, the medium was replaced with DME containing 1% FBS. After an additional 24 h, the cells were quiescent as indicated by the increase in [^{3}H]thymidine incorporation after the addition of purified mitogens in serum-free medium (data not shown).

$^{86}Rb^+$ Uptake

The uptake of $^{86}Rb^+$ (a K^+ tracer) was measured mostly as described previously (50, 57). Confluent and quiescent cultures of Swiss 3T3 cells in 33-mm dishes were washed two times with DME at 37°C. After incubation of variable length (see individual experiments) in 2 ml of experimental media, 30–50 μl of $^{86}RbCl$ containing $4–6 \times 10^6$ cpm were added. After 10 min, the cultures were washed rapidly six times with 0.1 M $MgCl_2$ at 4°C (58). The radioactivity was extracted with 5% trichloroacetic acid and assayed in a liquid scintillation counter to measure Cerenkov radiation. Ouabain-sensitive $^{86}Rb^+$ uptake was calculated by subtracting $^{86}Rb^+$ uptake in the presence of 2 mM ouabain from $^{86}Rb^+$ uptake in the absence of the inhibitor and was taken as a measure of Na^+/K^+ pump activity.

$^{22}Na^+$ Uptake

Confluent and quiescent Swiss 3T3 cells in 33-mm dishes were washed twice at 37°C with a modified DME in which the NaCl (110 mM) was replaced by either 220 mM sucrose or 110 mM choline chloride (see individual experiments). The cultures were incubated in modified DME at 37°C for 5 min in the presence of various additions as indicated in the individual experiments. The replacement of NaCl with sucrose or choline chloride increased the specific activity of the isotope by reducing the concentration of Na^+ in the medium to 50 mM. All media contained 2 mM ouabain to inhibit $^{22}Na^+$ efflux via the Na^+/K^+ pump. The cultures were incubated with a tracer amount of $^{22}Na^+$ ($6–8 \times 10^6$ cpm/ml) for 3 min during which $^{22}Na^+$ uptake by 3T3 cells is linear (57). The uptake reaction was stopped by washing each dish rapidly six times with 0.1 M $MgCl_2$ at 4°C (10 s) and the radioactivity was extracted and measured as described (57).

Intracellular Na^+ Content

The intracellular Na^+ content was measured essentially as previously described (28, 29, 57, 58). After treatment, the dishes were washed as described above for stopping the uptake of $^{22}Na^+$. When the dishes were dry, 0.5 ml of 5% trichloroacetic acid containing 15 mM LiCl was added to each dish. Cell Na^+ was measured in a flame photometer (Corning EEL, Model 430) with the LiCl serving as the internal standard.

Intracellular pH

Intracellular pH (pH_i) was measured with the weak acid 5,5 dimethyl [2-^{14}C] oxazolidine-2,4-dione (^{14}C-DMO) using a modification of a previously described method (8, 24, 56). Briefly, cultures were washed with Solution B (see legend for Fig. 5) and then incubated at 37°C for 1 h in that solution with or without bombesin. After this incubation, 75 μl of ^{14}C-DMO (final concentration 150 μM and $3–4 \times 10^6$ cpm/dish) were added to the cultures. After an additional 15 min, the medium was aspirated and the cultures were washed rapidly (the washing of each dish taking 5–5.5 s) by sequentially dipping the dish once in each of four beakers containing 200 ml of 0.1 M $MgCl_2$/10 mM Tris-HCl (pH 7.0) at 4°C. The monolayers were then dried and the cells solubilized with 0.6 ml of 0.1 N NaOH/2% Na_2CO_3/0.1% SDS. The radioactivity was measured using a liquid scintillation spectrometer. Protein was determined as described by Lowry et al. (25).

pH_i was calculated from extracellular pH, ^{14}C-DMO in washed monolayers and media, protein, and intracellular water using the formula of Waddell and Butler (67). Correction for ^{14}C-DMO trapped in extracellular water was made by subtracting the radioactivity remaining in pre-cooled cultures washed immediately after addition of ^{14}C-DMO. Four of five replicates were determined for each experimental value. A pKa of 6.3 was used for calculations. Intracellular water was determined using ^{14}C-urea as previously described (8, 24).

$^{45}Ca^{2+}$ Efflux

Quiescent cultures of Swiss 3T3 cells grown on 33-mm Nunc Petri dishes were incubated with $^{45}Ca^{2+}$ for 12–24 h in conditioned medium containing 4–8 μCi/ml of $^{45}Ca^{2+}$. Efflux of $^{45}Ca^{2+}$ was carried out at 37°C in an atmosphere of 10% CO_2/90% air into 2 ml of DME after removing the isotope from the medium by washing as described in the individual experiments. At the times indicated, 200-μl samples were removed from the medium and replaced by 200 μl of fresh medium. At the end of the efflux period, cultures were washed rapidly seven times with DME at 37°C and the radioactivity remaining in the cells was extracted with 0.1 M NaOH/2% Na_2CO_3/1% SDS. The sum of the radioactivity in each sample plus the radioactivity remaining in the cells at the end of the efflux period was taken as the total $^{45}Ca^{2+}$ in the cells at the beginning of the efflux period.

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

$[Ca^{2+}]_i$ was measured with the fluorescent Ca^{2+} indicator, quin 2, using a modification of the procedure of Tsien et al. (62, 63). At the beginning of the experiment, quiescent cells on Cytodex 2 beads were washed twice with serum-free DME. The beads were then resuspended in 180 ml of DME by gentle shaking. 10-ml aliquots were transferred to plastic tubes and incubated for 45 min at 37°C with 15 μM quin 2 tetraacetoxymethyl ester. The stock solution of quin 2 tetraacetoxymethyl ester was 7.5 mM dissolved in dimethyl sulfoxide. After this incubation, the beads were washed three times with Solution A, which contained 140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.9 mM $MgCl_2$, 25 mM glucose, 16 mM Hepes, 6 mM Tris, and a mixture of amino acids at the same concentrations as those in DME (pH 7.20). The beads were suspended in Solution A (final volume, 2.0 ml) and transferred to a 1-cm^2 quartz cuvette. The suspension was stirred continuously and maintained at 37°C. Fluorescence was monitored in a Perkin-Elmer LS-5 luminescence spectrometer with an excitation wavelength of 339 nm and an emission wavelength of 492 nm.

