Vol. 176, No. 1, 1991
April 15, 1991

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 79-86

DISTINGUISHING BOMBESIN RECEPTOR SUBTYPES USING THE OOCYTE ASSAY

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Received January 29, 1991

SUMMARY: Physiological responses to mammalian bombesin-like peptides were studied in Xenopus oocytes injected with mRNA isolated from Swiss 3T3 cells and rat esophagus in order to identify and characterize bombesin receptor subtypes. Both groups respond similarly to either gastrin releasing peptide or neuromedin B, but only the response to neuromedin B in oocytes expressing the esophagus mRNA is not blocked by a specific gastrin releasing pepride receptor antagonist, des-Met-[D-Phe^6]Bn(6-13) ethyl ester. Complete desensitization of gastrin releasing peptide-evoked responses in oocytes expressing esophagus mRNA does not abolish neuromedin B-evoked responses. A single application of neuromedin B abolishes responses to subsequently applied gastrin releasing peptide in oocytes expressing esophagus, but not Swiss 3T3, mRNA. RNA blot hybridization studies using a Swiss 3T3 gastrin releasing peptide receptor cDNA probe show no detectable hybridization in esophagus mRNA samples. These data suggest that a gastrin releasing peptide receptor is expressed in the esophagus and that it is distinct from that expressed in Swiss 3T3 cells and may represent a third subtype of mammalian bombesin receptor.

Mammalian bombesin-like peptides, gastrin-releasing peptide and neuromedin B, as well as their receptors, are distributed in various tissues and cell types, where they mediate a wide range of biological activities. These include neurotransmission, stimulation of secretion and cell proliferation (for review see 1). Agonist preference and the effects of antagonists on both binding and biologic responses have identified two receptor subtypes selectively expressed in different tissues. Esophageal smooth muscle expresses a NMB preferring receptor type, while pancreatic acinar cells and Swiss 3T3 cells possess a GRP preferring receptor type (2-7).

Abbreviations: NMB, neuromedian B; GRP, gastrin-releasing peptide.

0006-291X/91 $1.50

Bombesin receptors are coupled to a signal transduction mechanism which includes G-protein activation, polyphosphoinositide breakdown, IP_3 elevation and calcium mobilization (8-11). Activation of this pathway in Xenopus oocytes results in a depolarizing current due to Ca^{2+}-dependent chloride channels opening in the plasma membrane (12-14). Activation of exogenous bombesin receptors in oocytes induce Cl^- currents and Ca^{2+} efflux (15-16). In this study bombesin receptors from different sources are expressed in Xenopus oocytes to define the functional characteristics of different receptor subtypes.

MATERIALS AND METHODS

Oocyte preparation: Adult Xenopus females, purchased from Xenopus I, were kept at 19-21°C in a 12 hrs light/12 hrs dark cycle, and fed diced chicken once a week. The frogs were anesthetized on ice and ovary fragments were dissected in ND96 calcium-free medium (96 mM NaCl, 2 mM KCl, 1 mM MgCl, 5 mM Na-Hepes pH 7.5). Defolliculation was carried out by incubating the oocytes for 1-2 hrs in collagenase (2mg/ml, in ND96 Ca-free) at room temperature with shaking. Selected stage 5-6 oocytes were kept in NDE (ND96 +1.8mM $CaCl_2$ + 100U/ml penicillin and 100ug streptomycin) at 20°C.

RNA preparation and blot analysis: Poly A^+ mRNA was prepared from rat esophagus tissue and the Swiss 3T3 fibroblast cell line after homogenization in guanidine thiocyanate (17) using methods previously described (18). After oligo dT chromatography, mRNA was resuspended at 1 ug/ul in water, stored at -70°C and used for microinjections. For RNA blot analysis, 3 ug of poly A^+ mRNA was resolved by electrophoresis on a 1% formaldehyde agarose gel, capillary blotted to nitrocellulose, and hybridized to nick-translated cDNA fragments encoding either the Swiss 3T3 GRP receptor (19, 20), or rat esophageal NMB receptor (21), using methods described previously (18). After hybridization filters were washed twice for 15 minutes at room temperature in 0.3 M NaCl, 0.03 M Na-Citrate pH 7.0, 0.1% SDS, followed by two high stringency washes for 15 minutes at 60°C in 0.015 M NaCl, 0.0015 N Na-Citrate pH 7.0, 0.1% SDS. Filters then were exposed to Kodak XAR film for two days at -70°C using intensifying screens to detect hybridizing RNA species.

