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Neuromedin B receptor, expressed in *Xenopus laevis* oocytes, selectively couples to $G_{\alpha q}$ and not $G_{\alpha 11}$

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Abstract

G-proteins of the q family have been implicated as mediators of bombesin receptors action. We cloned *Xenopus* $G_{\alpha q}$ and $G_{\alpha 11}$ and specifically disrupted the synthesis of either protein with selective antisense oligonucleotides. $G_{\alpha q}$ antisense inhibited responses mediated by neuromedin B receptor (NMB-R) by 74%, though not by gastrin-releasing peptide receptor (GRP-R). $G_{\alpha 11}$ antisense had little effect on either GRP-R- or NMB-R-mediated responses. This suggests that NMB-R couples to $G_{\alpha q}$, and that GRP-R and NMB-R show distinct G-protein coupling preferences in the *Xenopus* oocyte.

Key words: G-protein; Neuromedin B receptor; Gastrin-releasing peptide receptor; *Xenopus* oocyte

1. Introduction

Members of the G_q class of G protein α-subunits [1,2], have been implicated as transducers of the pertussis toxin-insensitive activation of phosphoinositide-specific phospholipase C (PI-PLC) [3–8]. This signal transduction pathway has been shown to be activated by bombesin receptor subtypes, gastrin releasing peptide receptor (GRP-R) [9–11] and neuromedin B receptor (NMB-R) [12]. $G_{\alpha q}$ and $G_{\alpha 11}$, the most abundant members of the G_q family, show high degree of homology (88% identity at the protein level) and appear to be co-expressed in most tissues [1,2,13,14]. Transfection experiments demonstrated PI-PLC activation by either $G_{\alpha q}$ or $G_{\alpha 11}$ [15]. Activation by IL-8 or muscarinic m1 or m3 receptors of these G proteins failed to functionally distinguish between $G_{\alpha q}$ and $G_{\alpha 11}$ [16–19].

Mammalian bombesin-like peptides, GRP and NMB, regulate numerous physiologic processes including stimulation of hormone secretion, smooth muscle contraction, thermoregulation (for review see [20]) and also the pathogenesis and progression of human small cell lung cancer [21]. GRP-R and NMB-R have been cloned and pharmacologically characterized after expression in *Xenopus* oocytes [22–24]. Both GRP-R [9–11] and NMB-R [12] use a signal transduction pathway involving coupling to a PTX-insensitive G protein(s) that activates PI-PLC. Based on these similarities in pharmacology and signal transduction, we chose the *Xenopus* oocyte expression system combined with the antisense approach to study the interactions of *Xenopus* $G_{\alpha q}$ and $G_{\alpha 11}$ with mammalian GRP-R and NMB-R in an intact cell.

Antisense oligonucleotides have been successfully used in rat pituitary GH_3 cells to demonstrate that $G_{\alpha 01}$ and $G_{\alpha 02}$ couple inhibitory responses mediated by muscarinic and somatostatin receptors, respectively [25] and in *Xenopus* oocytes, to determine the subtypes of endogenous muscarinic receptors [26]. These studies provided precedents for using the antisense-induced protein depletion approach to define the specificity of receptor–G protein coupling.

2. Materials and methods

2.1. *Cloning of* Xenopus $G_{\alpha q}$ *and* $G_{\alpha 11}$

Total RNA was isolated from *Xenopus laevis* oocytes and the polyadenylated fraction was selected by oligo(dT)-cellulose chromatography using established methods [27]. A hexamer-primed cDNA library was constructed in λgt10 using established methods [27], except for the size-fractionation of the cDNA prior to the ligation to the λgt10 vector, which was carried out by Sepharose 4B chromatography rather than by polyacrylamide gel electrophoresis. Approximately 0.5×10^6 plaques were screened with a ^{32}P-labeled mouse $G_{\alpha q}$ probe, obtained from a murine Swiss 3T3 poly(A)$^+$ RNA by reverse transcription followed by gene-specific PCR amplification. The probe included the entire 359 amino acid open reading frame, with no flanking 5′ and 3′ untranslated sequences. Low stringency hybridization was performed overnight at 37°C as previously described [24]. Filters were washed three times at room temperature for 15 min in 1 × SSC, 0.1% SDS and then at 37°C for 20 min in 0.1 × SSC, 0.1% SDS, dried and autoradiographed. Subcloned cDNA inserts were sequenced on both strands in a thermal cycler using both vector and insert-specific primers as recommended by the supplier (FMOL, Promega, Madison, WI). Nucleotide sequence analysis was performed using the Sequence Analysis Software Package of the University of Wisconsin Genetics Groups and a Vax computer [28].

