Volume 311, number 1, 29–32 FEBS 11615 October 1992

Amino acids 356–372 constitute a G_i-activator sequence of the α_2-adrenergic receptor and have a Phe substitute in the G protein-activator sequence motif

Tsuneya Ikezu[a], Takashi Okamoto[b], Etsuro Ogata[a,*] and Ikuo Nishimoto[b]

[a]*Life Science Laboratory, Fourth Department of Internal Medicine, University of Tokyo School of Medicine, 3-28-6 Mejirodai, Bunkyo-ku, Tokyo 112, Japan* and [b]*Cardiovascular Research Center, Harvard Medical School, Massachusetts General Hospital-East, 4th Floor, 149 13th Street, Charlestown, MA 02129, USA*

Received 30 July 1992; revised version received 25 August 1992

The human α_2-adrenergic receptor contains the sequence KASRWRGRQNREKRFTF (amino acids 356–372) at the C-terminal end of its third intracellular loop. This sequence satisfies the structural criteria for G protein-activating sequences [(1992) J. Biol. Chem. 267, 8342–8346] except that the C-terminal sequence is B-B-X-X-Phe instead of B-B-X-B or B-B-X-X-B (B: basic residue, X: non-basic residue). Nevertheless, the synthetic peptide corresponding to this sequence (peptide α_2-F) was found to activate G_i and G_o strongly with a saturated effect at 1–3 μM. Furthermore, the substitution of the C-terminal Phe of peptide α_2-F with Arg, Trp, and Tyr (but not Ala or Asp) did not appreciably affect the G_i-activating potency. It is suggested that the C-terminal basic residue of the B-B-X-X-B motif in G_i-activating sequences can be replaced by an aromatic residue.

α_2-Adrenergic receptor; G_i-coupling mechanism; G_i-activator sequence; G protein-activator sequence motif; Aromatic amino acid

1. INTRODUCTION

Hormones, neurotransmitters, growth factors, and cytokines initiate the cytoplasmic signals by binding to their own receptors on the cell surface. Some of the receptors exert their function by activating intracellular effectors, such as enzymes and ion channels, via the intermediary role of GTP-binding G proteins [1]. Among these G-coupled receptors, there are at least seven types of adrenergic receptors, which mediate the effect of epinephrine and norepinephrine. The α_2AR (α_2-adrenergic receptor) belongs to this family, the function of which is mainly mediated by G_i activation [2].

Sequence analysis predicts that most of the G-coupled receptors consist of the membrane-spanning structure with seven transmembrane domains, which forms three cytoplasmic loops and one C-terminal tail [2]. The mutagenesis studies of the receptor-G protein pairs, βAR (β-adrenergic receptor)-G_s [3–6], rhodopsin-G_t [7–10], and mAChR (muscarinic acetylcholine receptor)-G_i [11], have specified short regions in the receptors that are required for interaction with G proteins. In particular, the C-terminal portions of I_3 (the third intracellular loop) of G-coupled receptors have been proposed to be the most likely region involved in G protein coupling. However, it remained undetermined whether this is the case with α_2AR.

Our previous study [12–14] clarified that IGF-IIR (insulin-like growth factor II receptor) interacts with and activates G_i in an environment of phospholipid vesicles as well as in that of native BALB/c 3T3 cell membranes. The mode of their interaction was found to be the same as that between conventional G-coupled receptors and G proteins, despite the fact that IGF-IIR is a receptor with a single transmembrane domain. We subsequently found that the Arg^{2410}–Lys^{2423} sequence of IGF-IIR can activate G_i directly in the same manner as conventional G-coupled receptors [15,16]. Notably, the function of this sequence was found to depend entirely upon the presence of: (i) at least two basic residues at the N-terminal side and (ii) the sequence B-B-X-B or B-B-X-X-B (where B is a basic residue, and X is a non-basic residue) at the C-terminus. Therefore, it is reasonable to assume that G-coupled receptors contain G protein-activator sequences that satisfy (i) and (ii) in addition to (iii) $10 \leqslant$ residue length $\leqslant 26$. Based on these criteria, we have identified a G_s-activator region in the C-terminal end of I_3 of β_2AR and a G_i/G_o-activator region in the comparable portion of M_4AChR (M_4 subtype mAChR) [17]. Furthermore, α_2AR was found to possess a G_s-activator region in its second intracellular loop and a region in the N-terminal end of I_3 that activates all of G_i, G_o, and G_s non-selectively. The former fact is consistent with the study indicating that α_2AR

Correspondence address: I. Nishimoto, Cardiovascular Research Center, Harvard Medical School, Massachusetts General Hospital-East, 4th Floor, 149 13th Street, Charlestown, MA 02129, USA.

