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]), 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-
sensitive Na\(^+\)/H\(^+\) antiport. This increases intracellular Na\(^+\) and causes cytoplasmic alkalization (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 45Ca\(^{2+}\) 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\(^{2}\), D-Pro\(^{3}\), 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 and stirred continuously at the slowest rate which partially inhibited by the bombesin antagonist (o-Arg t, D-Pro\(^{2}\), D-Trp\(^{7,9}\), Leu\(^{11}\)) substance P (SP).

86Rb\(^+\) Uptake

The uptake of 86Rb\(^+\) (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 twice with DME at 37°C. After incubation of variable length (see individual experiments) in 2 ml of experimental media, 30-50 ml of 86RbCl containing 4.6 x 10\(^{10}\) 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 in a flame photometer (Corning EEL, Model 430) with the LiCl serving as the internal standard.

Intracellular pH

Intracellular pH (pHi) was measured with the weak acid 5,5 dimethyl [2-\(^{14}\)C] oxalazine-2,4-dione (\(^{14}\)C-DMO) using a modification of a previously described method (28, 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 ml of 0.1 M NaOH containing 150 nM and 3-4 x 10\(^{10}\) 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\(_2\)CO\(_3\)/0.1% SDS. The radioactivity was measured using a liquid scintillation spectrometer. Protein was determined as described by Lowry et al. (25).

\(^{14}\)C-DMO 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-area 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 \(^{14}\)C/ 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 \(\mu\)l samples were removed from the medium and replaced with 0.5 ml of fresh medium. After 24 h. Quiescent cells on Cytodex 2 beads were washed twice with serum-free DME. The beads were then resuspended in 2 ml of DME by gentle shaking. 10-ml aliquots were transferred to plastic tubes and incubated for 45 min at 37°C with 15 \(\mu\)m quin 2 tetracetoxyxymethyl ester. The stock solution of quin 2 tetracetoxyxymethyl ester was 7.5 mM dissolved in dimethyl sulfoxide. After incubation, the beads were washed three times with Solution A, which contained 140 mM NaCl, 5 mM KCl, 1.8 mM Ca\(_2\)Cl\(_2\), 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 7.2). 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 359 nm and an emission wavelength of 492 nm.

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 \(\mu\)m quin 2 tetracetoxyxymethyl ester. The stock solution of quin 2 tetracetoxyxymethyl ester was 7.5 mM dissolved in dimethyl sulfoxide. After incubation, the beads were washed three times with Solution A, which contained 140 mM NaCl, 5 mM KCl, 1.8 mM Ca\(_2\)Cl\(_2\), 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 7.2). 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 359 nm and an emission wavelength of 492 nm.

\[^{45}\]Ca\(^{2+}\) was calculated using the formula of Tsien et al. (62).
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 x 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 x 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.

\[ F = \text{fluorescence at the unknown [Ca$^{2+}$]} \]
\[ F_{0.02} = \text{fluorescence after the trapped quin 2 is released by the addition of 0.02% Triton X-100} \]
\[ F_{	ext{Ca}^{2+}} = \text{fluorescence remaining after the Ca$^{2+}$ in the solution is chelated with 100 mM EGTA} \]

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 (PB$_{12}$) 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$^3$, D-Pro$^2$, D-Trp$^7$,9, Leu$^{10}$)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 tetracetoxymethyl ester were obtained from Lancaster Synthesis (Morecambe, 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. 1A). 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$^+$ for 4 h. This peptide had no effect on the rate of $^{86}$Rb$^+$ efflux from these cells (Fig. 1B). 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. 1C 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.
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 \(^{86}\text{Rb}\) uptake 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 \(^{86}\text{Rb}\) uptake were similar. A half-maximal increase in Na\(^+\) content was seen at 0.3 nM bombesin and a half-maximal increase of \(^{86}\text{Rb}\) uptake occurred at 0.4 nM bombesin. The effect of bombesin on Na\(^+\) entry was also assessed by measuring the uptake of \(^{22}\text{Na}\) from a solution containing 50 mM Na\(^+\) and 2 mM ouabain with choline chloride as the osmotic replacement. Bombesin (6.2 nM) increased \(^{22}\text{Na}\) uptake from 19.7 ± 0.8 to 27.9 ± 1.0 nmoI 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 \(^{14}\text{C}-\text{DMO}\) 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 \(^{14}\text{C}-\text{urea}\) exchange) did not change significantly in bombesin-treated cells. Bombesin increased \(^{14}\text{C}-\text{DMO}\) 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\(^{2+}\)], 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