2.2. *RNA analysis*

Groups of 50 *Xenopus* oocytes were microinjected with 50 ng of either $G_{\alpha q}$ or $G_{\alpha 11}$ antisense S-oligo (Oligos Etc. Ltd.). Control group was uninjected. Three hours later they were transferred into 8 ml of cold

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The full sequences of oocyte $G_{\alpha q}$ and $G_{\alpha 11}$ will be submitted to the Gene Bank upon the acceptance of this manuscript for publication.

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guanidinisothiocyanate (GIT) buffer and RNA was isolated on CsCl gradient, as described elsewhere [27]. RNA samples (10 μg of total RNA) were resolved by electrophoresis on agarose/formaldehyde gel and blotted onto nitrocellulose membranes using standard methodology [24]. Filters were hybridized to ^{32}P-labeled *Xenopus* oocyte $G_{\alpha q}$ and $G_{\alpha 11}$ cDNA probes as described previously [24]. Blots were washed at high stringency (3 × 10 min washes at room temperature in 1 × SSC/0.1% SDS, and one 15 min wash at 60°C in 0.1 × SSC/0.1% SDS), dried and autoradiographed. The mobility of 28S (5 kb) and 18S (2 kb) RNAs were determined by ethidium bromide staining.

2.3. Functional assay

Oocytes were excised from *Xenopus laevis* frogs (*Xenopus* I) and defolliculated with 2 mg/ml collagenase (Boehringer-Mannheim) in ND96 calcium-free buffer as described previously [29]. Oocytes were microinjected with 1–10 ng of RNA in vitro transcribed from cDNAs encoding either NMB-R [24] or GRP-R [23] and kept in ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.5) at 20°C. 24 h later, the same oocytes were injected with 5–50 ng of $G_{\alpha q}$ of $G_{\alpha 11}$ antisense S-oligos (Oligos Etc. Inc.). Control oocytes were injected with water. Three hours following the injection of antisense oligonucleotides, individual oocytes were placed in a perfusion chamber, voltage-clamped at −100 mV and membrane currents were continuously recorded [29]. 10^{-6} M neuromedin B (an agonist of both bombesin receptor subtypes) in ND96 was added directly to the perfusion chamber.

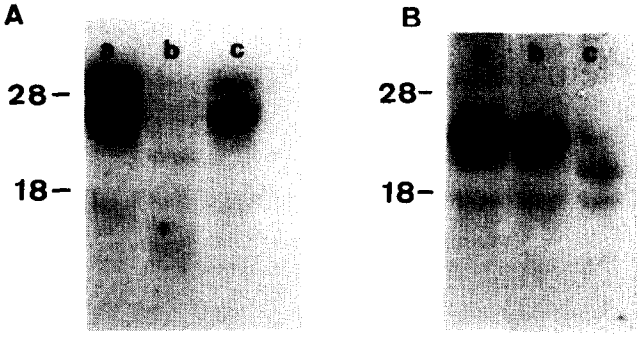

Fig. 2. Specific degradation of *Xenopus* oocyte $G_{\alpha q}$ or $G_{\alpha 11}$ mRNA induced by corresponding antisense S-oligos, as determined by RNA blot hybridization analysis. Total RNA from control oocytes (lane a) and oocytes microinjected with $G_{\alpha q}$ (lane b) or $G_{\alpha 11}$ (lane c) antisense S-oligos were resolved on agarose/formaldehyde gel and hybridized with oocyte $G_{\alpha q}$ (A) or $G_{\alpha 11}$ (B) probes. A 4.2 kb band of $G_{\alpha q}$ (Aa) and a 3.4 kb band of $G_{\alpha 11}$ (Bb) are present in control oocytes. $G_{\alpha q}$ antisense caused the degradation of the $G_{\alpha q}$ band (Ab) and not the $G_{\alpha 11}$ band (Bb). Conversely, $G_{\alpha 11}$ antisense caused the degradation of the $G_{\alpha 11}$ (Bc) band and not the $G_{\alpha q}$ band (Ac). In both cases, small amounts of a breakdown product of about 2.7–2.8 kb were detected.