**Present address:* Cancer Research Institute, 1-37-1 Kami-Ikebukuro, Toshima-ku, Tokyo 170, Japan.

activates G_s in response to high concentrations of the α_2-adrenergic agonists [18,19].

Based on the criteria (i)–(iii), on the other hand, it was impossible to find regions in α_2AR that can specifically activate G_i, a G protein to which this receptor is coupled in native conditions. The C-terminal end of I_3 of α_2AR had consisted of a sequence quite different from that satisfying these criteria [17,20]. Recently, the sequence of α_2AR has been partially corrected [21], which gave rise to the sequence similar to the sequence that satisfies (i)–(iii). It is KASRWRGRQNREKRFTF located in its C-terminal end of I_3, which satisfies (i) and (iii), but has a B-B-X-X-F structure at its C-terminus. A Phe residue is substituted for the extreme C-terminal basic residue. This study was conducted to find a specific G_i-activator region in α_2AR and to see whether or not other residues can be substituted for its extreme C-terminal residue.

2. EXPERIMENTAL

2.1. *GTPγS binding assay*

GTPγS binding to G proteins was assayed at 37°C in the presence of 20 μM Mg^{2+} and 60 nM [^{35}S]GTPγS (GTPγS, guanosine-5′-*o*-(3-thiotriphosphate)), as described previously [15]. GTPγS binding to polypeptide was negligible. The total amount of G proteins was measured as maximal GTPγS binding at room temperature, as described [22]. [^{35}S]GTPγS was purchased from Du Pont-New England Nuclear. Binding of GTPγS to G proteins obeyed the first-order kinetics according to the equation $\ln[(B_T-B)/B_T]=-k_{app}t$, where B is the binding at time t and B_T is the total binding observable at an infinite time. Thus, the apparent first-order rate constant for GTPγS binding (k_{app}), which is equal to the slope of the tangent to the GTPγS-binding curve at time 0 and represents the actual GTPγS binding rate, was calculated from this equation and the value of B at 2 min for G_o, 5 min for G_i and 10 min for G_s.

2.2. *Materials*

The receptor polypeptides used in this study were synthesized by the solid phase method and purified to near homogeneity by high-performance liquid chromatography, as described [15]. The lyophilized synthetic polypeptides were dissolved in distilled water. G_i and G_o, which were purified from bovine brain to homogeneity, were provided by Dr. Toshiaki Katada (Tokyo Institute of Technology, Yokohama, Japan) [23]. The G_i used in this study was trimeric G_{i2}. G_{i2} was purified from Sf9 cells transfected with $G_{\alpha 2}$-encoding baculovirus as described [24] with slight modifications using Mono Q column chromatography.

3. RESULTS AND DISCUSSION

We tested the ability of KASRWRGRQNREKRFTF, which is located at residues 356–372 of α_2AR, to activate G_i. This polypeptide was referred to as peptide α_2-F. Peptide α_2-F activated G_i as assessed by determining the rate constant value, k_{app}, of GTPγS binding (Fig. 1). The action of peptide α_2-F reached saturation at 1–3 μM of this peptide with an EC_{50} of ≈300 nM. This sequence is one of the receptor sequences that are the most potent in activation of G_i. A sequence with similar potency of the activity was reported in M_4AChR, another native G_i-coupled receptor [17]. It is RNQVRKKRQMAARERKVTR, which has a C-terminal structure very similar to the present sequence.

To clarify the G protein specificity, we examined the dose–response relationship for the action of peptide α_2-F on each of G_i, G_o and G_s. As shown in Fig. 2, peptide α_2-F activated G_o with larger efficacy and less potency. The efficacy of the peptide action on G_o was ≈1.3-fold of its G_i-activating ability. The EC_{50} value of the peptide action on G_o was estimated to be 500 nM, which is significantly higher than the EC_{50} value, 300 nM, of its G_i-activating ability. In contrast, this peptide indicated a weak action on G_s with smaller efficacy and lower potency. The efficacy of the peptide action on G_s was ≈2/3 of its G_i-activating ability and ≈1/2 of its G_o-activating activity. The EC_{50} was estimated to be 1 μM. This suggests that the peptide α_2-F sequence is a G_i/G_o-activator region of α_2AR. It has been reported [25] that purified α_2AR couples to G_o as potently as to G_i in phospholipid vesicles, which is consistent with the present study. It was also reported that the C-terminal MIII region of I_3 of M_4AChR possesses a potent activity to activate G_o as well as G_i [17]. The characteristic that G_i/G_o-activator regions are present in the C-terminal end of I_3 may be commonly shared by G_i-coupled receptors with multiple membrane-spanning configuration.

