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GRP Receptors Are Present in Non Small Cell Lung Cancer Cells

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Abstract Previously, GRP receptors were characterized in small cell lung cancer cells and here non-small cell lung cancer (NSCLC) cells were investigated: (^{125}I-Tyr^4) bombesin (BN) or ^{125}I-GRP bound with high affinity to NCI-H720 (lung carcinoid) and NCI-H1299 (large cell carcinoma) cells. Binding was specific, time dependent, and saturable. Specific (^{125}I-Tyr^4)BN binding to NCI-H1299 cells was inhibited with high affinity by GRP, BN, GRP$^{14-27}$, (D-Phe^6)BN$^{6-13}$methyl ester, moderate affinity by NMB, and low affinity by GRP$^{1-16}$. BN (10 nM) transiently elevated cytosolic calcium in a dose dependent manner. BN caused translocation of protein kinase C from the cytosol to the membrane and the translocation caused by BN was reversed by (D-Phe^6)BN$^{6-13}$methylester. BN stimulated arachidonic acid release and the increase caused by BN was reversed by (D-Phe^6)BN$^{6-13}$methylester. Using a clonogenic assay, BN stimulated the growth of NCI-H720 cells, and the number of colonies was reduced using (D-Phe^6)BN$^{6-13}$methylester. These data suggest that GRP receptors that are present in lung carcinoid and NSCLC cells may regulate proliferation. © 1996 Wiley-Liss, Inc.*

Key words: GRP receptor, cytosolic calcium, growth, arachidonic acid, protein kinase C

Numerous peptide receptors have been found in small cell lung cancer (SCLC) cells including bradykinin, cholecystokinin, gastrin releasing peptide, neuromedin B, neurotensin, opiate, somatostatin, vasopressin, and vasoactive intestinal peptide [1]. Two members of the bombesin (BN) family which function as autocrine growth factors in SCLC include gastrin releasing peptide (GRP) and neuromedin B (NMB). High levels of GRP immunoreactivity and mRNA are present in some classic SCLC cells such as NCI-H345 [2–4]. GRP is secreted from SCLC cells into conditioned medium [5]. GRP or BN binds with high affinity to NCI-H345 cells [6]. When GRP receptors are activated, phosphatidylinositol (PI) is turned over and cytosolic calcium (Ca^{2+}) is transiently elevated [7,8]. Also, BN may activate protein kinase C. While it is unknown which second messengers lead to proliferation, BN stimulates the clonal growth of SCLC cells in vitro and xenografts in vivo [9,10]. The actions of BN or GRP are reversed with high affinity by GRP receptor antagonists such as (D-Phe^6)BN$^{6-13}$methylester and (Psi^{13,14}, Leu^{14}) BN [11,12] and low affinity by substance P antagonists [13,14].

The GRP receptor has been cloned and is comprised of 7 hydrophobic domains and 384 amino acid residues [15,16]. It has 56% sequence homology with the NMB receptor which binds NMB but not GRP with high affinity [17]. Recently it was found that SCLC cells have NMB immunoreactivity and mRNA [18,19] and NMB receptors. NMB elevates the cytosolic Ca^{2+} and stimulates the growth of SCLC cells [20]. The actions of NMB are reversed with low affinity by (D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}) substance P but not GRP receptor antagonists.

GRP receptors have also recently been found on other tumor cell lines including breast cancer and prostate cancer [21,22]. Also, GRP immuno-

Abbreviations used: AA, arachidonic acid; BN, bombesin; Ca^{2+}, calcium; EGF, epidermal growth factor; (F)BN$^{6-13}$ME, (D-Phe^6)BN$^{6-13}$methylester; GRP, gastrin releasing peptide; NMB, neuromedin B; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer.

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reactivity is present in some NSCLC and lung carcinoid cell lines [19]. Further BN elevates the cytosolic Ca^{2+} in some NSCLC cell lines [23]. Here GRP receptors were characterized using lung carcinoid and non-small cell lung cancer (NSCLC) cell lines.

METHODS

SCLC cells were cultured as floating aggregates. They were cultured primarily in RPMI-1640 containing 10% heat inactivated fetal bovine serum [24]. The cells were split weekly by dilution 1/1 in new medium. The cells were mycoplasma free and were used when they were in exponential growth phase. NSCLC cells were cultured in RPMI-1640 containing 10% heat inactivated fetal bovine serum. When a monolayer formed, the adherent cells were washed with PBS and treated with trypsin/EDTA. The cells were pelleted and resuspended in serum supplemented medium and incubated at 37°C in 5% CO_2/95% air.

Receptor binding assays were conducted using ^{125}I-GRP or (^{125}I-Tyr^4) BN. The SCLC cells and NCI-H720 were harvested one day after a medium change and cells (2×10^6) were incubated with ^{125}I-GRP (0.1 nM) in the presence or absence of competitor; the receptor binding medium was SIT medium (RPMI-1640 containing 5 μg/ml bovine insulin, 10 μg/ml transferrin, and 3×10^{-8} M sodium selenite) with 0.25% bovine serum albumin and 250 μg/ml bacitracin. After incubation at 25°C for 20 min bound ^{125}I-GRP was separated from free using the centrifugation techniques described previously [6]. NSCLC cells such as NCI-H1299 (5×10^4) were placed in 24 well plates coated with human fibronectin (20 μg) as described previously [25]. When a monolayer of cells formed they were washed 4 times with SIT buffer followed by incubation in receptor binding medium. The cells were incubated with (^{125}I-Tyr^4)BN for 30 min at 37°C. Then they were washed 4 times in receptor binding medium at 4°C. The cells that contained bound peptide were dissolved in 0.2 N NaOH and counted in a gamma counter.