3. Results and discussion

To design specific antisense oligonucleotides, we have cloned *Xenopus* oocytes cDNAs encoding $G_{\alpha q}$ and $G_{\alpha q}$. We screened 5×10^5 members of a *Xenopus laevis* oocyte cDNA library with a mouse $G_{\alpha q}$ probe [1] at low stringency, and plaque-purified hybridizing cDNAs which were subsequently subcloned and sequenced. Alignment of the predicted amino acid sequences of the corresponding *Xenopus* proteins with mouse $G_{\alpha q}$ and $G_{\alpha 11}$ revealed very high degree of homology ($G_{\alpha q}$, 96% identity; $G_{\alpha 11}$, 92% identity). The *Xenopus* $G_{\alpha q}$ and $G_{\alpha 11}$ amino acid sequences (Fig. 1) were 89% identical, the same level of identity observed in mouse $G_{\alpha q}$ and $G_{\alpha 11}$ [1]. At the nucleotide sequence level, the two cDNAs were identical in 78% of the bases in the 1077 base open reading frame (data not shown). Northern analysis of *Xenopus* oocytes poly(A)$^+$ RNA, using *Xenopus* $G_{\alpha q}$ and $G_{\alpha 11}$ cDNA probes and high stringency hybridization conditions, detected hybridizing bands of about 4.2 kb for $G_{\alpha q}$ (Fig. 2A, lane a) and 3.4 kb for $G_{\alpha 11}$ (Fig. 2B, lane a), confirming that both transcripts are present at detectable levels in *Xenopus* oocyte mRNA.

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Q  1 MTLESIMACCLSEEAKEARRINDEIERQLRRDKRDARRELKLLLLGTGES 50
     |||:|.|||||||||.||.:|||.|||:|||||||:|.|||||||||||||
11 1 MTLDSTMACCLSEEVKESKRINAEIEKQLRRDKKDSRRELKLLLLGTGES 50

  51 GKSTFIKQMRIIHGSGYSDEDKRGFTKLVYQNIFTAMQAMIRAMETLKIP 100
     ||||||||||||||||||:|||:|||||||:||||||||.||||||||||
  51 GKSTFIKQMRIIHGSGYSEEDKKGFTKLVFQNIFTAMQSMIRAMETLKIL 100

 101 YKYEHNKGHALLVREVDVEKVASFENPYVDAIKYLWNDPGIQECYDRRRE 150
     ||||:||::| :||||||||||..||.|||:||| ||.|||||||||||||
 101 YKYEQNKANAQVVREVDVEKVCTFEQPYVNAIKNLWSDPGIQECYDRRRE 150

 151 YQLSDSTKYYLNDVDRIATQGYLPTQQDVLRVRVPTTGIIEYPFDLQSVI 200
     ||||||||||.||||||...|||||||||||||||||||||||||:.:|
 151 YQLSDSTKYYLTDVDRISKPGYLPTQQDVLRVRVPTTGIIEYPFDLENII 200

 201 FRMVDVGGQRSERRKWIHCFENVTSIMFLVALSEYDQVLVESDNENRMEE 250
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 201 FRMVDVGGQRSERRKWIHCFENVTSIMFLVALSEYDQVLVESDNENRMEE 250

 251 SKALFRTIITYPWFQNSSVILFLNKKDLLEEKIMYSHLVDYFPEYDGPQR 300
     |||||||||||||||||||||||||||||:||||||||||||||:|||||
 251 SKALFRTIITYPWFQNSSVILFLNKKDLLEDKIMYSHLVDYFPEFDGPQR 300

 301 DAQAAREFILKMFVDLNPDSDKIIYSHFTCATDTENIRFVFAAVKDTILQ 350
     ||..|||||||||||||||||||||||||||||||||||||||||||||||
 301 DAATAREFILKMFVDLNPDSDKIIYSHFTCATDTENIRFVFAAVKDTILQ 350

 351 LNLKEYNLV* 360
     |||||||||
 351 HNLKEYNLV* 360
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Fig. 1. Predicted amino acid sequences of *Xenopus* oocyte $G_{\alpha q}$ (upper lane) and $G_{\alpha 11}$ (lower lane).