$[Ca^{2+}]_i$ was calculated using the formula of Tsien et al. (62),

$$[Ca^{2+}]_i \text{ in nM} = 115 \frac{(F - F_{min})}{(F_{max} - F)},$$

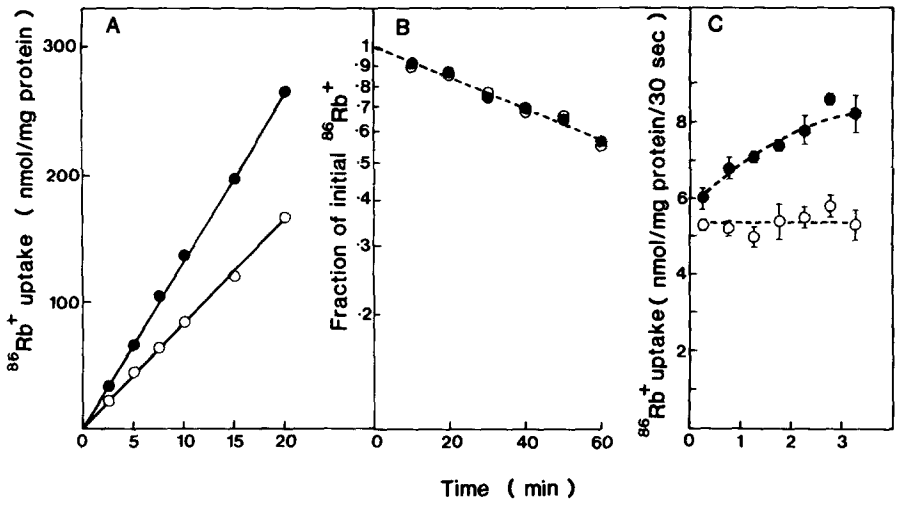

Figure 1. Bombesin stimulates $^{86}Rb^+$ influx but does not affect $^{86}Rb^+$ efflux. Quiescent cultures of Swiss 3T3 cells were incubated in DME containing trace amounts of $^{86}Rb^+$ (2.5×10^6 cpm/ml; 5.4 mM K^+) either with (solid circles) or without (open circles) 6.2 nM bombesin (*A*). The incubations were at 37°C in an atmosphere of 90% air/10% CO_2 for the times indicated. The results shown are the mean of duplicate cultures. For studies of $^{86}Rb^+$ efflux (*B*), quiescent 3T3 cells were loaded with isotope by adding 10^6 cpm of $^{86}Rb^+$ per ml of culture medium for 4 h. The cultures were then rapidly washed three times with prewarmed DME and incubated at 37°C in 2 ml of DME either with (solid circles) or without (open circles) 6.2 nM bombesin. At the times indicated, the remaining intracellular $^{86}Rb^+$ was measured as described in Materials and Methods. The radioactivity still present at each time is expressed as a fraction of zero time radioactivity (15,700 cpm per 33-mm dish). The cellular protein varied from 90 to 100 μg per dish and was corrected for in each instance. To study the kinetics (*C*), the growth medium of quiescent cultures of 3T3 cells was replaced with DME with (solid circles) or without (open circles) 6.2 nM bombesin at time zero. $^{86}Rb^+$ (2.5×10^6 cpm/ml) was added for 30-s intervals (plotted at the midpoint of that interval) and the uptake reaction terminated as described in Materials and Methods. The data represent mean ± SEM; $n = 3$.

where F is the fluorescence at the unknown $[Ca^{2+}]_i$; F_{max} is the fluorescence after the trapped quin 2 is released by the addition of 0.02% Triton X-100; and F_{min} is the fluorescence remaining after the Ca^{2+} in the solution is chelated with 100 mM EGTA.

Statistics were calculated using either Student's t test or the rank sum test. The data are presented as mean ± SEM.

Materials

Bombesin, litorin, bovine serum albumin (essentially fatty acid and globulin free), ouabain, and phorbol 12,13-dibutyrate (PBt_2) were obtained from Sigma Chemical Co. (St. Louis, MO). The NH_2-terminal fragment of gastrin-releasing peptide, GRP(1–16), and neuromedin B were from Peninsula Laboratories, Inc. (Belmont, CA). GRP(1–27) and (D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]) SP were obtained from Bachem (Saffron Walden, Essex, U.K.). Highly purified porcine platelet-derived growth factor (PDGF) was obtained from Bioprocessing Ltd. (Durham, U.K.). FBS was purchased from Gibco Europe (U.K.). Quin 2 and quin 2 tetraacetoxymethyl ester were obtained from Lancaster Synthesis (Morcambe, Lancs, U.K.). $^{22}Na^+$, $^{86}Rb^+$, and $^{45}Ca^{2+}$ were obtained from the Radiochemical Centre (Amersham, U.K.). ^{14}C-Urea and ^{14}C-DMO were from New England Nuclear (Boston, MA). All other chemicals were reagent grade.

Results

Bombesin Stimulation of $^{86}Rb^+$ Uptake: Time Course and Ouabain Sensitivity

Addition of 6.2 nM bombesin to confluent and quiescent cultures of Swiss 3T3 cells caused a marked increase (70 ± 3%; $n = 49$) in the initial rate of total $^{86}Rb^+$ uptake. The uptake of this cation was linear for at least 20 min either with or without bombesin (Fig. 1*A*). To measure $^{86}Rb^+$ efflux, quiescent cultures of Swiss 3T3 cells were incubated with $^{86}Rb^+$ for 4 h. Then, the cultures were washed and transferred to medium without $^{86}Rb^+$ either in the absence or presence of 6.2 nM bombesin. This peptide had no effect on the rate of $^{86}Rb^+$ efflux from these cells (Fig. 1*B*). To determine the time course of stimulation of $^{86}Rb^+$ uptake by bombesin, quiescent cultures of Swiss 3T3 cells were incubated in the presence of the peptide for various times before the addition of the isotope. The uptake reaction was terminated after 30 s of further incubation. Fig. 1*C* shows that the rate of $^{86}Rb^+$ uptake increased within seconds after bombesin addition and reached a maximum after ~2 min. The stimulation of $^{86}Rb^+$ uptake by bombesin was mediated by the Na^+/K^+ pump since it was virtually abolished by the specific inhibitor ouabain (Fig. 2). The concentration dependence of ouabain for inhibiting $^{86}Rb^+$ uptake was the same with and without bombesin stimulation.

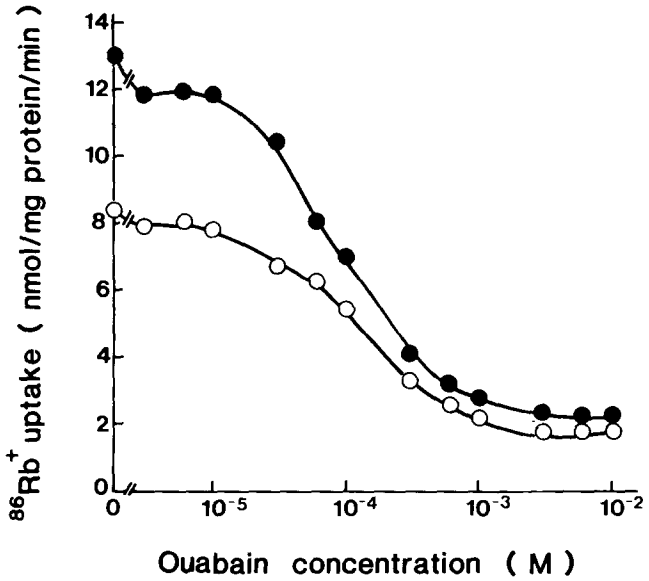

Figure 2. Inhibition of $^{86}Rb^+$ uptake as a function of ouabain concentration in the absence or presence of bombesin. Quiescent cultures of Swiss 3T3 cells were incubated in DME with the indicated concentration of ouabain either with (solid circles) or without (open circles) 6.2 nM bombesin. After 5 min, $^{86}Rb^+$ (2×10^6 cpm/ml) was added and the incubation continued for an additional 10 min. Experimental details are described in Materials and Methods. Each point is the average of duplicates.