For the protein kinase C assay, cells (10^7) were harvested and washed twice in SIT medium (RPMI-1640 containing 3×10^{-8} M Na_2SeO_3, 5 μg/ml insulin and 10 μg/ml transferrin) with 20 mM HEPES.NaOH (pH 7.4). BN (10^{-8} M) was added and after 5 min at 37°C the cells centrifuged, washed once in PBS and lysed in ice-cold 20 mM Tris, 2 mM EDTA, 0.5 M EGTA, 5 mM dithiothreitol, 100 μM PMSF, and 10 μg/ml leupeptin (pH 7.4). The cells were centrifuged at 12,000*g* for 20 min at 4°C and the supernatant boiled for 5 min. The pellet was solubilized in 0.2% SDS, 20 mM Tris.HCl, and 10 μg/ml leupeptin (pH 7.4) at 4°C for 30 min and after a 5 min centrifugation at 12,000*g* for 30 min, the membrane extract was boiled for 5 min. Protein (100 μg) was separated on an 8% SDS polyacrylamide gel [26], transferred to nitrocellulose, and treated with protein kinase C antiserum (monoclonal antibody clone MC5, Amersham Corp., Arlington Heights, IL) using Western blot techniques [27].

For arachidonic acid release assays, (^{3}H-5,6,8,9,11,12,14,15)arachidonic acid (2.5×10^6 cpm) was added to NCI-H1299 cells in 24 well plates. After 16 h the cells were washed twice in 1 ml of SIT medium containing 0.2% fatty acid-free bovine serum albumin (SIT/BSA). New medium was added containing BN-like peptides. After 40 min, 100 μl of media was removed from each well, placed in a vial, scintillation fluid added and sample counted in a β-counter.

Calcium studies were performed using Fura-2AM and cell line NCI-H720. Cells were harvested and the cells (2.5×10^6/ml) incubated with Fura 2 AM at 37°C for 30 min [7]. The cells that contained loaded Fura 2 were centrifuged at 150*g* for 10 min and resuspended at the same concentration in new SIT medium. The fluorescence intensity was continuously monitored using a spectrofluorometer equipped with a magnetic stirring mechanism and temperature (37°C) regulated cuvette holder prior to and after the addition of peptide.

Using NCI-H1299 cells the cytosolic Ca^{2+} was assessed in individual cells. NCI-H1299 cells (10^4) were cultured on fibronectin-treated Lab-Tek coverslip chambers. The cells were fed one day prior to the experiment and on the day of the experiment the cells were rinsed with 1 ml of buffer (150 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl, 10 mM glucose, 1 mM $CaCl_2$, and 20 mM HEPES.NaOH (pH 7.4) containing 1% BSA. After 5 min at 37°C, the buffer was removed and new buffer added containing 5 μM Indo-1AM. The cells were incubated for 30 min at 37°C and the old buffer removed and the cells treated with new buffer which was $CaCl_2$ free. After 5 min at 37°C the cells were treated with new buffer and assayed for Ca^{2+} using an ACAS 570 Interactive Laser Cytometer (Meridian Instruments, Okemos, MI). The excitation wavelength was

320 nm and the fluorescence emission was monitored at 485 nm (Ca^{2+}-free emission) and 405 nm (Ca^{2+}-bound emission). The emission ratio was determined every 30 sec prior to and after the addition of peptide.

Growth studies were performed using cell lines NCI-H720 cells and the agarose cloning system described previously [12]. The base layer consisted of 3 ml of 0.5% agarose in SIT medium containing 5% fetal bovine serum in 6 well plates. The top layer consisted of 3 ml of SIT medium in 0.3% agarose, the peptide(s) doubly concentrated and 6×10^4 single viable cells. For each cell line and peptide concentration, triplicate wells were plated. After 2 weeks 1 ml of 0.1% p-iodonitrotetrazolium violet was added and after 16 h at 37°C the plates were screened for colony formation; the number of colonies larger than 100 μm in diameter were counted using an Omnicon image analysis system.

RESULTS

Table I shows that 42% of the SCLC cell lines and 32% of the NSCLC cell lines examined bound (^{125}I-Tyr^4)BN with high affinity. In contrast none of the seven SCLC cell lines examined bound ^{125}I-EGF with high affinity whereas 12 out of 13 NSCLC cell lines had a high density of ^{125}I-EGF binding sites. These data indicate that moderate densities of GRP receptors are present on both SCLC and NSCLC cell lines whereas high densities of EGF receptors are present on NSCLC cells.

Because cell line NCI-H720 and H1299 bound (^{125}I-Tyr^4)BN best their binding properties were examined in more detail. At 25°C, total ^{125}I-GRP binding to NCI-H720 increased rapidly the first 45 min and slowly thereafter (Fig. 1, left). In contrast, nonspecific binding increased slowly at all times examined. Also, the dissociation of bound ^{125}I-GRP was examined. After the addition of 1 μM GRP, specific binding was slowly reversed (Fig. 1, right). After approximately 90 min half of the specifically bound ^{125}I-GRP was reversed. These data indicate that ^{125}I-GRP binding to NCI-H720 cells is time dependent and reversible. Similar data were observed using NCI-H1299 cells.

