Vol. 147, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1987 Pages 189-195

BOMBESIN-LIKE PEPTIDES ELEVATE CYTOSOLIC CALCIUM IN SMALL CELL LUNG CANCER CELLS

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Received July 6, 1987

The ability of bombesin-like peptides to elevate intracellular Ca^{2+} levels in small cell lung cancer cells was investigated using the fluorescent Ca^{2+} indicator, Fura 2. Nanomolar concentrations of bombesin elevated cytosolic Ca^{2+} levels in the absence or presence of extracellular Ca^{2+}. Potent bombesin receptor agonists, such as gastrin releasing peptide (GRP) or $(GRP)^{14-27}$ elevated cytosolic Ca^{2+} levels whereas inactive compounds such as $(D\text{-}Trp^{8})$bombesin or $(GRP)^{1-16}$ did not. Furthermore, the bombesin receptor antagonist $(D\text{-}Arg^{1}, D\text{-}Pro^{2}, D\text{-}Trp^{7,9}, Leu^{11})$substance P (30 μM) had no effect on the Ca^{2+} levels by itself but antagonized the increase in Ca^{2+} caused by 10 nM or 100 nM bombesin. These data suggest that bombesin receptors may regulate the release of Ca^{2+} from intracellular organelles in small cell lung cancer cells.

The peptide bombesin (BN) and the structurally related gastrin releasing peptide (GRP) represent a family of polypeptides biologically active in normal and malignant cells. In the normal CNS and periphery, BN-like peptides may function in a paracrine manner to regulate neural activity and hormone secretion (1). In tumor cells, high concentrations of BN-like peptides have been detected in small cell lung cancer (SCLC) cells (2-5). These peptides are secreted from SCLC cell lines and elevated levels of immunoreactive BN are present in the plasma of patients with extensive disease (6-8). Protein receptors which bind BN or GRP with high affinity have been detected on SCLC cells (9) and recently it was demonstrated that BN stimulates the growth of SCLC cell lines *in vitro* (10). Because the growth of SCLC *in vitro* and *in vivo* is inhibited by anti-BN monoclonal antibody, BN-like peptides may function as autocrine growth factors (11).

Recently, it was demonstrated in Swiss 3T3 cells, which have 100,000 BN receptors/cell (12), that BN stimulates phosphatidylinositol turnover (13). In particular, BN (1 nM) was shown to elevate the levels of inositol 1, 4, 5-

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0006-291X/87 $1.50

trisphosphate, inositol 1, 5-bisphosphate and inositol 1-phosphate. Recently, BN has also been shown to elevate the cytosolic free Ca^{2+} concentration of Swiss 3T3 cells (14), which is consistent with the known role of inositol trisphosphate in triggering the release of Ca^{2+} from intracellular stores (15). Here we investigated if BN-like peptides elevate cytosolic Ca^{2+} in SCLC cells.

MATERIALS AND METHODS

SCLC cell line NCI-H345 was cultured in HITES medium (RPMI-1640 containing 10^{-8} M hydrocortisone, 5 µg/ml bovine insulin, 10 µg/ml human transferrin, 10^{-8} M β-estradiol and 3 x 10^{-8} M Na_2SeO_3) supplemented with 2.5% heat inactivated fetal bovine serum as described previously (16). The cells were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Two days after a medium change, the cells were harvested by centrifugation at 1,000 x g for 10 min. The cells were resuspended in HITES medium and the centrifugation repeated.

The cells were resuspended in HITES medium which contained 20 mM HEPES/NaOH (pH 7.4) for loading of the fluorescent Ca^{2+} indicator Fura 2 AM (17). Cell suspensions at 2.5 X 10^6 cells/ml (5 ml) were incubated in flasks in the presence of 5 µM Fura 2 AM (Calbiochem Inc., La Jolla, CA) at 37°C for 30 min in a shaking water bath. Unloaded Fura 2 AM was removed by centrifugation at 150 x g for 10 min. The cells were then resuspended (2.5 x 10^6 cells/ml) and transferred to a spectrofluorometer equipped with a magnetic stirring mechanism and a temperature (37°C) regulated cuvette holder. The excitation and emission wavelengths were 340 and 510 nm respectively. Cytosolic Ca^{2+} concentrations were calculated employing methods similar to those used for Quin 2 (18) with correction for the presence of extracellular Fura 2. Synthetic BN-like peptides were obtained from Penninsula Laboratories, San Carlos, CA.