Microinjection: Oocytes were microinjected with 50 ng of mRNA, using Picospritzer II (General Valve, New York) and incubated for 2-4 days in NDE medium at 20°C.

Electrophysiology: Oocytes were placed in a 0.250 ml perfusion bath and voltage clamped at -60 mv, using Axoclamp-2A (Axon Instrument) as described elsewhere (12-13). Ligands were added rapidly and directly to the bath. When treated with antagonist, oocytes were incubated for 30 minutes with 10 uM of the antagonist before exposure to a mixture of agonist (1 uM) and antagonist (10 uM). The antagonist itself has no effect on the membrane potential. All experiments were repeated several times in oocytes from different frogs.

Chemicals: Collagenase (type II) was purchased from Sigma, gastrin-releasing peptide (GRP_{14-27}) and neuromedin B from Peninsula, and penicillin-streptomycin from Gibco Laboratories. The antagonist des-Met-[D-Phe^6]Bn(6-13) ethyl ester was synthesized as described (22). All other chemicals were of analytical grade.

RESULTS AND DISCUSSION

Antagonist studies: Oocytes injected with Swiss 3T3 mRNA respond to micromolar concentrations of either GRP or NMB (fig. 1A,B - left tracings). These responses are similar to those evoked by bombesin (15) and are generally composed of two depolarizing phases (14): rapid (D1) and slow (D2) with or without oscillations (see fig. 1A). Exposure of the oocytes to 10 uM of des-Met [D-Phe^6]Bn(6-13) ethyl ester, a specific antagonist for the GRP receptor in the pancreas (22), completely blocks both the GRP- and the NMB-evoked responses in these oocytes (fig. 1A,B, right tracings). These observations are consistent with a previous report (7) identifying the bombesin receptor subtype on Swiss 3T3 as a GRP-preferring receptor type.

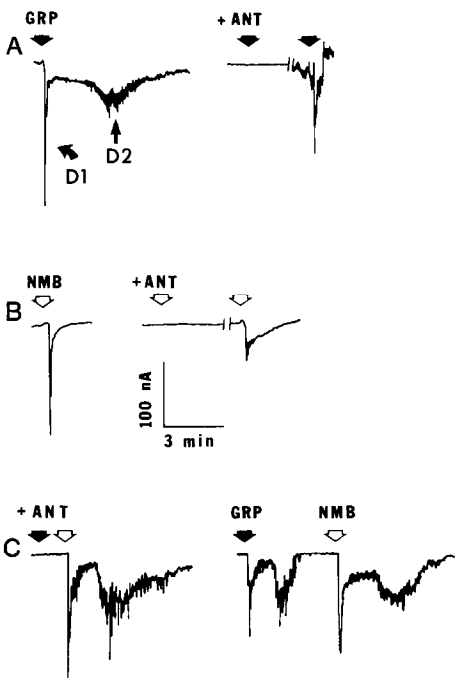


Fig. 1. The effect of des-Met [D-Phe^6]BN(6-13) ethyl ester on GRP- and NMB-evoked responses in oocytes injected with either Swiss 3T3 (A,B) or rat esophagus (C) mRNA. Applications of 1 μM GRP or NMB are denoted by black and white arrows, respectively. +ANT marks the presence of 10 µM des-Met [D-Phe^6]BN(6-13) ethyl ester, after 30 minutes of preincubation. A - GRP-evoked responses in Swiss 3T3 mRNA injected oocytes: left tracing - control; right tracing - in the presence of the antagonist and again without antagonist, after three hours of washing. B - NMB-evoked responses in Swiss 3T3 mRNA injected oocytes: left tracing - control; right tracing - in the presence of the antagonist and again without antagonist, after four hours of washing. C - GRP- and NMB-evoked responses in esophagus mRNA injected oocyte: left tracing - in the presence of antagonist; right tracing - after seven hours of washing.