^{125}I-GRP binding to NCI-H1299 cells was saturable. Figure 2 shows that ^{125}I-GRP was a linear function of radiolabeled GRP concentration at low concentrations (e.g., 1 nM) whereas at high concentrations such as 10 nM specific ^{125}I-GRP binding was saturable. A Scatchard plot of the specific binding data was linear. ^{125}I-GRP bound with high affinity (Kd = 1.5 nM) to a single class of sites (Bmax = 1,400/cell). Similar data were observed using NCI-H720 cells.

TABLE I. Binding to Lung Cancer Cell Lines*

Cell line	(^{125}I-Tyr^4)BN bound	^{125}I-EGF bound
SCLC		
NCI-H146	1.4	<5
NCI-H187	<1.0	<5
NCI-H209	<1.0	<5
NCI-H345	5.4	<5
NCI-H510	1.1	<5
NCI-H1092	<1.0	<5
NCI-H1694	<1.0	<5
NSCLC		
NCI-H157	<1.0	108
NCI-H838	<1.0	32
NCI-H1299	11.2	49
NCI-H1435	<1.0	27
NCI-H1573	1.1	347
NCI-H1623	<1.0	115
NCI-H1792	5.6	80
NCI-H2073	<1.0	29
NCI-H2342	<1.0	69
NCI-H2409	<1.0	97
NCI-H2444	<1.0	225
Carcinoid		
NCI-H720	6.0	<5
NCI-H727	<1.0	71

*The density of specific binding sites (fmol/mg protein) is indicated using 1 nM (^{125}I-Tyr^4)BN or (^{125}I-EGF) The mean value of 3 determinations is indicated. The S.E. was 10% of the mean value.

The specificity of (^{125}I-Tyr^4)BN binding was investigated using NCI-H1299 cells. Figure 3 shows that little specific (^{125}I-Tyr^4)BN binding was inhibited by 0.1 nM BN whereas almost all specific binding was inhibited by 1,000 nM BN. The IC_{50} value to inhibit half of the specific (^{125}I-Tyr^4)BN binding was 2 nM for BN. Also, GRP, GRP$^{14-27}$ and (D-Phe^6)BN$^{6-13}$methylester were potent inhibitors of (^{125}I-Tyr^4)BN binding with IC_{50} values of 5, 3, and 5 nM, respectively. GRP$^{1-16}$ did not inhibit specific (^{125}I-Tyr^4)BN binding and NMB had an IC_{50} value of 200 nM (data not shown). These data indicate that the C-terminal of BN or GRP is essential for high affinity binding activity to NCI-H1299 cells and similar data were obtained for NCI-H720 cells.

Previously it was shown that BN inhibited binding of ^{125}I-EGF to Swiss 3T3 cells [28]. Figure 4 shows that BN inhibited binding of ^{125}I-EGF to NCI-H1299 cells in a dose dependent manner and 1 μM BN inhibited approxi-

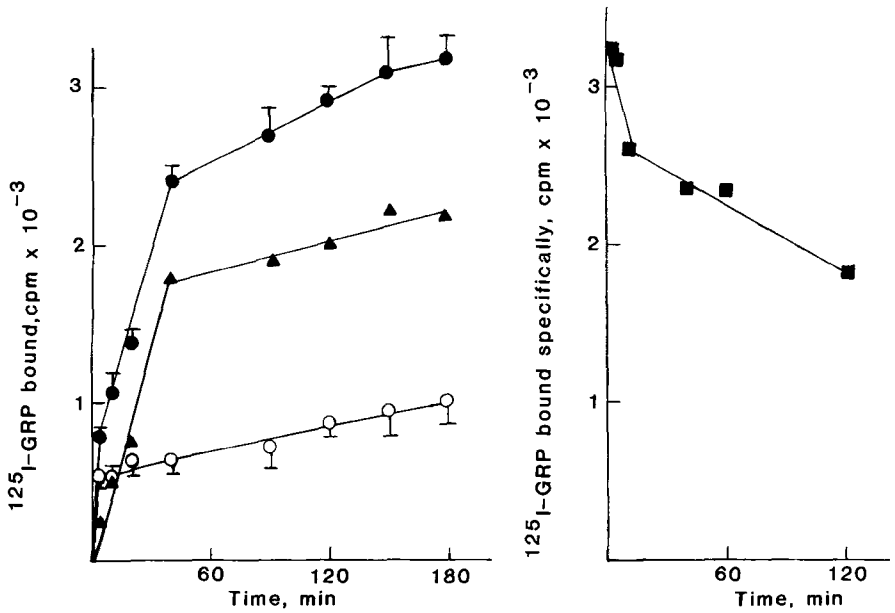


Fig. 1. Binding as a function of time. **Left:** The association of ^{125}I-GRP was determined in the absence (●) and presence (○) of 1 μM BN using NCI-H720 cells. The difference between the two represents specific binding (▲). **Right:** The dissociation of ^{125}I-GRP binding (■) was determined as a function of time after the addition of 1 μM BN.