Bombesin Enhancement of Na^+ Entry and Na^+/H^+ Antiport Activity

The activity of the Na^+/K^+ pump in intact and quiescent fibroblasts is limited and regulated by the availability of cytosolic Na^+ (for review see reference 51). A variety of mitogens including serum (28, 57), vasopressin (29), phorbol esters (13), and PDGF (24) stimulate the Na^+/K^+ pump by increasing Na^+ entry into the cell. To test whether bombesin stimulates the Na^+/K^+ pump in a similar way, the Na^+ content of quiescent 3T3 was measured after incubation with varying concentrations of bombesin in the presence of ouabain. The rate of $^{86}Rb^+$ influx was measured in parallel cultures of quiescent 3T3 cells at identical concentrations of bombesin. Fig. 3 shows that the concentration dependence of the increase in intracellular Na^+ and the stimulation of $^{86}Rb^+$ uptake were similar. A half-maximal increase in Na^+ content was seen at 0.3 nM bombesin and a half-maximal increase of

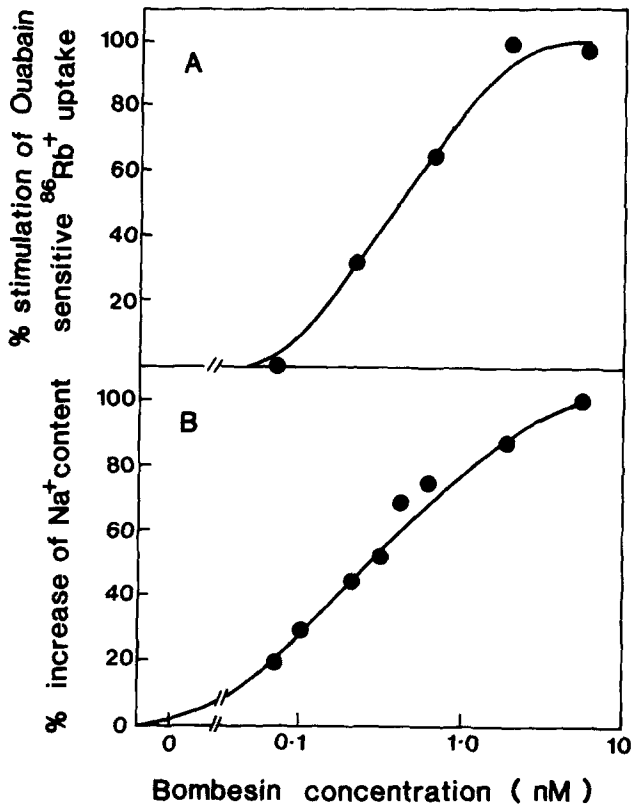

Figure 3. Effect of bombesin concentration on ouabain-sensitive $^{86}Rb^+$ uptake and intracellular Na^+ content. (*A*) Quiescent Swiss 3T3 cells were incubated at 37°C for 30 min with varying concentrations of bombesin in DME, both with and without 2 mM ouabain. $^{86}Rb^+$ (3×10^6 cpm/ml) was then added for 10 min and the uptake reaction terminated as described in Materials and Methods. Each point is the average of two samples. (*B*) Parallel cultures were incubated with varying concentrations of bombesin in DME for 30 min at which time ouabain (2 mM) was added. After an additional 30 min at 37°C, the intracellular Na^+ content was measured as described in Materials and Methods. Each point is the mean of four measurements except for the cultures without bombesin where eight dishes were used. The Na^+ content in the absence of ouabain was 50 nmol/mg protein. This value was subtracted from the results in the presence of ouabain to give 190 nmol Na^+ per milligram protein in the absence of bombesin and 290 nmol Na^+ per milligram protein at 6.2 nM bombesin.

$^{86}Rb^+$ uptake occurred at 0.4 nM bombesin. The effect of bombesin on Na^+ entry was also assessed by measuring the uptake of $^{22}Na^+$ from a solution containing 50 mM Na^+ and 2 mM ouabain with choline chloride as the osmotic replacement. Bombesin (6.2 nM) increased $^{22}Na^+$ uptake from 19.7 ± 0.8 to 27.9 ± 1.0 nmol Na^+/mg protein per min ($n = 6$).

Na^+ entry into Swiss 3T3 cells occurs, at least in part, through an electroneutral Na^+/H^+ antiport system (56). The activity of the antiport is stimulated by a variety of mitogens (7, 9, 24, 56, 65). If bombesin stimulates the Na^+/H^+ antiport, an increase in pH_i should occur simultaneously with the increase in Na^+ entry. To test this, we determined the distribution of ^{14}C-DMO between the extra- and intracellular spaces in quiescent 3T3 cells with and without bombesin and related peptides. The measurements were performed in an electrolyte solution containing 50 mM Na^+ with sucrose as the osmotic replacement and lacking HCO^-_3, thereby eliminating Cl^-/HCO_3^- exchange. The intracellular water (determined with ^{14}C-urea) did not change significantly in bombesin-treated cells. Bombesin increased ^{14}C-DMO uptake and pH_i. In seven experiments, pH_i increased by 0.12 ± 0.01 (Table I). Bombesin also increased pH_i by 0.10–0.15 U when the peptide was added to solutions containing 140 mM Na^+ (data not shown). Under identical experimental conditions, addition of epidermal growth factor (5 ng/ml), insulin (1 μg/ml), and vasopressin (20 ng/ml), a mitogenic combination that induces maximal cytoplasmic alkalinization in Swiss 3T3 cells (7, 8), increased pH_i by 0.21 ± 0.014 U.

To assess the role of the Na^+/H^+ antiport in bombesin-induced increases in pH_i, measurements of intracellular pH were carried out in the absence of extracellular Na^+ (Table I). When Na^+ was omitted from the bathing solution, bombesin did not cause cytoplasmic alkalinization, indicating that the bombesin-induced loss of H^+ from the cell requires extracellular Na^+. In fact, in the absence of extracellular Na^+, bombesin caused a significant fall in intracellular pH. It is possible that this results from an association between bombesin-mediated Ca^{2+} efflux and H^+ uptake. 50 nM A23187, which increases $[Ca^{2+}]_i$ as much as 6.2 nM bombesin (see below), had no significant effect on pH_i in the presence of Na^+ but markedly decreased pH_i in the absence of extracellular Na^+ (by 0.45 ± 0.06 U; $n = 5$). The fact that intracellular alkalinization by bombesin requires extracellular Na^+ provides strong support for the hypothesis that the peptide stimulates the activity of the Na^+/H^+ antiport.

Table I. Effect of Bombesin on Intracellular pH When Extracellular Na^+ was 50 mM or 0

	pH_i		
Extracellular Na^+	Control	Bombesin	P
50 mM ($n = 35$)	7.109 ± 0.015	7.228 ± 0.019	< 0.001
0 ($n = 15$)	6.927 ± 0.030	6.693 ± 0.030	< 0.001
P	< 0.001	< 0.001	

In these experiments, quiescent cultures of Swiss 3T3 cells were washed and incubated in either Solution B (see legend to Fig. 5) or Solution B in which the NaCl was replaced with an additional 100 mM sucrose for 1 h in the presence or absence of 6.2 nM bombesin. After this incubation, ^{14}C-DMO was added and intracellular pH measured as described in Materials and Methods. The data presented as mean ± SEM. n, number of separate cultures studied representing seven independent experiments at 50 mM Na^+ and three independent experiments at 0 Na^+.