| Extracellular Na\(^+\) | Control | Bombesin | P     |
|-----------------------|---------|----------|-------|
| 50 mM (n = 35)        | 7.107 ± 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, \(^{14}\text{C}-\text{DMO}\) 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\(^+\).
**Figure 4.** Stimulation of ouabain-sensitive $^{86}\text{Rb}^+$ 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. $^{86}\text{Rb}^+$ (2.5 x 10^6 cpm/ml) was then added, the incubation was continued for an additional 10 min, and $^{86}\text{Rb}^+$ uptake measured as described in Materials and Methods. Ouabain-sensitive $^{86}\text{Rb}^+$ uptake was determined by subtracting the $^{86}\text{Rb}^+$ 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 $^{86}\text{Rb}^+$ 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, [45C]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, [45C]DMO was added for an additional 15 min and intracellular pH measured as described in Materials and Methods.

**Stimulation of $^{86}\text{Rb}^+$ Uptake and Increase in pH, by Bombesin-like Peptides**

The concentration dependence of the stimulation of $^{86}\text{Rb}^+$ 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 $^{86}\text{Rb}^+$ 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 $^{86}\text{Rb}^+$ 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 alkalization correlated with their ability to stimulate $^{86}\text{Rb}^+$ 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, or the increment in pH, induced by PB2 (results not shown). It did, however, partially but significantly ($P < 0.01$) inhibit the increase in pH, caused by bombesin (Fig. 5). This supports the conclusion that bombesin and related peptides increase pH, after binding to their high-affinity receptor.

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

Quiescent cultures of Swiss 3T3 cells were incubated with $^{45}\text{Ca}^+$ 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 $^{45}\text{Ca}^+$ efflux at the earliest time point (15 s) after the addition of bombesin (Fig. 6A). 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 $^{45}\text{Ca}^+$ efflux by bombesin (Fig. 6B). Thus, bombesin appeared to stimulate the efflux of $^{45}\text{Ca}^+$ from an intracellular pool(s). The efflux of $^{45}\text{Ca}^+$ was also measured in cultures washed and incubated in Ca$^{2+}$-free medium containing 50 µM EGTA. Since bombesin stimulated $^{45}\text{Ca}^+$ efflux under these conditions (Fig. 6C), the peptide increases $^{45}\text{Ca}^+$ efflux by a mechanism other
than $^{45}\text{Ca}^{2+}/^{45}\text{Ca}^{2+}$ exchange.

If bombesin stimulates net $^{45}\text{Ca}^{2+}$ efflux, it should decrease the steady-state level of $^{45}\text{Ca}^{2+}$ 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 $^{45}\text{Ca}^{2+}$ content of the cultures (Fig. 7A). This decrease was measurable within 1 min after the addition of bombesin, and cultures were maximally depleted of $^{45}\text{Ca}^{2+}$ after 2 min (50% decrease). This effect persisted for at least 1 h. Thus, the addition of bombesin caused a decrease in total intracellular $\text{Ca}^{2+}$ in quiescent Swiss 3T3 cells.

The effect of various concentrations of bombesin and structurally related peptides is shown in Fig. 7B. Half-maximal stimulation of $^{45}\text{Ca}^{2+}$ 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 $\text{Ca}^{2+}$ efflux occurs in part by a pathway dependent on external Na$^+$ (1), $^{45}\text{Ca}^{2+}$ efflux was measured in cultures washed with and incubated in a Na$^+$-free electrolyte solution. Bombesin stimulated $^{45}\text{Ca}^{2+}$ efflux into an Na$^+$-free solution (Fig. 7C). Thus, Na$^+$-dependent $\text{Ca}^{2+}$ efflux does not appear to be a major component of bombesin-stimulated $^{45}\text{Ca}^{2+}$ efflux in Swiss 3T3 cells.