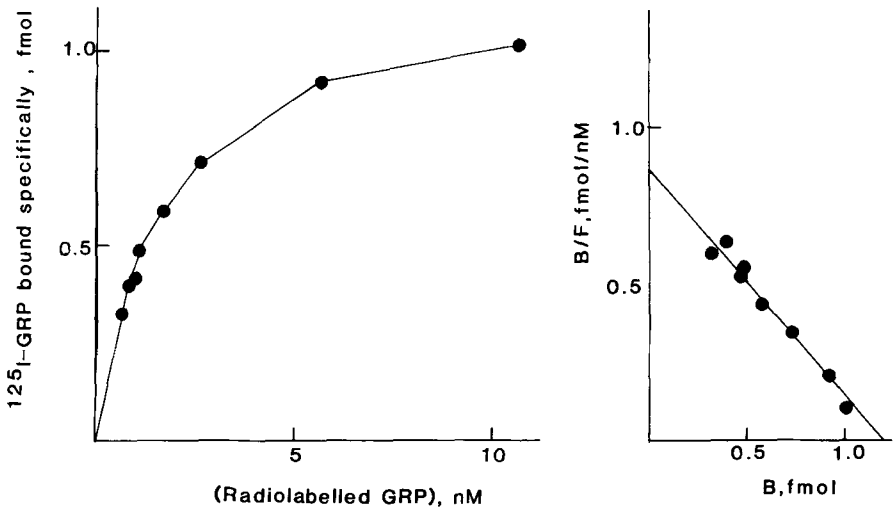


Fig. 2. Binding as a function of ligand concentration. **Left:** The amount of ^{125}I-GRP bound (●) was determined as a function of radiolabelled GRP concentration using NCI-H720 cells. **Right:** Scatchard plot of the specific binding data.

mately 40% of the ^{125}I-EGF binding at 37°C. In contrast, 300 nM EGF inhibited all specific ^{125}I-EGF binding and the IC_{50} value was 3 nM. Also, BN had no effect on ^{125}I-EGF binding at 4°C, whereas at EGF strongly inhibited ^{125}I-EGF binding at 4°C or 37°C (data not shown). These data suggest that the effects of BN on ^{125}I-EGF binding are indirect and occur only at 37°C whereas EGF competitively competes for ^{125}I-EGF binding sites.

BN may stimulate protein kinase C activity resulting in phosphorylation of the EGF receptor. Figure 5 shows that protein kinase C was normally localized to the cytosol. Addition of 10 nM BN caused translocation of some of the protein kinase C from the cytosol to the membrane whereas phorbol ester caused total translocation of PKC to the membrane [29]. The translocation of protein kinase C caused by BN was reversed by the GRP receptor antagonist (D-Phe^6)BN$^{6-13}$methylester.

Also BN released cytosolic Ca^{2+} from intracellular pools. Figure 6 shows that using Fura-2AM loaded SCLC cells BN increased the cyto-

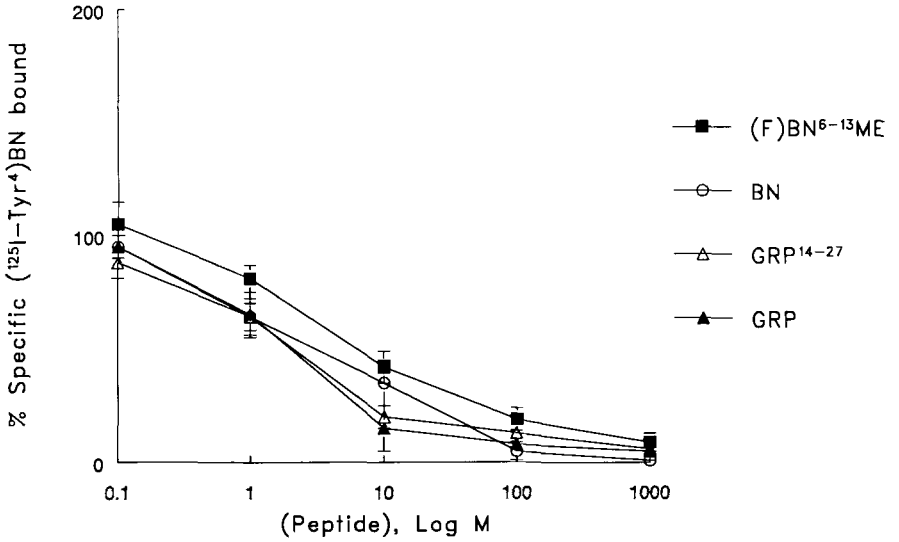


Fig. 3. Specificity of ^{125}I-GRP binding. The amount of specific ^{125}I-GRP bound was determined as a function of BN (○), GRP (▲), GRP$^{14-27}$ (△), and (D-Phe^6)BN$^{6-13}$methylester (■) concentration. The mean value ± S.E. of 3 determinations is indicated.