In order to assess the role of $G_{\alpha q}$ and $G_{\alpha 11}$ in mediating GRP-R and NMB-R-induced responses in the oocytes, we designed discriminating antisense oligonucleotides complementary to a region of the $G_{\alpha q}/G_{\alpha 11}$ cDNAs (sequence encoding amino acids 120–126; Fig. 1), whose nucleotide sequence was only 45% identical:

$G_{\alpha q}$ antisense: 5′-ATTCTCAAAAGAGGCGACC-3′
$G_{\alpha 11}$ antisense: 5′-CTGTTCAAAGGTACATACT-3′

To test antisense-induced specific RNA degradation, oocytes were microinjected with 50 ng of either $G_{\alpha q}$ or $G_{\alpha 11}$ antisense phosphorothioate oligonucleotides (S-oligos). Three hours after injection of the relevant antisense, Northern analysis did not detect bands of either $G_{\alpha q}$ (Fig. 2A, lane b) or $G_{\alpha 11}$ (Fig. 2B, lane c). The ablation of transcripts was specific. $G_{\alpha q}$ antisense did not interfere with $G_{\alpha 11}$ RNA (Fig. 2A, lane c), while $G_{\alpha 11}$ antisense did not interfere with $G_{\alpha q}$ RNA (Fig. 2B, lane b).

Oocytes expressing either the NMB-R or the GRP-R (10 ng of transcripts were microinjected the day before) were microinjected with either S-oligo antisense and 3–7 h later were tested for characteristic agonist-induced membrane electrical responses, as previously described

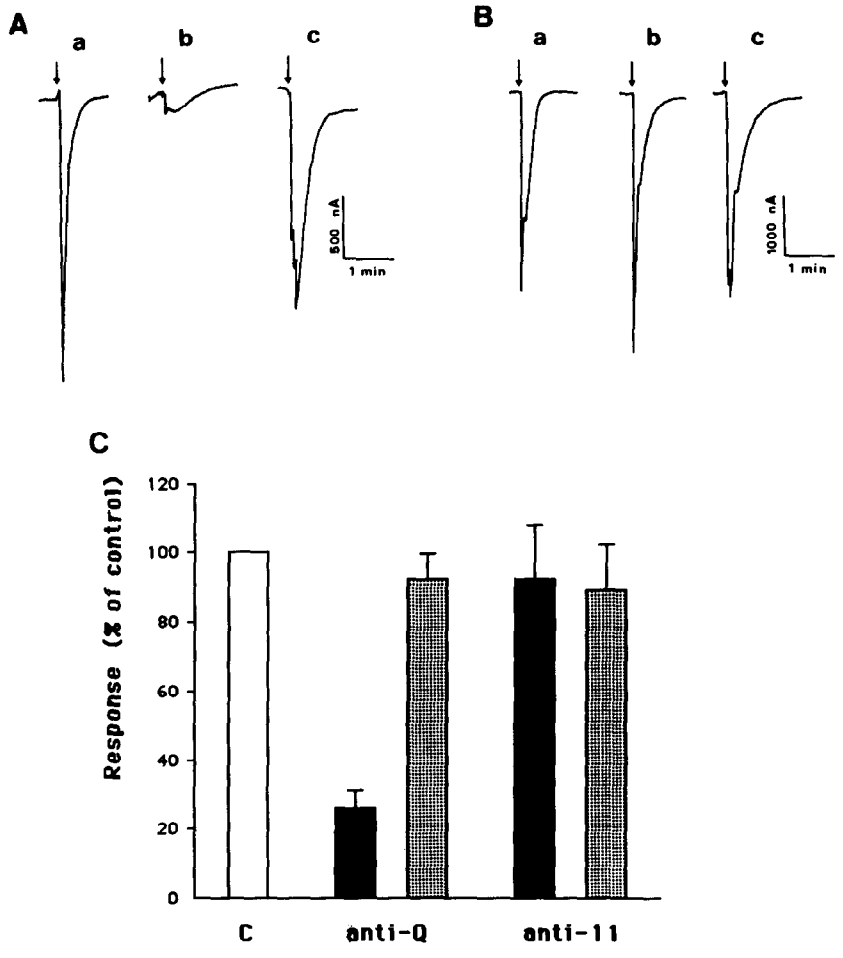

Fig. 3. Effects of specific $G_{\alpha q}$ or $G_{\alpha 11}$ antisense oligonucleotides on NMB-induced chloride currents, in oocytes expressing either NMB-R or GRP-R. (A,B) Traces of representative individual responses; A = NMB-R, and B = GRP-R-mediated responses, respectively. (a) Control; (b) antiQ-S-oligo-injected; c anti11-S-oligo-injected oocyte. (C) Summary of all experiments. In panel C, the open bar represents control responses mediated by either receptor (C). Black bars = responses in oocytes injected with NMB-R RNA; stippled bars = responses in oocytes injected with GRP-R RNA.