Next we carried out a series of experiments to address the question of whether other residues can substitute the extreme C-terminal Phe of peptide α_2-F. The G_i-activator peptide α_2-F sequence possesses the B-B-X-X-F structure at its C-terminus, whereas G protein-activator regions in M_4AChR, β_2AR, and IGF-IIR share the B-B-X-B or B-B-X-X-B motif at their C-terminus. Thus,

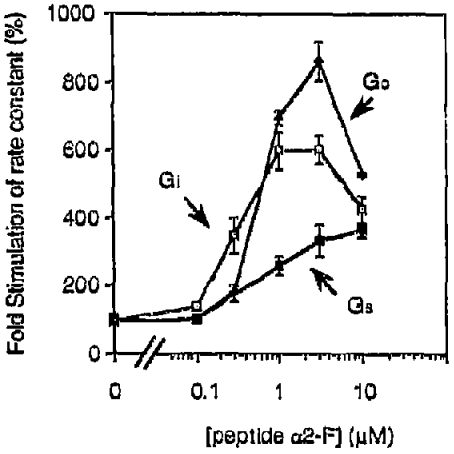

Fig. 1. Effect of peptide α_2-F on GTPγS binding to various G proteins. By incubating G proteins (G_i, □; G_o, ◆; G_s, ■) with peptide α_2-F (KASRWRGRQNREKRFTF, residues 356–372 of human α_2AR), GTPγS binding was assayed under the conditions described in section 2. The GTPγS binding rate was assessed as the rate constant, k_{app}, and the extent of stimulation was expressed as the degree of stimulation of the rate constant relative to the basal rate. The method used for calculation is described in section 2. The basal values of k_{app} were 0.070 ± 0.001 for G_i, 0.177 ± 0.022 for G_o, and 0.029 ± 0.005 for G_s (min^{-1}, mean ± S.E.) at these experiments.

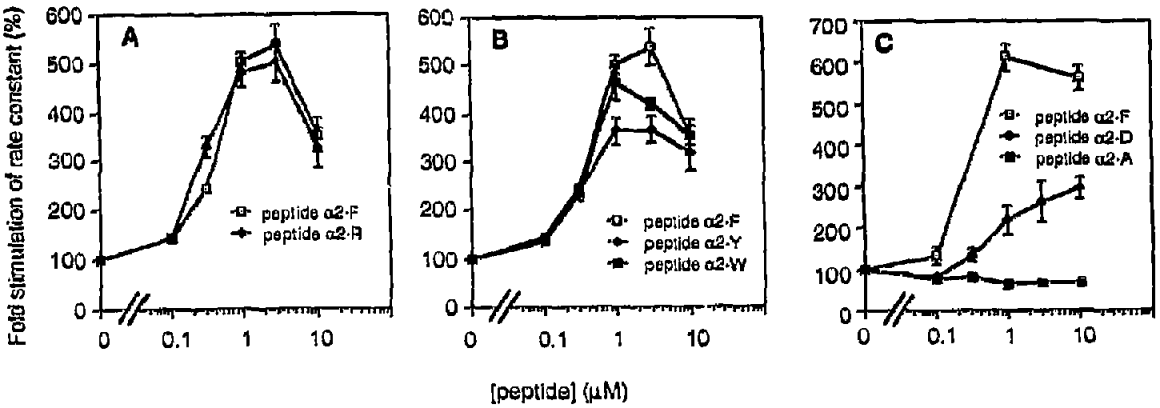

Fig. 2. Effect of peptide α_2-F variants with its extreme C-terminal residue substituted on GTPγS binding to G_i. (A) Effect of Arg substitution, (B) effect of aromatic acid substitutions, (C) effect of Ala and Asp substitutions. By incubating G_i with peptide α_2-R in A (□, peptide α_2-F; ◆, peptide α_2-R (KASRWRGRQNREKRFTR)) or peptide α_2-Y and -W; in B (□, peptide α_2-F; ◆, peptide α_2-Y (KASRWRGRQNREKRFTY); ■, peptide α_2-W (KASRWRGRQNREKRFTW)), or peptide α_2-D and -A; in C (□, peptide α_2-F; ◆, peptide α_2-D (KASRWRGRQNREKRFTD; ■, peptide α_2-A (KASRWRGRQNREKRFTA)), GTPγS binding was assayed under the same conditions as those described in the legend for Fig. 1. Values represent the mean ± S.E. of six experiments. Since the experiments described in A and B were done together, the dose-response curve of peptide α_2-F as a positive control was the same between A and B, whereas the experiments in C were done separately. The basal values of k_{app} for G_i were 0.071 ± 0.002 in A and B, and 0.069 ± 0.002 in C (min^{-1}, mean ± S.E.).

it is intriguing to examine whether the C-terminal Phe can be substituted with other residues, including basic residues. As shown in Fig. 2A, the substitution of Arg for the C-terminal Phe resulted in almost identical potency and efficacy of the action on G_i, suggesting that basic residues can be substituted for the extreme C-terminal Phe without altering the potency of the peptide action.