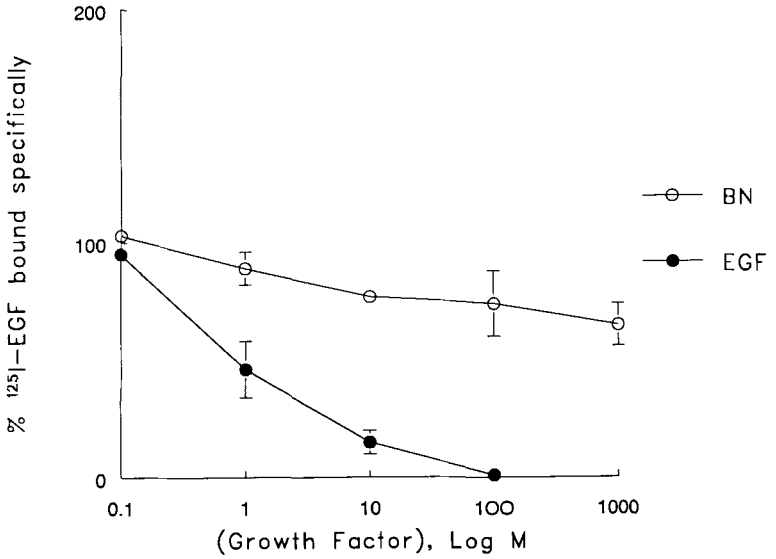


Fig. 4. ^{125}I-EGF binding. The amount of ^{125}I-EGF bound was determined as a function of EGF (●) and BN (○) concentration using NCI-H1299 cells.

solic Ca^{2+} in a dose dependent manner using NCI-H720 cells. The basal Ca^{2+} was 150 nM and 100 nM BN transiently increased the cytosolic Ca^{2+} from 150 to 180 nM. Even 0.1 nM BN, however, caused a weak Ca^{2+} response. The ability of other BN-like peptides to increase cytosolic Ca^{2+} was investigated. Table II shows that BN, GRP, GRP$^{14-27}$, and Ac-GRP$^{20-28}$ strongly increased cytosolic Ca^{2+} suggesting that the C-terminal octapeptide of GRP was the smallest fragment which could cause a strong biological response. In contrast, BN$^{8-14}$ caused a weak Ca^{2+} response whereas BN$^{9-14}$ or GRP$^{1-16}$ were inactive.

The ability of BN to alter Ca^{2+} in individual NCI-H1299 was assessed. Cells were loaded with Indo-1 AM and the cytosolic Ca^{2+} increased in most cells 30 sec after addition of 10 nM BN (Fig. 7). After 60 sec almost all cells responded to BN. Figure 8 shows that when the Ca^{2+} was quantitated in individual NCI-H1299 cells a steady baseline was obtained prior to the addition of BN. After the addition of BN at 2.5 min, the cytosolic Ca^{2+} was increased in 6 of 8 cells after 30 sec and it increased in the remaining 2 cells 60 and 90 sec after the addition of BN, respectively. At the 5 min timepoint the cyto-

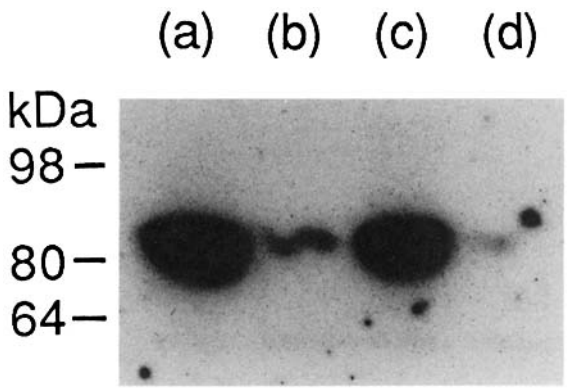


Fig. 5. Protein kinase C Western blot. The cytosol (a), and membrane extract (b) of NCI-H345 cells treated with 10 nM BN is indicated. If cells were treated with 10 nM BN + 1 μM (D-Phe^6)BN$^{6-13}$ME the amount of PKC in the membrane (d) but not cytosol (c) is reduced.

solic Ca^{2+} had returned to baseline in 3 cells and was still slightly elevated in the 5 other cells. The increase in cytosoic Ca^{2+} caused by BN was reversed by antagonists such as (D-Phe^6)BN$^{6-13}$methyl ester (data not shown). Routinely approximately 90% of the cells responded to BN.

BN analogues stimulated arachidonic acid release from NCI-H1299 cells. The rate of ^{3}H-arachidonic acid released increased approximately 6-fold by 100 or 1,000 nM BN (Table III). Also, GRP and GRP$^{14-27}$ but not GRP$^{1-16}$ increased significantly arachidonic acid release. These data indicate that the C-terminal of GRP is essential for arachidonic acid release. Also, (D-Phe^6)BN$^{6-13}$ME inhibited the increase in arachidonic acid release caused by 100 nM BN in a dose dependent manner. (D-Phe^6)BN$^{6-13}$ME had no effect on basal arachidonic acid release (data not shown).

BN stimulated the clonal growth of NCI-H720 cells. The effect was dose dependent with 1 and 10 nM BN but not 0.1 nM significantly stimulating colony formation (Table IV). Also, 1 μM (D-Phe^6)BN$^{6-13}$ME significantly inhibited the increase in colony formation caused by 10 nM BN.

Previously we found that GRP binds with high affinity to SCLC cells, elevates cytosolic Ca^{2+}, and stimulates the growth of SCLC cells [12]. Also, most classic SCLC cells have high levels of GRP whereas only a few NSCLC neuroendocrine and carcinoid cell lines have immunoreactive GRP. Because some lung carcinoids have GRP receptors and GRP immunoreactivity, GRP may be an autocrine growth factor for lung carcinoids [30]. Here we report that some NSCLC cells have biologically active GRP receptors.

