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Short Communication

Peptide fragment of the m3 muscarinic acetylcholine receptor activates G_q but not G_{i2}

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Abstract: G_q, a heterotrimeric guanine nucleotide-binding protein, plays important roles such as the regulation of calcium mobilization and cell proliferation. This protein is considered as a promising drug target for the treatment of cardiac hypertrophy. Selective activation of G_q would be quite useful for analyzing the role of G_q in signaling pathways. We synthesized m3i3c–a peptide with 16 amino acid residues that corresponds to the junction between the *C*-terminus of the third intracellular loop and the sixth transmembrane helix (TM-VI) of human m3 muscarinic acetylcholine receptor, which couples to G_q but not G_{i2}. At micromolar concentrations, this peptide was found to activate G_q but not G_{i2}. This peptide is the first small compound that selectively activates G_q but not G_{i2}. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: GTP-binding proteins; G protein-coupled receptors; muscarinic receptor m3; peptide fragments

INTRODUCTION

Heterotrimeric G proteins function in transducing signals from GPCRs to effector systems such as adenylyl cyclase, cyclic guanosine monophosphate (cGMP) phosphodiesterase, phospholipase C, and ion channels [1]. In addition to the classical roles (of G_s and G_i) of regulating adenylyl cyclase, many other functions of G proteins have been demonstrated thus far (e.g. regulation of transcriptional and motility machineries) [2]. Low-molecular-weight compounds that can directly and selectively activate one of the four G protein families (G_s, $G_{i/o}$, G_q, and G_{12}) would be quite useful in analyzing the involvement of that particular G protein in a given signaling pathway. In fact, mastoparan (a peptide of 14 amino acid residues that activates $G_{i/o}$ but not G_s or G_t) [3,4] has successfully been employed for demonstrating the involvement of $G_{i/o}$ in intracellular trafficking [5] and programmed cell death of cerebellar granule neurons [6].

Such a low-molecular-weight and direct activator has not yet been found for G_q, although this protein plays important roles such as the regulation of calcium mobilization and cell proliferation [7]. In fact G_q is considered as a promising drug target for the treatment of myocardial hypertrophy [8]. In the present study, we designed a 16-residue peptide, namely, m3i3c, as a candidate that can activate G_q by referring to the common motif observed in G_q-coupled muscarinic acetylcholine receptors (mAChRs). Here, we demonstrate that m3i3c potently activates G_q but not G_{i2} at all. Although many researchers report the activation of G proteins by the fragment peptide of GPCRs, the G proteins activated were limited to G_s [9,10] and $G_{i/o}$ [11]. The present report is the first one that demonstrates the activation of G_q by a peptide fragment of a GPCR.

MATERIALS AND METHODS

Extraction of G_q from Bovine Brain Membranes

As it is difficult to prepare recombinant G_q proteins in large amounts [12] and recombinant G_q may lack modifications normally occurring in natural proteins, we used bovine brain membranes as the source of G_q proteins. Bovine brain membranes were prepared as described previously [13] and extracted in an extraction buffer [100 mM Hepes-KOH (pH 8.0), 50 mM NaCl, 0.1 mM $MgSO_4$, 10 mM dithiothreitol (DTT), 50 μM guanosine diphosphate (GDP), 5 μg/ml pepstatin A, 5 μg/ml E-64, and 0.7% CHAPSO (Dojindo)] at 4 °C for 60 min. The mixture was ultracentrifuged at 70 000 × G for 60 min, and the resultant supernatant was recovered and stored at −80 °C until use.

GTPγS-Binding Assay of G_q

Activation of G_q (enhancement of GDP release and subsequent GTPγS binding) was assayed by quantifying a trypsin-resistant

Abbreviations: G protein, guanine nucleotide-binding protein; GPCR, G protein-coupled receptor; AChR, acetylcholine receptor; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]2-hydroxypropanesulfonate; i2, second intracellular loop; i3N, *N*-terminal portion of the third intracellular loop; i3C, *C*-terminal portion of the third intracellular loop; TM-VI, sixth transmembrane helix.