[29]. Representative individual traces are shown in Fig. 3A,B and the summary of experiments is shown in Fig. 3C. $G_{\alpha q}$ antisense inhibited NMB-R-mediated responses by 74% (26.3 ± 5.0% of control response, 56 oocytes from 9 donors; Fig. 3C). In most cases, the rapid component of the response (D1) was completely abolished and the amplitude of the slow component (D2) was significantly inhibited, as shown in Fig. 3A. GRP-R mediated responses were not affected by the $G_{\alpha q}$ antisense (91.4 ± 8.1% of control responses, 127 oocytes from 14 donors; Fig. 3C). The magnitude of the effects observed with either S-oligo was not affected by the amplitude of the response to NMB, nor by the amounts of RNA injected (1–10 ng/oocyte, not shown). Hence, inhibition of $G_{\alpha q}$ synthesis affected the responses mediated by NMB-R but not by GRP-R. $G_{\alpha 11}$ antisense S-oligo had virtually no effect on either GRP-R- or NMB-R-mediated responses (89.3 ± 13.0% and 92.4 ± 15.3% of control responses, respectively; Fig. 3C). To test whether the NMB-R-induced response was mediated by both $G_{\alpha q}$ and $G_{\alpha 11}$, we injected a mixture of both S-oligos. This treatment, however, did not further inhibit the response observed with $G_{\alpha q}$ antisense alone (not shown). Hence, efficient NMB-R coupling was sensitive to $G_{\alpha q}$ depletion, but not $G_{\alpha 11}$ depletion.

Our results strongly suggest that two bombesin receptor subtypes that utilize a similar signal transduction pathway, appear to couple to different G proteins. Furthermore, despite their remarkable structural similarity (89% amino acid identity), endogenous $G_{\alpha q}$ and $G_{\alpha 11}$ do not appear to be functionally interchangeable for NMB-R coupling.

The identity of the G-protein used to couple the GRP-R to PI-PLC is unclear. Despite extensive mRNA degradation, it is still possible that the residual protein level adequately supports GRP-R coupling. Another possibility is that GRP-R also couples through $G_{\alpha q}$, but that the affinity of the receptor/G protein interaction is higher for GRP-R than NMB-R. Thus, a small amount of residual $G_{\alpha q}$ remaining after antisense depletion was sufficient

to couple GRP-R but not NMB-R. Alternatively, the GRP-R may use neither $G_{\alpha q}$ nor $G_{\alpha 11}$ for coupling. Moriarty et al. [30] and Blitzer et al. [31], using microinjection of the purified protein subunits into *Xenopus* oocytes, have proposed that $G_{\alpha o}$ mediates responses to serotonin and α_{1B}-adrenergic receptors expressed in oocytes. Their conclusions were supported by an antisense experiment, in which oocytes were assayed 24 h after its injection. We have, therefore, injected an antisense S-oligo complementary to a unique oocyte $G_{\alpha o}$ sequence [32] (encoding the first 6 amino acids at the amino-terminus). This treatment had no effect on either NMB-R- or GRP-R-mediated responses up to 7 h after the injection of the oligonucleotide (not shown). Although in many target cells the effect of bombesin is PTX-insensitive [9], Moriarty et al. [33] reported that in *Xenopus* oocytes bombesin-evoked responses were inhibited by PTX. In our hands, functional assay for GRP-R and NMB-R in oocytes was PTX-insensitive (not shown), and exhibited a pharmacological profile similar to that observed in the tissues of origin [23,24,29]. We have no ready explanation for these discrepancies.

In an earlier study, Lipinsky et al. [34] examined the effects of co-expressing either mouse $G_{\alpha q}$ or $G_{\alpha 11}$ with mouse thyrotropin releasing hormone (TRH) receptor in *Xenopus* oocytes, where co-expression of mouse $G_{\alpha 11}$, but not $G_{\alpha q}$, potentiated responses. These observations are consistent with the conclusion of our study; although $G_{\alpha q}$ and $G_{\alpha 11}$ are structurally very similar, they are not interchangeable for receptor coupling. Hence, this is the first demonstration of functional differences between native $G_{\alpha q}$ and $G_{\alpha 11}$ in an intact cell.

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