Stimulation of $^{86}Rb^+$ Uptake and Increase in pH_i by Bombesin-like Peptides

The concentration dependence of the stimulation of $^{86}Rb^+$ uptake by bombesin and structurally related peptides is shown in Fig. 4. The concentration of bombesin required for 50% stimulation was 0.31 nM. This is virtually identical to the concentration (0.3 nM) required for half-maximal stimulation of DNA synthesis by this peptide in the presence of insulin (52). Litorin (5, 45), GRP(1–27) (27, 48), and neuromedin B (30), which share with bombesin a common carboxyl-terminal heptapeptide (see reference 69 for sequences), also stimulated $^{86}Rb^+$ in quiescent 3T3 cells; the concentrations of litorin, GRP(1–27), and neuromedin B that produced half-maximal stimulation were 0.19 nM, 2.2 nM, and 11 nM, respectively (Fig. 4). In contrast, GRP(1–16), which has no homology with bombesin, did not stimulate $^{86}Rb^+$ uptake above basal levels (data not shown).

Intracellular pH was also increased by GRP(1–27), litorin, and neuromedin B, but not by GRP(1–16) (Fig. 5). Thus, the ability of bombesin analogues to induce intracellular alkalinization correlated with their ability to stimulate $^{86}Rb^+$ uptake (see above) as well as with their ability to bind to the specific high-affinity receptors and to cause DNA synthesis in quiescent Swiss 3T3 cells (70). The effect of the peptide (D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]) SP (26), which is a bombesin antagonist in other systems (16, 68) and which inhibits the binding of bombesin to its specific receptor and bombesin-stimulated DNA synthesis in Swiss 3T3 cells (70), was tested. This peptide did not alter basal pH_i or the increment in pH_i induced by PBt_2 (results not shown). It did, however, partially but significantly ($P < 0.01$) inhibit the increase in pH_i caused by bombesin (Fig. 5). This supports the conclusion that bombesin and related peptides increase pH_i after binding to their high-affinity receptor.

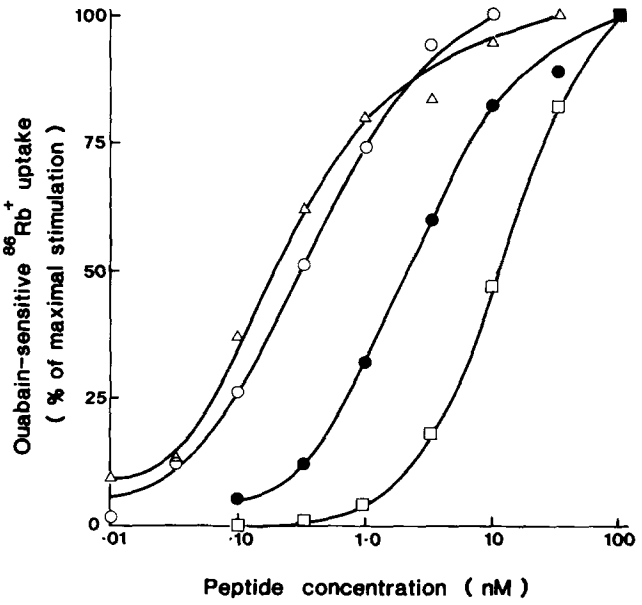

Figure 4. Stimulation of ouabain-sensitive $^{86}Rb^+$ uptake by bombesin-related compounds. Quiescent cultures of Swiss 3T3 cells were incubated for 30 min at 37°C in DME containing the concentration of peptide indicated. $^{86}Rb^+$ (2.5×10^6 cpm/ml) was then added, the incubation was continued for an additional 10 min, and $^{86}Rb^+$ uptake measured as described in Materials and Methods. Ouabain-sensitive $^{86}Rb^+$ uptake was determined by subtracting the $^{86}Rb^+$ uptake in the presence of 2 mM ouabain from that without ouabain. The peptides studied included bombesin (open circles), litorin (open triangles), GRP(1–27) (solid circles), and neuromedin B (squares). The results are the percent of the maximum stimulation by that peptide. The values shown are the mean of three experiments. The mean maximum ouabain-sensitive $^{86}Rb^+$ uptake in nmol/ng protein per minute was 8.9 with bombesin, 9.6 with litorin, 8.7 with GRP(1–27), and 8.4 with neuromedin B.

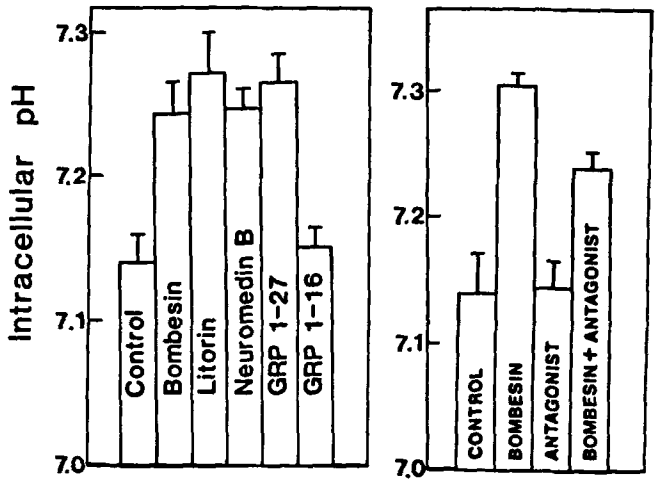

Figure 5. Effect of bombesin agonists and antagonists on intracellular pH. (*Left*) In this experiment, quiescent Swiss 3T3 cells were washed twice, and then incubated for 1 h in Solution B containing the indicated addition. Solution B contained 50 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.9 mM $MgCl_2$, 25 mM glucose, 200 mM sucrose, 16 mM Hepes, 6 mM Tris base (pH 7.0). The peptide concentrations were bombesin, 6.2 nM; litorin, 12 nM; GRP(1–27), 20 nM; neuromedin B, 40 nM; GRP(1–16), 1 μM. After this incubation, [^{14}C]-DMO was added for an additional 15 min and intracellular pH measured as described in Materials and Methods. (*Right*) In this experiment, quiescent cultures of Swiss 3T3 cells were washed twice and incubated for 1 h in Solution B containing the indicated addition. The bombesin concentration was 6.2 nM. The concentration of (D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]) SP (*Antagonist*) was 100 μM. After this incubation, [^{14}C]-DMO was added for an additional 15 min and intracellular pH measured as described in Materials and Methods.

Bombesin Stimulation of $^{45}Ca^{2+}$ Efflux: Kinetics and Effect of Bombesin-like Peptides

Quiescent cultures of Swiss 3T3 cells were incubated with $^{45}Ca^{2+}$ for 12–24 h, washed with nonradioactive DME containing 1.8 mM Ca^{2+}, and then transferred to this medium in the presence or absence of 6.2 nM bombesin. The peptide increased the rate of $^{45}Ca^{2+}$ efflux at the earliest time point (15 s) after the addition of bombesin (Fig. 6*A*). In cultured cells, there is a rapidly exchanging pool of Ca^{2+} which is extracellularly bound. This pool can be eliminated almost completely in Swiss 3T3 cells by washing the cultures with DME containing 3 mM EGTA (21, 22). Washing the cultures in this manner did not affect the stimulation of $^{45}Ca^{2+}$ efflux by bombesin (Fig. 6*B*). Thus, bombesin appeared to stimulate the efflux of $^{45}Ca^{2+}$ from an intracellular pool(s). The efflux of $^{45}Ca^{2+}$ was also measured in cultures washed and incubated in Ca^{2+}-free medium containing 50 μM EGTA. Since bombesin stimulated $^{45}Ca^{2+}$ efflux under these conditions (Fig. 6*C*), the peptide increases $^{45}Ca^{2+}$ efflux by a mechanism other

than $^{45}Ca^{2+}/^{40}Ca^{2+}$ exchange.