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band of G_q in the GTPγS-bound state with an antibody specific for G_q. A similar assay was used to analyze the effects of mutations in G_t on the interaction with rhodopsin [14]. These assays are based on the property of α subunits to resist trypsin digestion in the GTPγS-bound state and be recognized as a band slightly smaller (by approximately 3 kDa) than the native protein, in contrast to the protein in the GDP-bound state that can be extensively digested by the protease.

The membrane extract was preincubated in a binding buffer [100 mM Hepes-KOH (pH 8.0), 150 mM NaCl, 10 mM DTT, and 0.1 mM GDP] supplemented with 10 mM $MgSO_4$ and 0.0005% Lubrol PX (Nacalai) (unless otherwise specified) at 37 °C for 60 min. GTPγS (Roche Diagnostics, 0.1 mM) and various concentrations of the peptide were then added, and the mixture was incubated at 37 °C for 2 h to facilitate the binding of GTPγS to G_q. The concentration of CHAPSO in this reaction mixture was 0.35% because the membranes were mixed in the ratio 1 : 1 (v/v) with 2× binding buffer. The reaction mixtures were then treated with tosyl-phenylalanyl-chloromethyl ketone-treated trypsin (0.1 mg/ml) in the presence of additional GDP (2.5 mM; for the termination of GTPγS binding) on ice for 2 h. After the addition of soybean trypsin inhibitor (0.5 mg/ml) and 4-Amidinophenylmethanesulfonyl fluoride hydrochloride (2.5 mg/ml), the reaction mixtures were separated on a 9.5% SDS-PAGE gel. The gel was blotted on nitrocellulose membranes, and the membranes were visualized with anti-G_q (Sigma) or anti-G_{i2} (LabVision) antibodies. The trypsin-resistant bands were quantified using NIH-image software. Intensities in the presence of m3i3c were compared with that in the absence of m3i3c. *P*-values <0.05 (Student's paired *t*-test) were considered significant.

RESULTS

Design of the G_q-Activating Peptide

Figure 1(a) shows the amino acid sequences of m1–m5 AChRs at the junction between the *C*-terminal portion of the third intracellular loop (i3C) and the TM-VI. The m1/m3/m5 AChRs, which couple to G_q, contain AA and LS amino acid pairs in common, whereas in the m2/m4 AChRs that couple to $G_{i/o}$, these AA and LS pairs are replaced by VT and IL(F) pairs, respectively [15]. For the latter AChRs, the VT/IL(F) motif is known to be the structural element that determines the specificity of the receptors to $G_{i/o}$ [16]. With regard to the m1/m3/m5 receptors, the AA/LS motif is known to be the element in i3C that determines the receptors' specificity to G_q in addition to the elements in the second intracellular loop (i2) and the *N*-terminal portion of the third intracellular loop (i3N) [16]. As the sequence encompassing this AA/LS motif has been suggested to form an amphiphilic helix [16] and since mastoparan has been demonstrated to form an amphiphilic helix when bound to G protein α subunits [3,17], the peptide containing the AA/LS motif is expected to activate G_q. The 16-residue peptide thus designed, m3i3c (Figure 1(b)), corresponds to the residues 483–493 of the human m3 AChR [18].

Requirement of Stabilizing Detergent for the GTPγS-Binding Assay of G_q

For detection of the GTPγS-bound trypsin-resistant band of G_q, the extracted G_q had to be supplemented with additional detergent; we used Lubrol PX, which was found to be optimal. The CHAPSO extract from brain membranes was incubated with GTPγS, treated with trypsin, and detected with anti-G_q antibody. Although the trypsin-resistant band was expected at a position slightly lower than the native protein (Figure 2(a), right lane), no band was observed (Figure 2(a), left lane). We suspected that G_q is not stable in the buffer containing CHAPSO (0.35%); hence, we added other detergents to the reaction mixture. Figure 2(b) shows the effects of various detergents on the appearance of the trypsin-resistant band. The detergents were added at a concentration slightly above their cmc (except for Lubrol PX) to confirm that the effects are due to detergent micelles rather than monomers. Although the trypsin-resistant band was not observed when the buffer supplemented with octylglucoside was