The lung carcinoid cell line NCI-H720, which is nonadherent, bound ^{125}I-GRP with high affinity. Binding was saturable, time dependent, and reversible at 25°C. Also, the adherent large cell lung cancer cell line NCI-H1299 bound ^{125}I-GRP saturably. At 4°C ^{125}I-GRP bound to the cell surface whereas at 37°C ^{125}I-GRP binding sites were readily internalized. Either ^{125}I-GRP or (^{125}I-Tyr^4)BN could be used as a receptor probe with similar results. Specific ^{125}I-GRP binding was inhibited with high affinity by BN, GRP, GRP$^{14-27}$ but not GRP$^{1-16}$, suggesting that the C-terminal of GRP is essential for high affinity binding activity. ^{125}I-GRP binding to NCI-H1299 or H720 cells was inhibited with high affinity by Ac-GRP$^{20-27}$, moderate affinity by BN$^{8-14}$, and low affinity by BN$^{9-14}$ suggesting that the C-terminal 8 amino acids of BN was the minimal fragment required for full biological potency. ^{125}I-GRP binding was inhibited with high affinity by antagonists such as (D-Phe^6)BN$^{6-13}$ME and moderate affinity by NMB (IC_{50} = 200 nM). Recently, we also found that some NSCLC cells such as NCI-H1299 bind (^{125}I-Tyr^0)NMB with high affinity (Moody, unpublished results). In contrast specific (^{125}I-Tyr^0) NMB binding is inhibited with high affinity by NMB and BN, moderate affinity by GRP and low affinity by (D-Phe^6)BN$^{6-13}$ME. These data suggest that NSCLC cells, similar to SCLC cells [6,20], have GRP and NMB receptors.

SCLC GRP receptors are coupled to a guanine nucleotide binding protein which stimulates phosphatidylinositol turnover. The inositol 1,4,5-trisphosphate, resulting from phosphatidylinositol-bisphosphate metabolism by phospholipase C, may release Ca^{2+} from intracellular organelles such as the endoplasmic reticulum. Here BN elevated cytosolic Ca^{2+} in both NCI-H720 and H-1299 cells. It is interesting to note that in NCI-H1299 cells cytosolic Ca^{2+} was increased after addition of 10 nM BN in almost all cells examined. These data suggest that GRP receptors are uniformly expressed in NCI-H1299 cells which like all lung cancer cells are derived from patient biopsy specimens. Also the diacylglycerol released from PI turnover may activate protein kinase C. Here we found that approximately 10% of the protein kinase C underwent

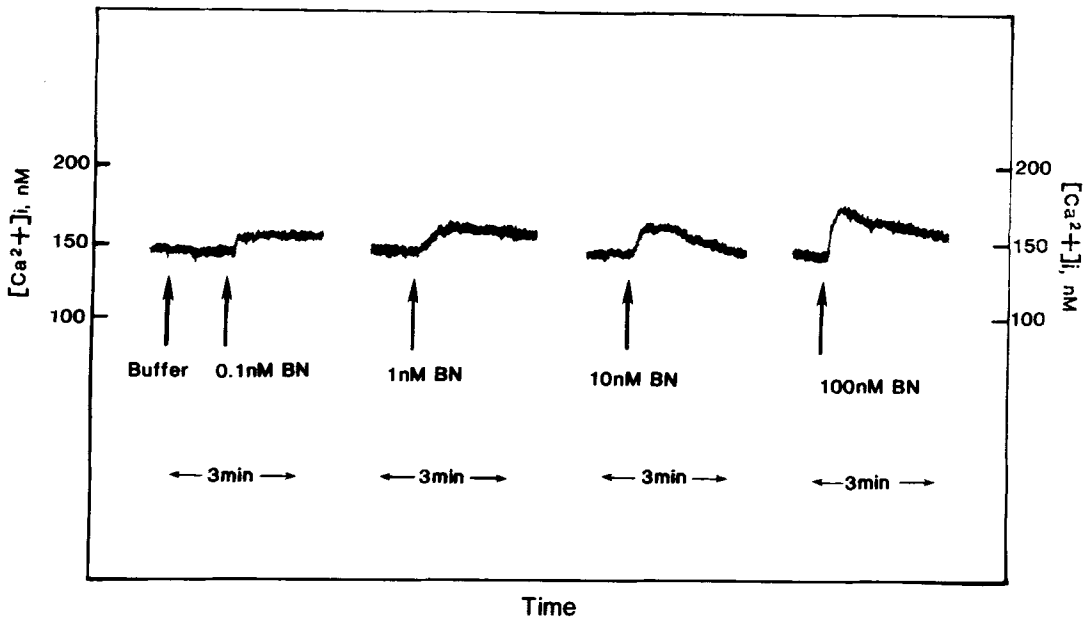


Fig. 6. Cytosolic Ca^{2+} in NCI-H720. The cytosolic Ca^{2+} was determined in Fura-2AM loaded NCI-H720 cells using 0.1 nM BN, 1 nM BN, 10 nM BN and 100 nM BN. This experiment is representative of 2 others.