**Effect of Bombesin on Intracellular $\text{Ca}^{2+}$ Concentration**

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

Although the studies on $^{45}\text{Ca}^{2+}$ efflux indicated that bom-
tion of bombesin is presented as the ratio between the peak [Ca\(^{2+}\)], and the [Ca\(^{2+}\)], 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\(^{9,10}\), Leu\(^1\)) SP on the response of [Ca\(^{2+}\)] to bombesin or PDGF. After a suitable control period, 75 μM (D-Arg\(^1\), D-Pro\(^2\), D-Trp\(^{9,10}\), Leu\(^1\)) 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\(^{2+}\)], were performed after sequential addition of Triton X-100 and EGTA as described in Materials and Methods.

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\(^{2+}\)], even at a concentration of 1 μM (Fig. 8, left). This peptide also did not alter the stimulation of [Ca\(^{2+}\)], by bombesin (data not shown). The stimulation of [Ca\(^{2+}\)], by bombesin was markedly inhibited by (D-Arg\(^1\), D-Pro\(^2\), D-Trp\(^{9,10}\), Leu\(^1\)) SP. In contrast, neither the increase in [Ca\(^{2+}\)], induced by addition of PDGF (Fig. 8, bottom right) nor the decrease in the steady-state level of \(^{45}\text{Ca}^{2+}\) was affected by this antagonist.

Effect of Pretreatment with PBt\(_2\) on the Stimulation of \(^{86}\text{Rb}^+\), \(^{22}\text{Na}^+\), and \(^{42}\text{Ca}^+\) Fluxes by Bombesin

Ca\(^{2+}\)-sensitive, phospholipid-dependent protein kinase (protein kinase C) may play a role in mediating a variety of cellular responses (58) 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 phospholipid-dependent kinase, and (c) the desensitization to further biological actions of PBt\(_2\) (11, 47, 47, 54). 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 \(^{86}\text{Rb}^+\) uptake (Table II), \(^{22}\text{Na}^+\) uptake (Table III), or \(^{42}\text{Ca}^+\) efflux (Fig. 9) could still be stimulated by bombesin.
Table III. Effect of Pretreatment of 3T3 Cells with PBt2 on the Stimulation of 22Na⁺ Uptake by PBt2 and Bombesin

| Addition | Without pretreatment | With pretreatment |
|----------|----------------------|-------------------|
|          | 22Na⁺ uptake (nmol/mg protein per min) |                      |
| None     | 19.1 ± 1.3           | 18.0 ± 1.2        |
| PBt2     | 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. 22Na⁺ 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 PBt2 and 6.2 nM bombesin. The data represent mean ± SEM (n = 6).