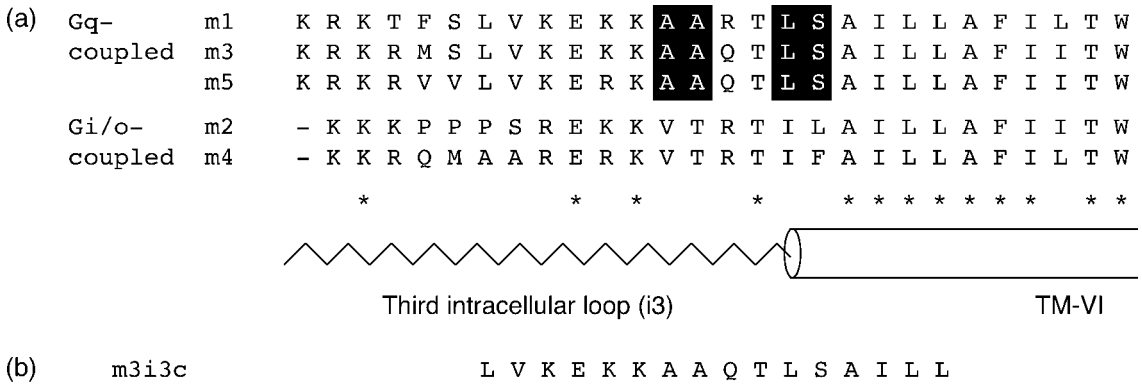

Figure 1 Human mAChR sequences at the i3 loop/TM-VI junction. (a) Residues conserved in all five subtypes are annotated with asterisks. Residues specifically conserved in G_q-coupled subtypes are indicated in white on a black background. The tentative position of the sixth transmembrane helix (TM-VI) is drawn as a cylinder. (b) Amino acid sequence of the m3i3c peptide. The NCBI accession numbers of human mAChR are as follows: m1, P11229; m2, P08172; m3, P20309; m4, P08173; and m5, P08912.

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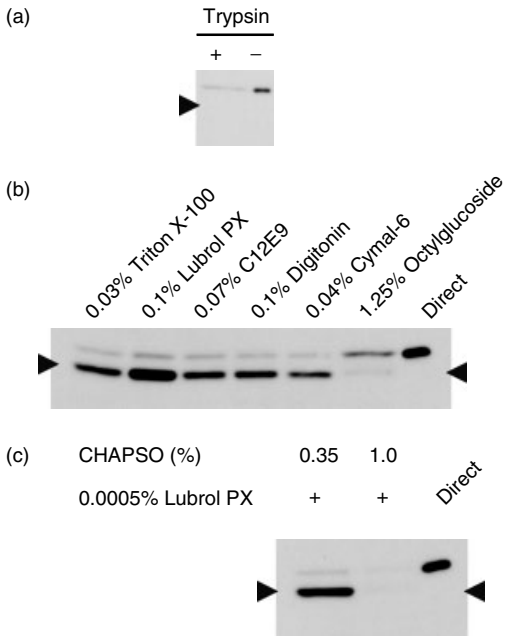

Figure 2 Effect of detergents on the intensity of the trypsin-resistant band of GTPγS-bound G_q. Bovine brain membrane extract was incubated with GTPγS in the presence of the indicated detergent (if any), digested with trypsin, electrophoresed, and detected with anti-G_q antibody. Bands slightly lower than that of the native protein (annotated with arrow heads) correspond to the trypsin-resistant GTPγS-bound G_q. (a) Absence of the trypsin-resistant band with no addition of the detergent. With (left) or without (right) trypsin treatment. (b) Effect of various detergents on the intensity of the resistant band. (c) Disappearance of the trypsin-resistant band with increasing CHAPSO concentration, ranging from 0.35 to 1.0%.

used, it was clearly observed with buffers supplemented with Triton X-100, Lubrol PX, C12E9, digitonin, and Cymal-6. Figure 2(c) shows the effects of Lubrol PX and additional CHAPSO on the trypsin-resistant band. The band appeared on addition of Lubrol PX at a concentration as low as 0.0005% (slightly above its cmc) (Figure 2(b)), whereas it disappeared on adding 1% CHAPSO to the buffer containing 0.0005% Lubrol PX (Figure 2(c)). The average micellar weight of the detergents used in this study is as follows: CHAPSO: 1.0×10^4 [19], octylglucoside: 7.9×10^3 [19], Triton X-100: 8.0×10^4 [20], Lubrol PX: 6.8×10^4 [20], C12E9: $>6.6 \times 10^4$ [20], digitonin: 7.0×10^4 [19], and Cymal-6: 3.2×10^4 [21]. The present observations suggest that detergents of low micellar weight tend to destabilize G_q, whereas those of high micellar weight tend to stabilize G_q.