If bombesin stimulates net $^{45}Ca^{2+}$ efflux, it should decrease the steady-state level of $^{45}Ca^{2+}$ in Swiss 3T3 cells labeled to specific activity equilibrium. Indeed, when 6.2 nM bombesin was added to such cells, there was a rapid decrease in the $^{45}Ca^{2+}$ content of the cultures (Fig. 7*A*). This decrease was measurable within 1 min after the addition of bombesin, and cultures were maximally depleted of $^{45}Ca^{2+}$ after 2 min (50% decrease). This effect persisted for at least 1 h. Thus, the addition of bombesin caused a decrease in total intracellular Ca^{2+} in quiescent Swiss 3T3 cells.

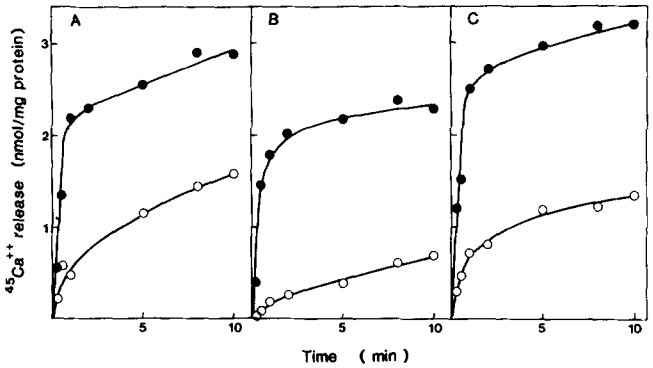

Figure 6. Bombesin-stimulated $^{45}Ca^{2+}$ efflux from quiescent cultures of Swiss 3T3 cells. Quiescent Swiss 3T3 cells in 33-mm dishes were incubated for 12 to 24 h in conditioned media containing 4 μCi/ml of $^{45}Ca^{2+}$. At the start of the experiment, the radioactive medium was aspirated and the cultures were rapidly washed as follows: seven times with 2 ml DME at 37°C (*A*); seven times with 2 ml of DME containing 3 mM EGTA at 37°C (*B*), and seven times with 2 ml of Ca^{2+}-free DME containing 50 μM EGTA, at 37°C (*C*). The cultures were subsequently incubated with 2 ml of DME (*A* and *B*) or 2 ml of Ca^{2+}-free DME with 50 μM EGTA (*C*), either in the absence (open circles) or the presence (solid circles) of 6.2 nM bombesin. The efflux of $^{45}Ca^{2+}$ was measured as described in Materials and Methods.

The effect of various concentrations of bombesin and structurally related peptides is shown in Fig. 7*B*. Half-maximal stimulation of $^{45}Ca^{2+}$ efflux occurred at the following concentrations: 0.16 nM litorin, 0.3 nM bombesin, 2.5 nM GRP(1–27), and 4.6 nM neuromedin B.

Since in some cell types Ca^{2+} efflux occurs in part by a pathway dependent on external Na^+ (1), $^{45}Ca^{2+}$ efflux was measured in cultures washed with and incubated in a Na^+-free electrolyte solution. Bombesin stimulated $^{45}Ca^{2+}$ efflux into an Na^+-free solution (Fig. 7*C*). Thus, Na^+-dependent Ca^{2+} efflux does not appear to be a major component of bombesin-stimulated $^{45}Ca^{2+}$ efflux in Swiss 3T3 cells.

Effect of Bombesin on Intracellular Ca^{2+} Concentration

The fluorescent indicator, quin 2, was used to estimate intracellular Ca^{2+} concentration. In quiescent Swiss 3T3 cells, $[Ca^{2+}]_i$ was 207 ± 9 nM (n = 139). This value was similar to those reported previously in fibroblasts (37). Bombesin caused an increase in $[Ca^{2+}]_i$ which occurred within 5 s after the addition of the peptide and was maximal in 15–30 s (Fig. 8, left). The effect of bombesin was transient with $[Ca^{2+}]_i$ usually returning to control levels in 3–5 min. Bombesin increased $[Ca^{2+}]_i$ in a concentration-dependent manner (Fig. 8, upper right). At concentrations above 6 nM, bombesin augmented $[Ca^{2+}]_i$ by threefold. The Ca^{2+} ionophore A23187 at 25 nM caused an increase in $[Ca^{2+}]_i$ similar to a submaximal response to bombesin (peak $[Ca^{2+}]_i$/control $[Ca^{2+}]_i$ = 2.18 ± 0.13; n = 6). At 50 nM, the effect of the ionophore was comparable to a maximal bombesin response (peak $[Ca^{2+}]_i$/control $[Ca^{2+}]_i$ = 3.14 ± 0.22; n = 6). Thus, 25–50 nM A23187 can be used to assess the role of changes in $[Ca^{2+}]_i$ in Swiss 3T3 cells.

Although the studies on $^{45}Ca^{2+}$ efflux indicated that bom-

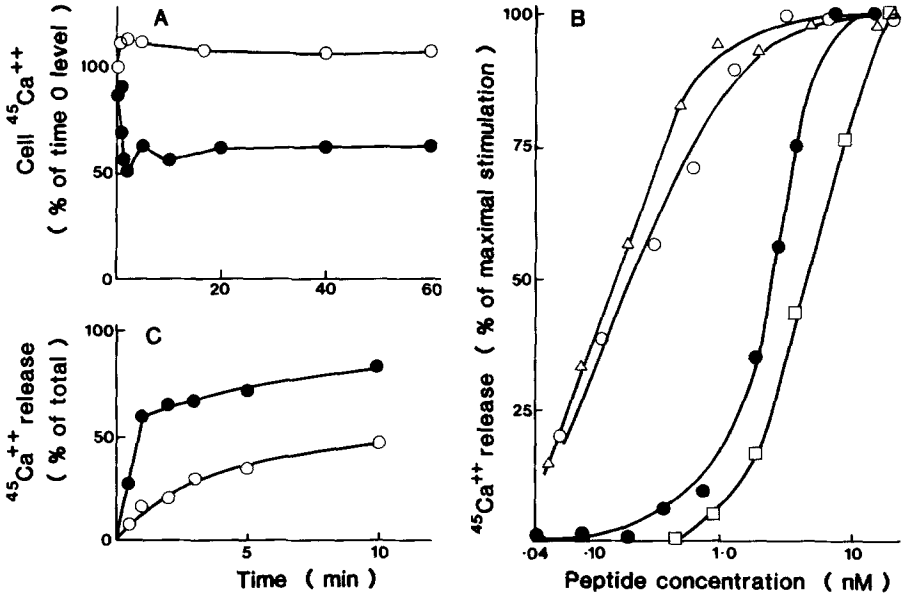

Figure 7. Other aspects of $^{45}Ca^{2+}$ efflux from quiescent 3T3 cells followed addition of bombesin or structurally related peptides. (*A*) Quiescent cultures of 3T3 cells, preincubated with $^{45}Ca^{2+}$ for 12 to 24 h were then incubated with (solid circles) or without (open circles) 6.2 nM bombesin in the presence of the isotope. At the times indicated, the radioactive medium was aspirated and the cultures were rapidly washed seven times with 2 ml of 3 mM EGTA-containing DME at 37°C. The radioactivity left in the cells was extracted as described in Materials and Methods. The results are expressed as percentage of the radioactivity present in the cells before the addition of bombesin. (*B*) Quiescent Swiss 3T3 cells were incubated with $^{45}Ca^{2+}$ as above and then washed seven times with 2 ml DME. The cultures were then exposed for 2 min to varying concentrations of the following peptides: bombesin (open circles), litorin (open triangles), GRP(1–27) (solid circles), and neuromedin B (open squares) in 2 ml of DME. At that point, the radioactivity in the medium and the cell-associated radioactivity were determined. The results are expressed as percent of the maximum stimulation given by bombesin. (*C*) Quiescent Swiss 3T3 cells were incubated with $^{45}Ca^{2+}$ as above. They were then washed seven times with 2 ml of the following solution: 45 mM choline bicarbonate, 105 mM choline chloride, 5 mM KCl, 1.8 mM $CaCl_2$, 25 mM glucose. The efflux of $^{45}Ca^{2+}$ into 2 ml of this solution with (solid circles) or without (open circles) 6.2 nM bombesin was measured as described in Materials and Methods.