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 86Rb⁺ 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 86Rb⁺ 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. 3B). 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 alkalization 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 (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 45Ca²⁺ 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 45Ca²⁺ efflux was seen after the cells were washed with DME containing 3 mM EGTA which markedly reduces the amount of extracellularly bound 45Ca²⁺ (21, 22) (Fig. 6B); (b) bombesin stimulated 45Ca²⁺ efflux into a Ca²⁺-free medium containing 50 μM EGTA indicating that the effect of the peptide cannot be explained by 45Ca²⁺/40Ca²⁺ exchange (Fig. 6C); and (c) bombesin caused a 50% decrease
in \( {\text{Ca}^{2+}} \) 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 \( {\text{Ca}^{2+}} \) from an intracellular pool into the cytoplasm. The increased \([{\text{Ca}^{2+}}]\) then stimulates \( {\text{Ca}^{2+}} \) efflux by a mechanism which requires neither extracellular \( {\text{Ca}^{2+}} \) (Fig. 6 C) nor extracellular \( {\text{Na}^{+}} \) (Fig. 7 C), presumably by the plasma membrane ATP-dependent \( {\text{Ca}^{2+}} \) pump (1). This model for the effect of bombesin on \( {\text{Ca}^{2+}} \) efflux predicted that the addition of the peptide would cause a rapid increase in the concentration of \( {\text{Ca}^{2+}} \) in the cytosol. This prediction was tested using the fluorescent \( {\text{Ca}^{2+}} \) indicator quin 2. It was found that bombesin and its biologically active analogues caused a rapid, concentration-dependent increase in \([{\text{Ca}^{2+}}]\) (Fig. 8). The effect started within 3–5 s and \([{\text{Ca}^{2+}}]\) 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 \( {\text{86Rb}^{+}} \) uptake and \( {\text{45Ca}^{2+}} \) efflux was similar to their ability to inhibit the binding of \( \text{[25I]}\)-GRP to high-affinity receptors (70). In addition, the antagonist (\( \text{d}-\text{Arg}^{\alpha}-\text{d}-\text{Pro}^{\alpha}-\text{d}-\text{Trp}^{5}\)-\( \text{Leu}^{9}\) SP, which inhibits the binding of bombesin to its receptor (70), also inhibited the increase in \( \text{pH}_{\text{i}} \) and the mobilization of \( {\text{Ca}^{2+}} \) caused by bombesin.

There has been considerable interest in the signal(s) that link receptor occupancy by an agonist with the mobilization of \( {\text{Ca}^{2+}} \) 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 \( {\text{Ca}^{2+}} \) mobilization from an intracellular source (3, 17, 18, 44, 59). It is likely that bombesin effects on \( {\text{Ca}^{2+}} \) 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 \([{\text{Ca}^{2+}}]\), 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 \( {\text{Na}^{+}} \) influx in human fibroblasts is secondary to an elevation in \([{\text{Ca}^{2+}}]\) (40, 66). This does not appear to be the case in quiescent Swiss 3T3 cells. The \( {\text{Ca}^{2+}} \) ionophore A23187 does not cause alkalization of the cells in the presence of \( {\text{Na}^{+}} \) at ionophore concentrations that increase \([{\text{Ca}^{2+}}]\) to levels comparable to a maximal bombesin-mediated increase in \([{\text{Ca}^{2+}}]\).

Recently, evidence has accumulated that stimulation of protein kinase C leads to activation of the \( {\text{Na}^{+}}/{\text{H}^{+}} \) 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 \( \text{PBt}^{2} \) 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 \( {\text{22Na}^{+}} \) and \( {\text{86Rb}^{+}} \) fluxes (see Tables III and IV in reference 65). Since the activity of the \( {\text{Na}^{+}}/{\text{H}^{+}} \) antiport and the \( {\text{Na}^{+}}/{\text{K}^{+}} \) pump are intact in \( \text{PBt}^{2} \)-treated cells, it was concluded that both the \( \text{PBt}^{2} \) and the synthetic diacylglycerol, 1-oleoyl-2-acetylglycerol, stimulate monovalent ion transport by stimulating protein kinase C (65).

Interestingly, \( \text{PBt}^{2} \) pretreatment did not affect either the stimulation of \( {\text{45Ca}^{2+}} \) efflux or the increase in \([{\text{Ca}^{2+}}]\) by bombesin, indicating that protein kinase C does not play a role in eliciting \( {\text{Ca}^{2+}} \) mobilization by bombesin. These findings indicate that the bombesin receptor and the processes involved in \( {\text{Ca}^{2+}} \) mobilization are functional after \( \text{PBt}^{2} \) 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 \( \text{PBt}^{2} \) partially inhibited ouabain-sensitive \( {\text{86Rb}^{+}} \) uptake and \( {\text{22Na}^{+}} \) 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 \( \text{PBt}^{2} \)-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 \( {\text{Na}^{+}}/{\text{H}^{+}} \) 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 \( {\text{Na}^{+}}/{\text{H}^{+}} \) antiport remains to be determined.

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