Activation of G_q by m3i3c

The intensity of the GTPγS-bound trypsin-resistant band markedly increased with increasing m3i3c concentration (Figure 3(a), annotated with a filled arrowhead). Quantification of the GTPγS-bound lower bands indicated that the EC_{50} and the maximum fold activation values are 4.9 μM and 1.7-fold (at 12.5–25 μM; Figure 3(c)), respectively. At a very high concentration (200 μM), the peptide exhibited lower activation for unknown reasons, similar to mastoparan with regard to G_o activation [3]. In contrast to G_q, G_{i2} was not activated by m3i3c at all (Figure 3(b)) as expected, based on the coupling specificity of m3 AChR (Figure 1). Thus, m3i3c was found to retain the functions of m3 AChR.

The appearance of the upper band of G_q in the presence of 6.25–200 μM peptide (Figure 3(a), annotated with an empty arrowhead) is not due to insufficient trypsin treatment (too low or too short trypsin treatment). If that were the case, the upper band would have been observed at a comparable intensity

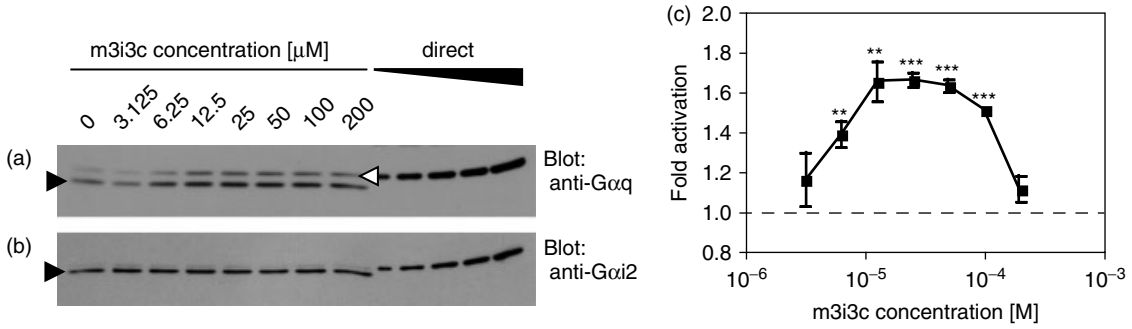

Figure 3 m3i3c activates G_q but not G_{i2}. (a) Dose-dependent appearance of the trypsin-resistant band of G_q in the presence of m3i3c (detected with anti-G_q antibody). Trypsin-resistant bands are annotated with a filled arrowhead. Bands not cleaved are annotated with an empty arrowhead. Lanes annotated 'Direct' contain 4, 8, 12, 20, and 32% (from left to right) of membrane extract that had not been digested with trypsin; the intensity of these direct bands was used to calibrate the intensity of the trypsin-resistant bands on the left. Representative results from four independent experiments are shown. (b) m3i3c did not activate G_{i2}. Trypsin-resistant bands (annotated with a filled arrowhead) were detected with anti-G_{i2} antibody. (c) Fold activation of G_q with increasing concentration of m3i3c. Each data point represents mean ± SEM of four independent experiments. Significance levels in comparison to the value in the absence of m3i3c were determined using Student's *t*-test. Double and triple asterisks denote $P < 0.01$ and $P < 0.001$, respectively.

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in the absence of the m3i3c peptide (extreme left lane). We found at least two states of the GDP-bound G_q with different sensitivities to trypsin (Kubota and Wakamatsu, unpublished results). The temperature and magnesium ion concentration influence the relative amounts of G_q in the two states. The appearance of the upper band in the presence of 6.25–200 μM peptide may be due to a shift of the GDP-bound G_q from the trypsin-sensitive to trypsin-resistant state due to the addition of the peptide.