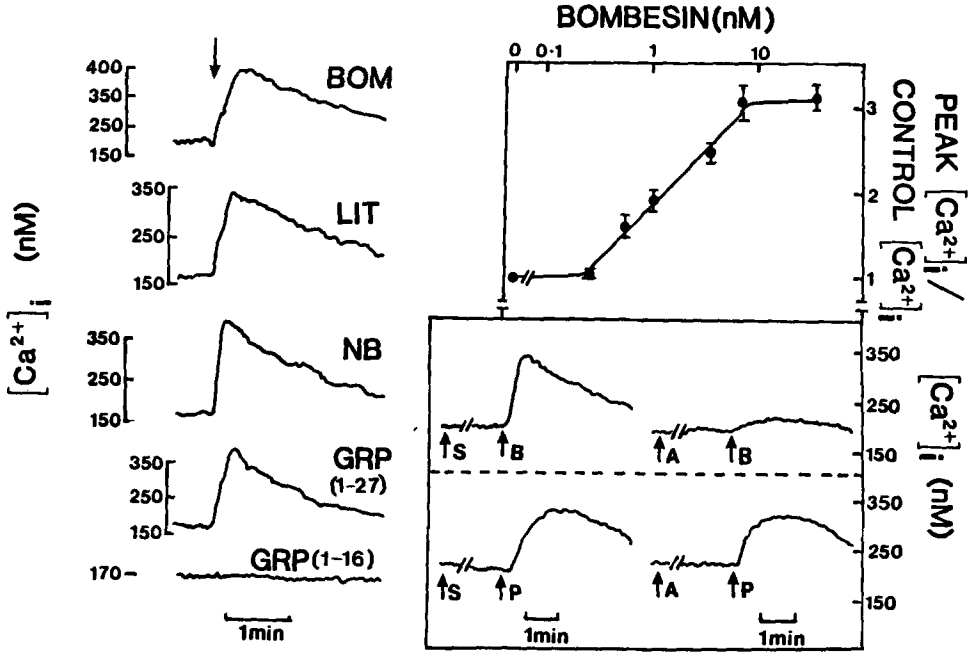

Figure 8. Effect of bombesin and structurally related peptides on $[Ca^{2+}]_i$. Quiescent Swiss 3T3 cells on Cytodex 2 beads were washed and incubated with the fluorescent indicator, quin 2, as described in Materials and Methods. They were then suspended in 2 ml of Solution A, placed in the fluorimeter, and stirred at 37°C. Fluorescence was recorded continuously. After a suitable control period, various peptides were added and fluorescence followed until it returned near control levels. The concentrations of the various peptides were as follows: bombesin (*BOM*), 3.1 nM; litorin (*LIT*), 3 nM; GRP(1–27), 10 nM; neuromedin B (*NB*), 20 nM; GRP(1–16), 1 μM. The tracings presented are typical of 4–5 studies of each peptide. (*Upper right*) Effect of various concentrations of bombesin on $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ after the addition of bombesin is presented as the ratio between the peak $[Ca^{2+}]_i$ and the $[Ca^{2+}]_i$ immediately preceding the addition of the peptide. The figures represent the mean ± SEM of 3–15 independent measurements at each concentration of bombesin. (*Lower right*) Effect of (D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]) SP on the response of $[Ca^{2+}]_i$ to bombesin or PDGF. After a suitable control period, 75 μM (D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]) SP (*A*) or an equivalent volume of solvent (*S*) were added. 3 min later, either 3.1 nM bombesin (*B*) or 0.3 nM porcine PDGF (*P*) were added. In all cases, the measurements of $[Ca^{2+}]_i$ were performed after sequential addition of Triton X-100 and EGTA as described in Materials and Methods.

besin mobilized Ca^{2+} from an intracellular pool (Figs. 6 and 7), it was possible that the increase of $[Ca^{2+}]_i$ could have resulted from an increase in the entry of Ca^{2+} into the cells along the steep electrochemical gradient. This was tested by adding 1.8 mM EGTA just before the addition of bombesin thereby lowering the free Ca^{2+} concentration to ~0.6 μM (46). In fact, addition of EGTA had little or no effect on the stimulation of $[Ca^{2+}]_i$ by bombesin.

The biologically active bombesin-like peptides litorin, GRP(1–27), and neuromedin B also stimulated $[Ca^{2+}]_i$. The time course and magnitude of these effects were identical to the response to bombesin. In contrast, GRP(1–16), which has no structural homology to bombesin and does not bind to the bombesin receptor (70), had no effect on $[Ca^{2+}]_i$ even at a concentration of 1 μM (Fig. 8, left). This peptide also did not alter the stimulation of $[Ca^{2+}]_i$ by bombesin (data not shown). The stimulation of $[Ca^{2+}]_i$ by bombesin was markedly inhibited by (D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]) SP. In contrast, neither the increase in $[Ca^{2+}]_i$ induced by addition of PDGF (Fig. 8, bottom right) nor the decrease in the steady-state level of $^{45}Ca^{2+}$ was affected by this antagonist.

Effect of Pretreatment with PBt_2 on the Stimulation of $^{86}Rb^+$, $^{22}Na^+$, and $^{45}Ca^{2+}$ Fluxes by Bombesin

Ca^{2+}-sensitive, phospholipid-dependent protein kinase (protein kinase C) may play a role in mediating a variety of cellular responses (38) including monovalent ion fluxes (65) and cell growth (53). In the preceding paper (69), we showed that bombesin causes a rapid activation of protein kinase C in intact 3T3 cells. Prolonged pretreatment of 3T3 cells with PBt_2 leads to (*a*) a marked reduction in the number of phorbol ester–binding sites (10, 11), (*b*) the disappearance of protein kinase C activity in cell-free extracts (47, 54), and (*c*) the desensitization to further biological actions of PBt_2 (11, 47, 54, 55). If the action of bombesin on ion fluxes is mediated through activation of protein kinase C, prolonged pretreatment of 3T3 cells with PBt_2 should block the stimulation of Na^+ uptake and of Na^+/K^+ pump activity caused by a subsequent challenge with bombesin. To test this possibility, cultures of quiescent 3T3 cells were treated with 800 nM PBt_2 for 40 h, washed extensively to remove residual PBt_2 (14), and then challenged with either PBt_2 or bombesin to determine whether $^{86}Rb^+$ uptake (Table II), $^{22}Na^+$ uptake (Table III), or $^{45}Ca^{2+}$ efflux (Fig. 9) could still be stimulated by bombesin.