Table 1. The effect of the antagonist des-Met [D-Phe^6]Bn(6-13) ethyl ester on the GRP- and NMB-evoked responses in oocytes injected with either Swiss 3T3 or rat esophagus mRNA

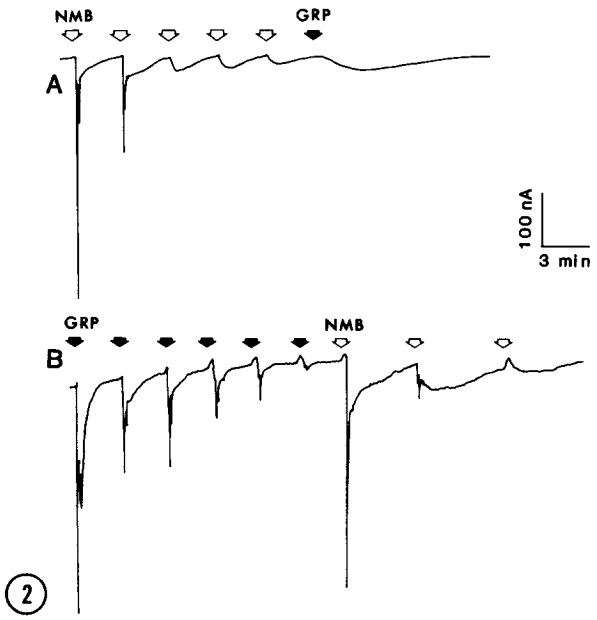
mRNA (50 ng)	Agonist (1μM)		Control oocytes		+Antagonist	
		phase	Amplitude (nA)	n:N	Amplitude (nA)	n:N
Swiss 3T3	GRP	D1	95±21	35:7	2±1	11:4
		D2	50±6		3±2	
Swiss 3T3	NMB	D1	126±32	13:3	0	3:1
		D2	45±19		0	
esophagus	GRP	D1	106±24	18:4	10±5	10:3
		D2	32±5		8±3	
	NMB	D1	255±47	66:5	128±35	9:4
		D2	90±18		77±15	

D1 and D2 are the fast and slow phases of the response, respectively (see fig. 1A). All experiments were repeated several times in oocytes from different frogs, injected with different samples of mRNAs. The number of oocytes assayed for each condition is denoted by n, and the number of different donors by N. The average amplitudes of the responses with SEM values were calculated.

Oocytes injected with rat esophagus mRNA similarly respond to 1 uM of either GRP or NMB (fig. 1C, right tracing). According to a previous report (22), the esophageal NMB-preferring receptor is not affected by the antagonist des-Met [D-Phe^6]Bn(6-13) ethyl ester. However, exposure of these oocytes to 10 uM of this antagonist abolishes the GRP-evoked responses but not the NMB-evoked responses (fig. 1C, left tracing).

Table 1 summarizes the responses to GRP and NMB in oocytes injected with either Swiss 3T3 or rat esophagus mRNA and the effect of the antagonist des-Met [D-Phe^6]Bn(6-13) ethyl ester on these responses. In Swiss 3T3 mRNA injected oocytes both GRP- and NMB-evoked responses are completely blocked by the antagonist. In contrast, the antagonist blocks 90% of the GRP-evoked response and 50% of the NMB-evoked response in esophagus mRNA injected oocytes. This difference implies that in addition to the antagonist-insensitive NMB-preferring subtype previously described in esophageal muscle (5), there may also be an antagonist-sensitive GRP-preferring subtype in the esophagus.