RESULTS

The basal cytosolic free Ca^{2+} concentration of SCLC cells was 150 nM ± 40 nM S.D. (n=4). The fluorescence signal obtained with unstimulated cells increased slowly (Fig. 1A), however, this was due to leakage of intracellular Fura 2 into the suspension medium rather than due to an increase in intracellular Ca^{2+}. The addition of 0.1 nM BN to SCLC cells induced a moderate rise in cytosolic Ca^{2+} which ceased after approximately 1 minute (Fig. 1B). In contrast, using 1 or 10 nM BN, the fluorescence rapidly increased within 15 sec to maximal values (Fig. 1C and 1D), then slowly decreased. The initial increase in the fluorescence reached a greater amplitude using 10 nM rather than 1 nM BN. These data indicate that BN in a dose dependent manner causes a transient increase in the cytosolic free Ca^{2+} levels in SCLC cells.

Subsequently, we investigated the source of the Ca^{2+} increase elicited by BN. The experiments described in Fig. 2 indicate the response of SCLC cells to BN in the presence and absence of extracellular Ca^{2+}. In

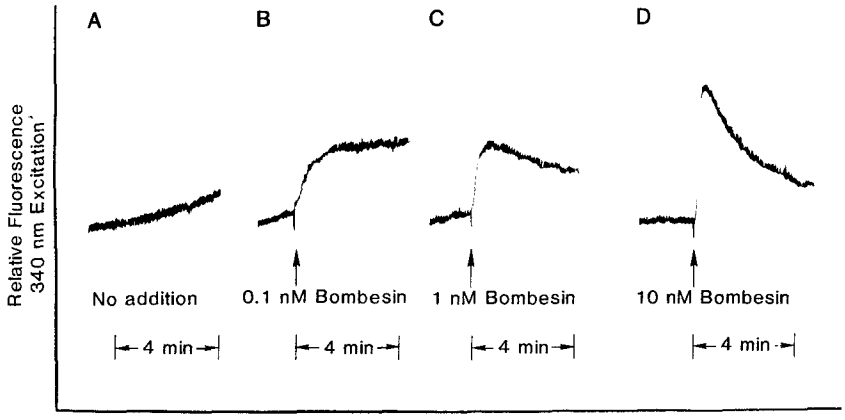

Figure 1. Dose dependency of bombesin on the cytosolic Ca^{2+} concentration of small cell lung cancer cells. Fura 2 measurements were made of cytosolic Ca^{2+} in SCLC cells in the absence (A) and the presence of 0.1 nM BN (B), 1.0 nM BN (C), and 10 nM BN (D). The conditions were as described in Materials and Methods.

the presence of 0.4 mM extracellular calcium (Fig 2A), the addition of 100 nM BN was followed by an initial rise and subsequent fall in cytosolic Ca^{2+} similar to that observed with 10 nM BN. The addition of 5 mM EGTA was made in the experiment shown in Fig 2B in order to chelate all extracellular Ca^{2+} and make it unavailable as a source of elevated cytosolic Ca^{2+}. The addition of EGTA caused an immediate decrease in the fluorescent signal due to

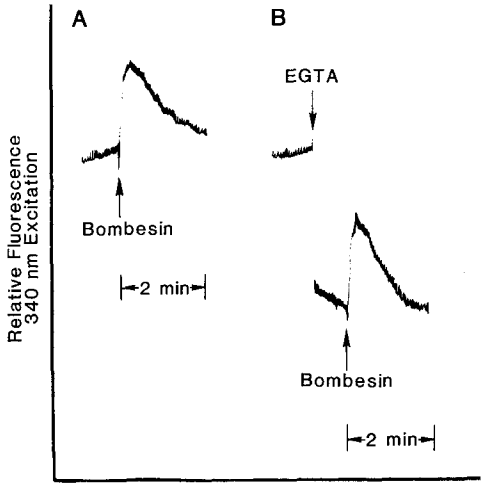

Figure 2. Response of cytosolic Ca^{2+} to bombesin in the presence and absence of extracellular free Ca^{2+}. Fura 2 measurements were made of cytosolic Ca^{2+} in SCLC cells in the presence of 0.4 mM $CaCl_2$ in the absence (A) and presence (B) of 5 mM EGTA. Bombesin was added at a concentration of 100 nM. A sufficient quantity of NaOH was added together with EGTA to counteract the acidification caused by the binding of Ca^{2+} to EGTA.