TABLE II. Effect of BN-Like Peptides on Cytosolic Ca^{2+}*

Peptide	Cytosolic Ca^{2+}
BN	+
GRP	+
GRP^{14-27}	+
Ac-GRP^{20-28}	+
BN^{8-14}	±
BN^{9-14}	−
GRP^{1-16}	−

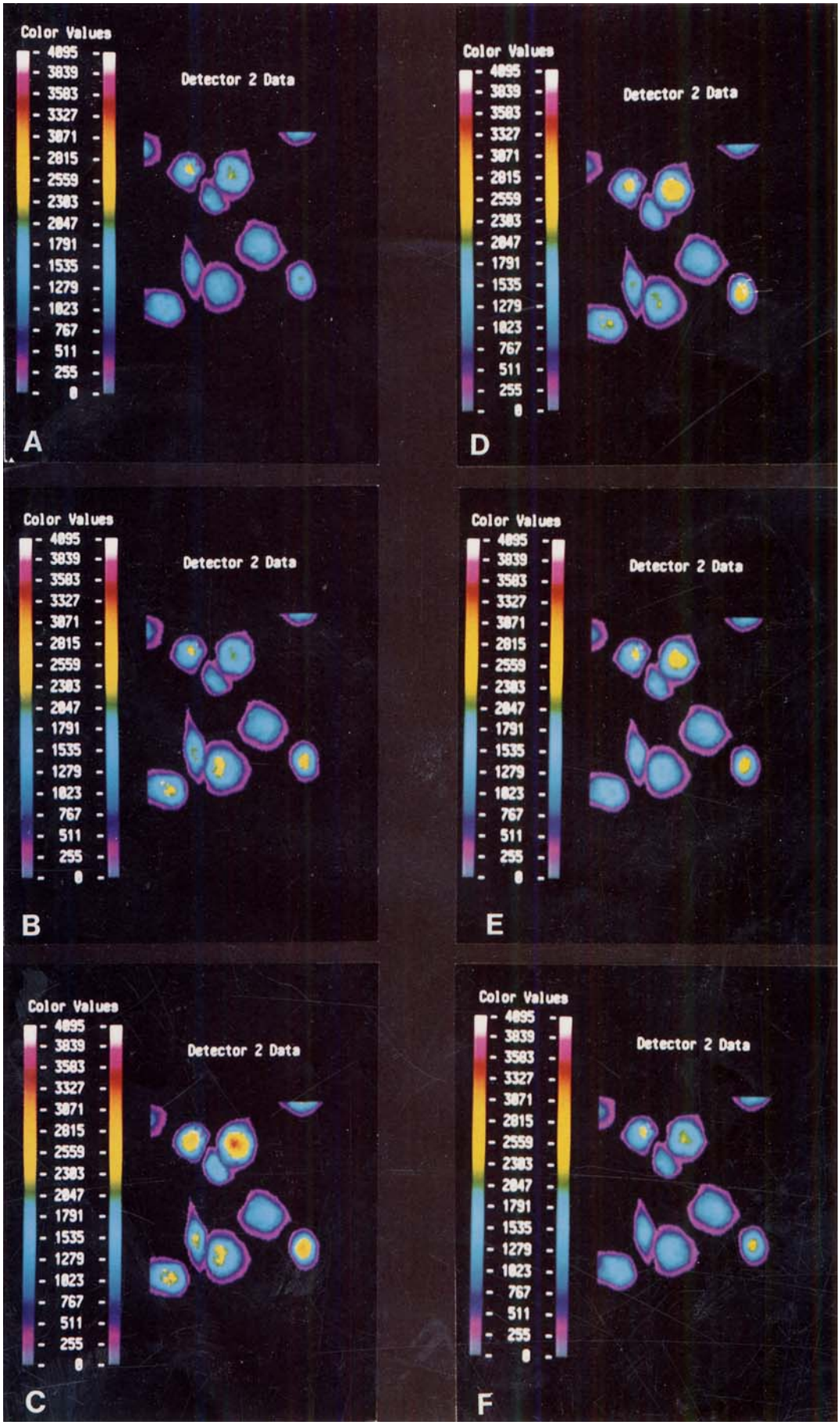
*The ability of BN-like peptides (10 nM) to elevate cytosolic Ca^{2+} in NCI-H720 cells was determined; strong response, +; weak response, ±; no response, −. Each peptide was tested on 3 different occasions using Fura-2 AM loaded NCI-H720 cells.

translocation from the cytosol to the membrane after addition of 10 nM BN and that the translocation caused by BN was reversed by (D-Phe^6)BN^{6-13}ME. Although the effects of BN on cytosolic Ca^{2+} and protein kinase C translocation are small they are highly reproducible and represent valuable assays to assay for biological activity of BN-like peptides. Previously, we found that all of the protein kinase C was translocated by 1 μM PMA and that the PKC translocated by PMA was reversed by H7 [29]. Similar Ca^{2+} and PKC results were observed for NCI-H345 and H720 cells.

The increase in cytosolic Ca^{2+} and protein kinase C translocation caused by BN was rapid and occurred with 5 min. In contrast, after 45 min BN stimulated phospholipase A_2 resulting in ^{3}H-arachidonic acid release. BN, GRP, GRP^{14-27} but not GRP^{1-16} significantly stimulated ^{3}H-arachidonic acid release. The increase in arachidonic acid release caused by 100 nM BN was significantly inhibited by 10 μM (D-Phe^6)BN^{6-13}ME. Recently, we found that BN stimulates c-fos gene expression after 1 h in SCLC cells [31] and it remains to be determined if BN elevates c-fos mRNA in NCI-H720 or H1299 cells.