Homologus and heterologus desensitization. In either Swiss 3T3 or esophageal mRNA injected oocytes, repeated challenges with the same agonist cause a gradual desensitization of the response (fig. 2). A second challenge with the same agonist yields responses with about half the magnitude of the first response (see also fig. 3a,b,d,e). The rapid phase of the response (D1) is usually the first to be desensitized. The slow phase (D2) shows less desensitization. In Swiss 3T3 mRNA injected oocytes, successful desensitization of D1, by repeated applications of one agonist,



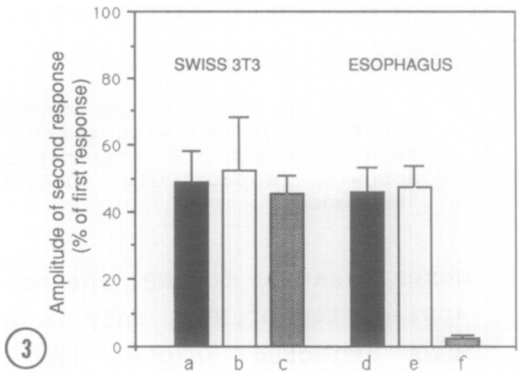


Fig. 2. Cross desensitization between NMB- and GRP-evoked responses in oocytes injected with either Swiss 3T3 (A) or rat esophagus (B) mRNA. Applications of 1 μM NMB are marked with white arrows, and of 1 μM GRP with black arrows. Oocytes were treated as described in "methods".

Fig. 3. Desensitization of the response by a second challenge with agonist in oocytes injected with Swiss 3T3 (a-c) or esophagus (d-f) mRNA. The response is expressed as a percent of the first response in the same oocyte. Black columns indicate two challenges with GRP. Empty columns indicate two challenges with NMB. Gray columns indicate alternate challenges: first to NMB and the second to GRP. Agonists concentrations were 1 μM. The second challenge was performed only after membrane current returned to basal level, but not more than 6 minutes after the first challenge. Every experiment is an average of at least 6 oocytes.

resulted in a fully desensitized D1 phase of the response to the other agonist (fig. 2A). In contrast, in oocytes injected with esophageal mRNA, a typical response to NMB can still be elicited after desensitization to GRP (fig. 2B). These data indicate that only one receptor type is found in Swiss 3T3 and both GRP and NMB receptor types are seen in rat esophagus.

Desensitization studies using alternating exposures to GRP and NMB show that the GRP receptor types in Swiss 3T3 and esophagus mRNA behave differently. When Swiss 3T3 mRNA injected oocytes are exposed to alternating applications of GRP and NMB, both GRP- and NMB-evoked responses are gradually desensitized (fig. 4A), as in the case of repeated applications of one agonist alone. In esophageal mRNA injected oocytes, however, although responding when first exposed to GRP, no further response to GRP can be elicited after a single application of NMB (fig. 4B). Despite complete

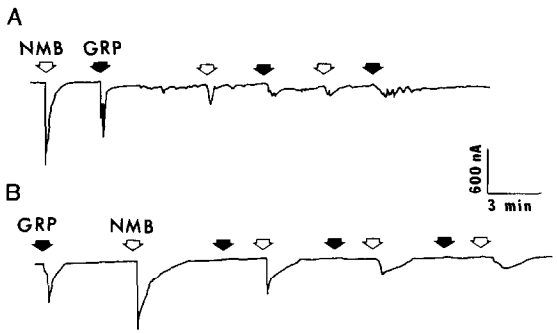


Fig. 4. Alternating applications of GRP and NMB (1 μM) in oocytes injected with either Swiss 3T3 (A) or rat esophagus (B) mRNA. GRP and NMB applications are marked with black and white arrows, respectively. Oocytes were treated as described in "methods".

desenitization to GRP, the oocytes remain responsive to subsequent application of NMB. Only in the esophagus (and not in Swiss 3T3) mRNA injected oocytes the GRP-evoked response is completely desensitized by a single application of NMB (fig. 3c,f). These functional differences suggest that the GRP-preferring receptor type in the Swiss 3T3 cells is not identical to the esophageal GRP receptor type. This conclusion is supported by the Northern blot analysis of the two receptor subtypes.