Table I. Effect of peptides on Ca^{2+} levels in SCLC cell line NCI-H345

Peptide	Ca^{2+} response
BN	+
GRP	+
GRP^{14-27}	+
GRP^{21-27}	+
GRP^{22-27}	+/-
GRP^{23-27}	-
GRP^{1-16}	-
$(D\text{-}Trp^{8})BN$	-
VIP	-
Somatostatin	-
$(D\text{-}Arg^{1}, D\text{-}Pro^{2}, D\text{-}Trp^{7,9}, Leu^{11})SP$	-

Peptides (1μM) were tested for their ability to strongly (+), weakly (+/-) or inability (-) to elevate intracellular Ca^{2+} levels.

the conversion of Ca^{2+} bound to Ca^{2+} free extracellular Fura 2. The subsequent addition of 100 nM BN was followed by a rapid rise in cytosolic Ca^{2+} that was identical in rate and magnitude to that observed in the presence of extracellular Ca^{2+}. This result strongly suggests that the source of BN-induced elevated cytosolic Ca^{2+} is from intracellular stores rather than due to plasmalemmal Ca^{2+} influx.

The peptide specificity of the increase in cytosolic Ca^{2+} in SCLC cells was also investigated. GRP and GRP^{14-27}, which are potent BN receptor agonists, at a 1 μM concentration cause a rapid increase in cytosolic Ca^{2+} (Table I). Similarly, GRP^{21-27} and GRP^{22-27}, which are weak BN receptor agonists, rapidly and slowly increased the Fura 2 fluorescence signal respectively. In contrast, GRP^{23-27}, GRP^{1-16} and $(D\text{-}Trp^{8})BN$, which are inactive, did not affect cytosolic Ca^{2+}. Peptides structurally unrelated to BN, such as vasoactive intestinal polypeptide (VIP) and somatostatin (SRIF), were also ineffective at increasing the cytosolic Ca^{2+}. These data indicate that in addition to BN, certain other BN-like peptides also elevate cytosolic Ca^{2+} in SCLC cells.

Further evidence for the involvement of BN receptors in agonist-induced elevations of cytosolic Ca^{2+} was obtained with the use of BN receptor antagonists. The addition of $(D\text{-}Arg^{1}, D\text{-}Pro^{2}, D\text{-}Trp^{7,9}, Leu^{11})$ substance P (30 μM) had no effect on the basal Fura 2 fluorescence signal of SCLC cells (Fig 3A). However, the presence of this BN receptor antagonist blocked completely the elevation of cytosolic Ca^{2+} normally

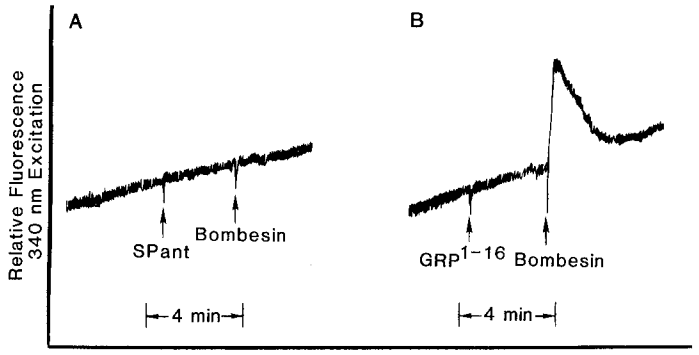

Figure 3. Inhibition of bombesin-induced increase in cytosolic Ca^{2+} by a bombesin receptor antagonist. Fura-2 measurements were made of the effect of 100 nM bombesin on the cytosolic Ca^{2+} concentration in the presence of 30 μM of the substance P antagonist (SPant), (D-Arg^1, D-Pro^2, D-$Trp^{7,9}$, Leu^{11})substance P (Panel A) or in the presence of 1 μM of the inactive peptide GRP^{1-16} (Panel B).

elicited by 100 nM BN. The presence of BN antagonists had no effect on the elevation of cytosolic Ca^{2+} observed using the Ca^{2+} ionophore ionomycin (data not shown). Peptides that are inactive, e.g. GRP^{1-16}, had no effect on the BN-induced Ca^{2+} signal (Fig. 3B).