BN stimulates the clonal growth of NCI-H720 cells and the increase in growth caused by BN is reversed by receptor antagonists such as (D-Phe^6)BN^{6-13}ME. Similar results [11] were previously obtained for SCLC cell line NCI-H345 in vitro. Also, BN stimulated SCLC xenograft formation in vivo and GRP receptor antagonists such as ($Psi^{13,14}$,Leu^{14})BN slowed xenograft formation in vivo. It remains to be determined if GRP receptor antagonists slow NSCLC xenograft formation in vivo.

BN weakly decreased ^{125}I-EGF binding to NCI-H1299 cells. Previously, we found that ^{125}I-EGF binding was strongly inhibited by monoclonal antibody (mAb) 108 [32]. MAb-108, in contrast to EGF, was not internalized by NSCLC cells and did not stimulate tyrosine kinase activity. Previously it was hypothesized that in Swiss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Detector 2 Data
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Figure 7.

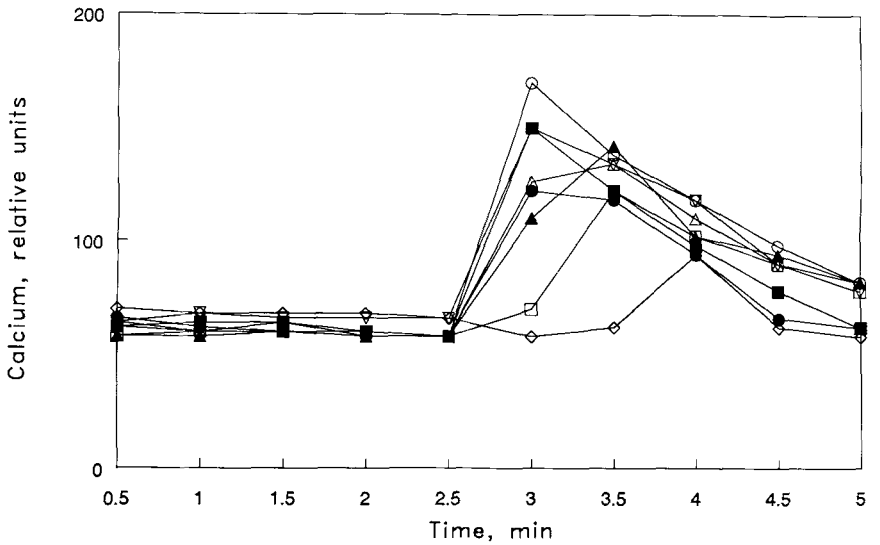

Fig. 8. Cytosolic Ca^{2+} in single NCI-H1299 cells. The cytosolic Ca^{2+} was determined in individual cells as a function of time. BN (10 nM) was added at 2.5 min.

TABLE III. Effect of BN Analogues on NCI-H1299 Arachidonic Acid Release

Addition	cpm
None	105 ± 19
BN, 1,000 nM	734 ± 122**
GRP, 1,000 nM	784 ± 112**
GRP^{1-16}, 1,000 nM	124 ± 29
GRP^{14-27}, 1,000 nM	523 ± 44**
NMB, 1,000 nM	299 ± 134*
BN, 100 nM	784 ± 149**
BN + (D-Phe^6)BN^{6-13}ME, 0.1 μM	701 ± 142**
BN + (D-Phe^6)BN^{6-13}ME, 1 μM	561 ± 51**
BN + (D-Phe^6)BN^{6-13}ME, 10 μM	309 ± 47*

*$P < 0.05$, **$P < 0.01$; the mean value ± S.D. of 4 determinations is indicated relative to control.

TABLE IV. Effect of BN on NCI-H720 Clonal Growth

Additions	Colonies
None	23.1 ± 7.5
BN, 0.1 nM	39 ± 3.9
BN, 1 nM	71.3 ± 12*
BN, 10 nM	101.4 ± 2.1**
BN, 10 nM + (D-Phe^6)BN^{6-13}ME, 1 μM	34.7 ± 3.0

*$P < 0.05$, **$P < 0.01$; the mean value + S.E. of 3 determinations is indicated.

3T3 cells, high densities of both GRP and EGF receptors (approximately 100,000/cell), BN stimulated PKC resulting in phosphorylation of Thr^{654} of the EGF receptor, causing EGF receptor desensitization [28]. Here even though cell line NCI-H1299 has only 1,400 GRP receptors and 100,000 EGF receptors/cell, BN still significantly inhibited EGF receptor binding. It remains to be determined if activation of GRP receptors alters the ability of EGF to stimulate cellular proliferation.

In summary these data indicate that GRP receptors are biologically active in NSCLC cells.

Fig. 7. Cytosolic Ca^{2+} in NCI-H1299. The cytosolic Ca^{2+} was determined in Indo-1 AM loaded NCI-H1299 cells. BN (10 nM) was added and the relative color value determined after **(A)** 0 s, **(B)** 30 s, **(C)** 60 s, **(D)** 90 s, **(E)** 120 s, and **(F)** 150 s.

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