DISCUSSION

Significance of G_q Activation by m3i3c

Although the 1.7-fold activation of G_q by m3i3c observed in this study (Figure 2(c)) might be considered weak when compared to the activation of $G_{o/i}$ by mastoparan, we believe that this activation is significant for the following reasons: (i) GTPγS-binding assays tend to yield lower fold activation values than steady-state GTPase assays. This is because unlike steady-state GTPase reaction (a linear reaction), GTPγS binding is a saturable reaction with time; the initial rate of binding tends to be underestimated when the binding value cannot be obtained at very early time points due to technical reasons. In the present case, the amount of bound GTPγS was determined at 2 h to accumulate signals. In addition, this tendency is more pronounced in faster reactions, i.e. activated states. In fact, for the activation of G_o reconstituted in lipid vesicles by mastoparan, the fold activation value determined by a GTPγS-binding assay was 75% than that determined by a steady-state GTPase assay, although the GTPγS binding was determined at as early as 1 min (compare Tables 1 and 2 in Higashijima, *et al.*, 1990) [3]. In fact, the activation of recombinant G_{i1} by mastoparan as determined by a GTPγS-binding assay we performed was 2.5-fold [22]. (ii) The EC_{50} value of the G_q activation (4.9 μM) is lower than the dissociation constant of mastoparan and G_{i1} (approximately 30 μM; Morikawa, to be published). (iii) This peptide did not activate G_{i2} at all (Figure 2(b)). (iv) The peptide fragment corresponding to the junction between the i3C and TM-VI of m4 AChR ($G_{i/o}$-coupled) did not activate G_q at all; this peptide contains a VT/IF motif instead of the AA/LS motif (Figure 1) (Kubota *et al.*, to be published). The low potency of m3i3c compared to that of the parent m3 AChR may be ascribed to the lack of other G protein-recognition sites [15].

Wess and coworkers demonstrated that the AA/LS motif in the i3C/TM-VI junction of m3 AChR is the structural element that discriminates between G_q and $G_{i/o}$ [23]. The present observation that m3i3c activates G_q but not G_{i2} agrees well with their findings.

Other G Protein-Activating Peptides

Our approach of using peptide fragment of GPCR as a G protein-activating compound is not new. Palm *et al.* [10] and Cheung *et al.* [9] found that i3c fragment of β-adrenergic receptor activates G_s, whereas Okamoto and Nishimoto found that i3c fragment of m4 muscarinic receptor activates $G_{i/o}$ more potently than G_s [11]. A G_q-activating peptide, however, has not been reported thus far. This may be due to the difficulty in preparing recombinant G_q as well as to the low abundance of G_q in tissues [12]. The present demonstration of the activation of G_q by m3i3c was made possible by using the highly sensitive immunodetection system of G_q that used an appropriate detergent.

Possible Application of m3i3c

The m3i3c peptide is the first low-molecular-weight compound that selectively activates G_q. Although there is one report that demonstrates the activation of G_q by mastoparan [24]. Mastoparan has also been known to potently activate $G_{i/o}$ [3]. In addition to G_q-coupled GPCRs, ric-8A [25] and the *Pasteurella multocida* toxin (PMT) [26] are known to activate G_q. However, ric-8A acts on the α subunit of $G_{q/i1/o}$ but not on the heterotrimeric αβγ form [25]. PMT is known to activate G_{12} as well as G_q [27]. As there is no report that shows the activation of G_{12} by m3 AChR, m3i3c is expected not to activate G_{12} and is considered more specific than PMT.

One advantage of low-molecular-weight activators over large proteinaceous ones is their easy application in structural analyses. CD analyses showed that the α-helix content of G_{i1} decreases upon activation by mastoparan or compound 48/80 [22]; such analyses would be difficult if proteinaceous activators are used because large signals arising from the activator proteins would obscure the subtle alterations in the signals of the G protein α subunits. Another advantage of m3i3c is that it is a short linear peptide that can be synthesized automatically.

The role of G_o in exocytosis in chromaffin cells has been demonstrated by using mastoparan in combination with GP Ant-2 [28], which antagonizes the activation of $G_{i/o}$ [5]. Thus, m3i3c would be useful for demonstrating the role of G_q in a given signaling pathway, particularly when employed in combination with YM-254 890, a G_q-selective blocker [29].

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