DISCUSSION

Receptors for BN-like peptides were previously characterized in dispersed guinea pig pancreatic acini (19). (^{125}I-Tyr^4)BN bound with high affinity (Kd = 2 nM) to a single class of sites (Bmax = 5000 cell). Also, nM concentrations of BN stimulated $^{45}Ca^{2+}$ efflux from the cells, elevated cellular cGMP levels and induced the release of amylase from the cells. Because the concentration of peptide required to inhibit specific (^{125}I-Tyr^4)BN binding was greater than the concentration to induce a biological response, it was hypothesized that there were spare receptors for BN and that occupation of only 25% of these receptors was sufficient to induce a biological response.

Here we investigated the effects of BN on cytosolic Ca^{2+} in a SCLC cell line. Previously, we observed that (^{125}I-Tyr^4)BN bound with high affinity (Kd = 0.5 nM) to a single class of sites (2,000/cell) (9). In particular, peptides such as BN, GRP, GRP^{21-27} and GRP^{22-27} inhibited specific (Tyr^4)BN binding with IC_{50} values of 1, 4, 300 and 10,000 nM respectively. Table I shows that each of these peptides at a 1 μM concentration elevated cytosolic Ca^{2+} levels. These data suggest that each of these peptides is a BN receptor agonist. Surprisingly, GRP^{22-27} (1 uM) and BN (0.1 nM) slowly elevate cytosolic Ca^{2+} levels, even though at these concentrations only approximately 10% of the receptors are

theoretically occupied (9). These data suggest that in SCLC cells, similar to the exocrine pancreas, there are spare BN receptors.

The effects of other peptides on the cytosolic Ca^{2+} levels was investigated. Previously, we determined that the C-terminal of GRP or BN was essential for high affinity binding activity (BN and GRP have the same C-terminal heptapeptide). In this regard, GRP^{14-27} but not GRP^{1-16} elevated the cytosolic Ca^{2+} levels. Peptides, which are structurally unrelated to BN, such as VIP or SRIF, did not alter the cytosolic Ca^{2+} levels. Previously, we determined that there are VIP and SRIF receptors on SCLC cell line NCI-H345 (8,20). The substance P analogue (D-Arg^1, D-Pro^2, D-$Trp^{7,9}$, Leu^{11})substance P functions as a pancreatic acinar and 3T3 BN receptor antagonist (14,21). Here (D-Arg^1, D-Pro^2, D-$Trp^{7,9}$, Leu^{11})substance P had no effect on the cytosolic Ca^{2+} levels but did antagonize the effects caused by BN. Thus, in SCLC cells (D-Arg^1, D-Pro^2, D$Trp^{7.9}$, Leu^{11})substance P may similarly function as a BN receptor antagonist. In this regard, we have recently determined that (D-Arg^1, D-Pro^2, D-$Trp^{7,9}$, Leu^{11})substance P inhibits specific (Tyr^4)BN binding activity with an IC_{50} value of 1 μM and it antagonizes the growth of SCLC in vitro induced by BN (22).

In summary, SCLC BN receptors, when activated rapidly elevate cytosolic Ca^{2+}. Because the increase in Ca^{2+} caused by BN is not dependent on the presence of extracellular Ca^{2+}, BN apparently induces the release of Ca^{2+} from intracellular stores. Based upon previous studies with Swiss 3T3 cells (13, 14) the effects of BN on Ca^{2+} levels in SCLC cells is most likely mediated by phosphatidylinositol turnover and the generation of inositol trisphosphate. Furthermore, the demonstration that BN elevates cytosolic Ca^{2+} in SCLC cells may provide one explanation for a mechanism by which BN stimulates the growth of these highly malignant cells.

ACKNOWLEDGEMENTS

This research is supported by NCI grants CA-33767 and CA-32946.

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