RNA blot analysis: A recently isolated cDNA clone, encoding the Swiss 3T3 GRP-preferring receptor (19), was used as a probe in RNA

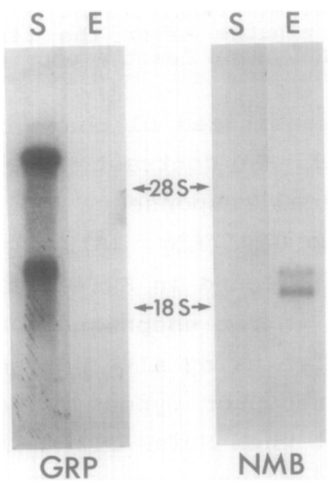


Fig. 5. Northern blot of Swiss 3T3 (S) and rat esophagus (E) mRNA, using cloned cDNA encoding either Swiss 3T3 GRP (left) or rat esophagus NMB (right) receptor types as probes. Two species (7.2 and 3.1 kb) which hybridize to the Swiss 3T3 GRP receptor probe in Swiss 3T3 mRNA (left, S) are not detectable in esophageal mRNA (left, E). On the other hand, two bands (3.0 and 2.8 kb) which hybridize to the esophageal NMB receptor probe in esophageal mRNA (right, E) are not seen in Swiss 3T3 mRNA (right, S).

blotting studies to identify transcripts enconding the Swiss 3T3 GRP receptor (fig. 5 left). Hybridization of mRNA isolated from Swiss 3T3 cells with the Swiss 3T3 GRP cDNA clone resulted in two bands of about 7.2 and 3.1 kb (fig. 5 left, S), as had been observed in previous studies (19, 20). Hybridization of mRNA isolated from rat esophagus with the same probe shows no detectable hybridized RNA (fig. 5 left, E). Ten-fold dilution of the Swiss 3T3 mRNA sample still shows the two hybridizing bands on the RNA blot, while no electrophysiologic response can be detected in the oocytes (not shown). These results indicate that the RNA blot is a more sensitive assay for small amounts of the Swiss 3T3 GRP receptor mRNA than the oocytes assay. Thus, the GRP-evoked response in oocytes injected with esophagus mRNA is probably not due to Swiss 3T3 GRP receptor mRNA undetectable in the RNA blot. When a cDNA clone derived from the NMB-preferring receptor (21) is used as a hybridizing probe (fig. 5 right), no mRNA hybridizing to the NMB-preferring probe is found in Swiss 3T3 (fig. 5 right, S), while hybridizing mRNA (3.2 and 2.8 kb) is clearly present in the esophagus (fig.5 right, E) as observed previously (21). These data are consistent with the conclusion that esophagus mRNA encodes an NMB-preferring bombesin receptor previously defined by cDNA cloning, but the GRP-preferring receptor in the esophagus differs from that identified in Swiss 3T3 cells.

Conclusions: Three criteria were used to identify bombesin receptor subtypes in oocytes injected with Swiss 3T3 or esophageal mRNA. These are: (1) sensitivity of the agonist-evoked responses to a specific GRP receptor antagonist, des-Met-[D-Phe^6]Bn(6-13) ethyl ester; (2) cross desensitization between GRP- and NMB-evoked responses; and (3) RNA blot hybridization using cDNA probes for previously identified Swiss 3T3 GRP and esophageal NMB receptors. By using a specific GRP receptor antagonist we have distinguished NMB-and GRP-preferring receptor types in the esophagus. However, cross desensitization and RNA hybridization analyses indicate that the GRP receptor type in the esophagus differs from the GRP receptor type in Swiss 3T3, suggesting that more than one GRP-preferring receptor subtype exists.

ACKNOWLEDGMENTS: We would like to thank Dr. Harold Gainer for many helpful discussions regarding the design of this study and preparation of the manuscript. In addition, we thank Dr. Ben Szaro for his suggestions on the manuscript.

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