Table II. Effect of Pretreatment of 3T3 Cells with PBt_2 on the Stimulation of $^{86}Rb^+$ Uptake by PBt_2 and Bombesin

Addition	Without pretreatment	With pretreatment
	Ouabain-sensitive $^{86}Rb^+$ uptake (nmol/mg protein per min)	
None	3.1 ± 0.3	3.8 ± 0.3
PBt_2	6.8 ± 0.5	3.9 ± 0.4
Bombesin	6.4 ± 0.4	4.7 ± 0.3

Quiescent 3T3 cells were incubated for 40 h in a mixture of their own conditioned medium, fresh DME, and Waymouth's medium in a ratio of 1:1.5:1.5 either in the presence or absence of 800 nM PBt_2. Cells were then washed three times in a modified DME in which NaCl (110 mM) was replaced with 220 mM sucrose, and which contained 1 mg/ml bovine serum albumin (fatty acid free). The cells remained in this medium for 20 min at 37°C before two final washes in the same medium. This washing removes residual PBt_2 (14). Ouabain-sensitive $^{86}Rb^+$ uptake was then measured as described in Materials and Methods. Additions during the uptake studies were either 400 nM PBt_2 or 6.2 nM bombesin. The experiment was done at low Na^+ concentration (50 mM) to facilitate comparison with the experiment presented in Table III. The data represent mean ± SEM (n = 8).

Table III. Effect of Pretreatment of 3T3 Cells wtih PBt_2 on the Stimulation of $^{22}Na^+$ Uptake by PBt_2 and Bombesin

Addition	Without pretreatment	With pretreatment
	$^{22}Na^+$ uptake (nmol/mg protein per min)	
None	19.1 ± 1.3	18.0 ± 1.2
PBt_2	28.7 ± 2.0	16.4 ± 0.9
Bombesin	35.5 ± 1.6	26.9 ± 2.0

During the experiment described in Table II, parallel cultures of quiescent 3T3 cells were pretreated and washed in exactly the same way. $^{22}Na^+$ uptake was then measured in these cultures as described in Materials and Methods. All uptake measurements were done in 2 mM ouabain. The concentrations of the additions were 400 nM PBt_2 and 6.2 nM bombesin. The data represent mean ± SEM ($n = 6$).

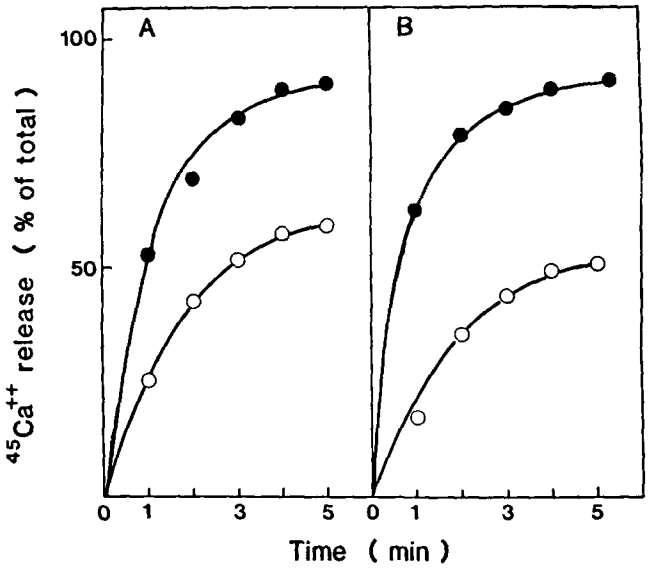

Figure 9. Effect of pretreatment of 3T3 cells with PBt_2 on the stimulation of $^{45}Ca^{2+}$ release by bombesin. Quiescent 3T3 cells were incubated for 40 h in a mixture of their own conditioned medium, fresh DME, and Waymouth medium in a ratio of 1:1.5:1.5. Half of these cultures were pretreated with 800 nM PBt_2 during this incubation (*B*). In the last 20 h of the incubation 8 μCi/ml of $^{45}Ca^{2+}$ was added to all cultures. At the end of the incubation, all of the cultures were washed three times with 2 ml of DME which contained 8 μCi/ml of $^{45}Ca^{2+}$. After a further 20-min incubation at 37°C, the cultures were rapidly washed three times with 2 ml of 3 mM EGTA-containing DME and four times with 2 ml of DME to completely remove the PBt_2 (14). The cultures were then incubated in 2 ml of DME with no addition (open circles) or 6.2 nM (solid circles) bombesin. The data presented are the mean of two experiments. The results without PBt_2 pretreatment are seen in *A* and with PBt_2 pretreatment in *B*. The efflux of $^{45}Ca^{2+}$ was measured as described in Materials and Methods.

Pretreatment with PBt_2 completely prevented the effect of the subsequent addition of PBt_2 on both $^{86}Rb^+$ and $^{22}Na^+$ uptake and markedly reduced the ability of bombesin to stimulate these transport processes (Tables II and III). The stimulation of ouabain-sensitive $^{86}Rb^+$ uptake by bombesin, after PBt_2 pretreatment, was only 27% of the stimulation without pretreatment (Table II). Increasing the concentration of bombesin by 10-fold caused no further increase in $^{86}Rb^+$ uptake (data not shown). Bombesin stimulation of $^{22}Na^+$ uptake in quiescent 3T3 cells was reduced 51% by PBt_2 pretreatment (Table III). The partial loss of ionic response to bombesin in 3T3 cells with greatly reduced number of phorbol ester-binding sites and protein kinase C (10, 11, 42, 53, 54) suggests that the stimulation of this phosphotransferase system by bombesin (69) is at least partly responsible for the increased $^{86}Rb^+$ and $^{22}Na^+$ uptake. In contrast, pretreatment with PBt_2 neither affected the stimulation of $^{45}Ca^{2+}$ efflux by bombesin (Fig. 9) nor the increase in $[Ca^{2+}]_i$ induced by this peptide.

Discussion

Bombesin and structurally related peptides that are potent mitogens for quiescent Swiss 3T3 cells (52, 70) caused rapid changes in monovalent ion fluxes. There was an increase in ouabain-sensitive $^{86}Rb^+$ uptake which was detected within seconds after the addition of bombesin (Figs. 1–3). This stimulation of the plasma membrane Na^+/K^+ pump was dependent on the concentration of bombesin and its analogues. The relative ability of the peptides of the bombesin family to stimulate ouabain-sensitive $^{86}Rb^+$ uptake (Fig. 4) was comparable to their relative potencies in increasing DNA synthesis in quiescent cells (70). In addition to stimulating the Na^+/K^+ pump, bombesin also increased Na^+ entry into the cells in a concentration-dependent manner (Fig. 3*B*). Since the half-maximal concentration for bombesin stimulation of Na^+ influx (0.4 nM) was similar to the half-maximal concentration for bombesin stimulation of the Na^+/K^+ pump (0.3 nM), it is likely that bombesin, like other mitogens (8, 13, 24, 28, 29, 51, 56, 57, 65) stimulated Na^+/K^+ pump activity as a consequence of increased Na^+ entry into the cells.

The entry of Na^+ into Swiss 3T3 occurs at least in part through a Na^+/H^+ antiport (9, 56). It is apparent that bombesin stimulated Na^+ entry through the Na^+/H^+ antiport. (*a*) The addition of bombesin under conditions in which the peptide caused increased Na^+ entry was associated with a rise in intracellular pH (Table I; Fig. 5). (*b*) The cellular alkalinization caused by bombesin was abolished when the peptide was added in the absence of extracellular Na^+ (Table I). Stimulation of the Na^+/H^+ antiport by bombesin could have resulted from either a primary stimulation of antiport activity or an increase in cellular metabolism generating additional protons which were then excreted by the Na^+/H^+ antiport. The fact that the peptide caused a reproducible increase in pH_i (Table I) is incompatible with the latter mechanism. It is concluded that the stimulation of monovalent ion flux by peptides of the bombesin family was triggered by activation of the Na^+/H^+ antiport. A variety of other mitogens which bind to specific receptors also enhance the activity of the Na^+/H^+ antiport in quiescent Swiss 3T3 cells (7–9, 24, 56, 65) and in other cell types (20, 35, 41, 42).

In addition to changes in monovalent ion fluxes, bombesin and structurally related analogues caused a rapid increase in the efflux of $^{45}Ca^{2+}$ from preloaded cells. This stimulation was concentration dependent and was demonstrated at the earliest time point studied (30 s). The efflux came from an intracellular pool. The following evidence supports this conclusion: (*a*) bombesin stimulation of $^{45}Ca^{2+}$ efflux was seen after the cells were washed with DME containing 3 mM EGTA which markedly reduces the amount of extracellularly bound $^{45}Ca^{2+}$ (21, 22) (Fig. 6*B*); (*b*) bombesin stimulated $^{45}Ca^{2+}$ efflux into a Ca^{2+}-free medium containing 50 μM EGTA indicating that the effect of the peptide cannot be explained by $^{45}Ca^{2+}/^{40}Ca^{2+}$ exchange (Fig. 6*C*); and (*c*) bombesin caused a 50% decrease

in $^{45}Ca^{2+}$ content in cells labeled to specific activity equilibrium (Fig. 7*A*). These findings were consistent with the model that bombesin, after binding to its receptor, causes release of Ca^{2+} from an intracellular pool into the cytoplasm. The increased $[Ca^{2+}]_i$ then stimulates Ca^{2+} efflux by a mechanism which requires neither extracellular Ca^{2+} (Fig. 6*C*) nor extracellular Na^+ (Fig. 7*C*), presumably by the plasma membrane ATP-dependent Ca^{2+} pump (1). This model for the effect of bombesin on Ca^{2+} efflux predicted that the addition of the peptide would cause a rapid increase in the concentration of Ca^{2+} in the cytosol. This prediction was tested using the fluorescent Ca^{2+} indicator quin 2. It was found that bombesin and its biologically active analogues caused a rapid, concentration-dependent increase in $[Ca^{2+}]_i$ (Fig. 8). The effect started within 3–5 s and $[Ca^{2+}]_i$ increased as much as threefold after the addition of bombesin at saturating concentrations.

Peptides of the bombesin family bind to a specific receptor in Swiss 3T3 cells (70). Stimulation of monovalent and divalent ion transport by these peptides is a consequence of binding to this receptor since the relative ability of bombesin and its analogues to increase ouabain-sensitive $^{86}Rb^+$ uptake and $^{45}Ca^{2+}$ efflux was similar to their ability to inhibit the binding of ^{125}I-GRP to high-affinity receptors (70). In addition, the antagonist (D-Arg^1-D-Pro^2-D-Trp^{7,9}-Leu^{11}) SP, which inhibits the binding of bombesin to its receptor (70), also inhibited the increase in pH_i and the mobilization of Ca^{2+} caused by bombesin.

There has been considerable interest in the signal(s) that link receptor occupancy by an agonist with the mobilization of Ca^{2+} from an intracellular store. It has been proposed that various hormones and neurotransmitters accelerate the breakdown of polyphosphoinositides resulting in the production of inositol 1,4,5 triphosphate, which acts as a second messenger inducing Ca^{2+} mobilization from an intracellular source (3, 17, 18, 44, 59). It is likely that bombesin effects on Ca^{2+} are mediated by inositol 1,4,5 triphosphate in quiescent Swiss 3T3 cells since the peptide increases the breakdown of phosphoinositides in these cells (6).

Since the binding of bombesin and its analogues to its high-affinity receptors rapidly activates protein kinase C (69) and increases $[Ca^{2+}]_i$, it was of interest to determine if either of these signalling mechanisms played a role in the stimulation of monovalent ion transport by bombesin. It has been suggested that stimulation of Na^+ influx in human fibroblasts is secondary to an elevation in $[Ca^{2+}]_i$ (40, 66). This does not appear to be the case in quiescent Swiss 3T3 cells. The Ca^{2+} ionophore A23187 does not cause alkalization of the cells in the presence of Na^+ at ionophore concentrations that increase $[Ca^{2+}]_i$ to levels comparable to a maximal bombesin-mediated increase in $[Ca^{2+}]_i$.

Recently, evidence has accumulated that stimulation of protein kinase C leads to activation of the Na^+/H^+ antiport (65) although the molecular mechanism of this effect of the phosphotransferase remains to be elucidated. Since bombesin stimulates protein kinase C activity (69), it was plausible that the effects of the peptide on monovalent ion transport were mediated by protein kinase C. This possibility was tested using cells which had been pretreated with PBt_2 for 40 h. Prolonged pretreatment with phorbol esters decreases the number of phorbol ester–binding sites in intact Swiss 3T3 cells (10, 11) and decreases the activity of protein kinase C in cell-free extracts (47, 54). Furthermore, this pretreatment desensitizes the cells to biological effects of phorbol esters (10, 11, 53, 55, 65), including stimulation of $^{22}Na^+$ and $^{86}Rb^+$ fluxes (see Tables III and IV in reference 65). Since the activity of the Na^+/H^+ antiport and the Na^+/K^+ pump are intact in PBt_2-treated cells, it was concluded that both the PBt_2 and the synthetic diacylglycerol, 1-oleoyl-2-acetylglycerol, stimulate monovalent ion transport by stimulating protein kinase C (65).

Interestingly, PBt_2 pretreatment did not affect either the stimulation of $^{45}Ca^{2+}$ efflux or the increase in $[Ca^{2+}]_i$ by bombesin, indicating that protein kinase C does not play a role in eliciting Ca^{2+} mobilization by bombesin. These findings indicate that the bombesin receptor and the processes involved in Ca^{2+} mobilization are functional after PBt_2 pretreatment. Thus, phorbol ester–pretreated cells provide a useful system to define the contribution of protein kinase C in the production of the biological responses elicited by peptides of the bombesin family. Using this approach, we found that prolonged pretreatment with PBt_2 partially inhibited ouabain-sensitive $^{86}Rb^+$ uptake and $^{22}Na^+$ uptake in the presence of bombesin. Thus, it appears that bombesin stimulates monovalent ion transport in part by activating protein kinase C. Since some stimulation of monovalent ion transport by bombesin persists in PBt_2-pretreated cells, it is likely that the peptide can stimulate monovalent ion transport by a second mechanism. Insulin and epidermal growth factor also stimulate the Na^+/H^+ antiport by a mechanism which is independent of protein kinase C (64). The nature of the protein kinase C independent mechanism(s) for stimulating the Na^+/H^+ antiport remains to be determined.

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