The Phe residue belongs to an aromatic amino acid family, which is apt to bind other residues through hydrophobic binding. Thus, we further examined whether aromatic residues Tyr and Trp can be substituted for the C-terminal Phe of peptide α_2-F without changing the G_i-activating activity. Fig. 2B shows that this is the case. When the peptide α_2-F variants, the extreme C-terminal residue of which was Tyr or Trp, were synthesized, they indicated G_i-activating activity with a potency similar to that of peptide α_2-F, although their efficacy was slightly diminished. In contrast, when Asp was substituted for the C-terminal residue, both the potency and efficacy of the peptide to activate G_i were markedly diminished (Fig. 2C). When Ala was substituted, the peptide completely lost its G_i-activating function. Therefore, it is reasonable to assume that basic residues or aromatic ones are allowable as the C-terminal residue of the G_i-activator region of α_2AR.

In summary, we localized a potent G_i/G_o-activator region of α_2AR to residues 356–372. We [21] previously reported that this receptor contains a region in the second intracellular loop that can activate G_s selectively, and a region in the N-terminal end of I_3 that activates plural G proteins with low potencies. Thus, it is likely that α_2AR activates G_i by using the α_2-F region presently identified in the C-terminal end of I_3 (Fig. 3). Furthermore, because the region that can activate G_i/G_o potently and selectively has been found in α_2AR, it is reasonable to assume that the N-terminal sequence of I_3 that weakly and non-selectively activates $G_i/G_o/G_s$ may specify the region that potently activates some other G protein.

It has been suggested that α_2-adrenergic stimulation of phospholipase A_2 in platelets is desensitized by protein kinase C activation [26]. Since the peptide α_2-F sequence contains a possible phosphorylation site for protein kinase C, it is an interesting question whether peptide α_2-F loses the G_i-activating function when it is

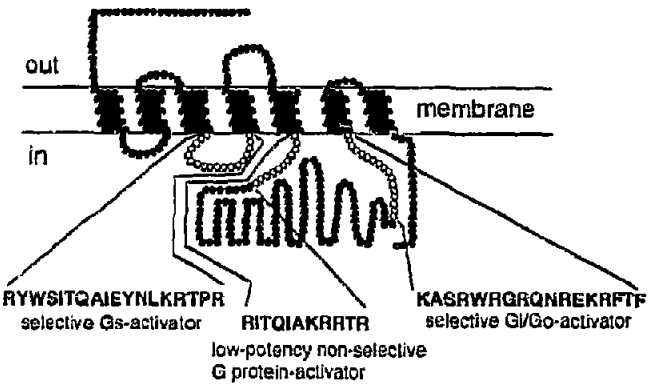

Fig. 3. Integrated scheme illustrating the cytoplasmic functional domains in α_2AR. This figure illustrates the location of three functional domains in α_2AR and their significance. We [17] have identified the second intracellular loop region RYWSITQAIEYNLKRTPR that specifically activates G_s, and the N-terminal region of the third intracellular loop RITQIAKRRTR that activates G_s, G_i and G_o with low potencies. In the present study, the C-terminal region of the third intracellular loop KASRWRGRQNREKRFTF has been found to be highly potent in activating G_i and G_o. The configuration of α_2AR is based on ref. 21.

phosphorylated by purified protein kinase C. We previously reported a similar phosphorylation-mediated regulation of the active peptide in a G-coupled receptor [22]. The G_s-activator region in I_3 of β_2AR is subject to protein kinase A-dependent phosphorylation, resulting in a drastic reduction in the G_s-coupling ability and potentiation of the G_i-activating function. The present study may be important in this regard as well, since investigators can focus on the limited region of residues 356–372 when they investigate the cellular mechanism that modulates the function of α_2AR.

Acknowledgements: We especially thank Dr. Toshiaki Katada (Tokyo Institute of Technology) for his gift of G proteins. We also thank Drs. Akira Kikuchi and Yoshimi Takai (Kobe University) and Dr. Yujiro Hayashi (Suntry Biomedical Institute) for their technical advice; Mr. Yoshiomi and Mrs. Yumi Tamai (Society For Educational Aid to Traffic Orphans) for indispensable support; Atomic Research General Center (University of Tokyo) for the use of their facilities; and Mr. Shuji Matsuda, Ms. Yasuko Homma, Ms. Misaki Nagashima (University of Tokyo), and Ms. Patricia Beekman for technical assistance. This work was supported in part by grants from the Ministry of Education, Science, and Culture of Japan, Teijin Institute for Biomedical Research, the Life Insurance Association of Japan, Napolex Corporation, the Japan Research Foundation for Clinical Pharmacology, the Sagawa Foundation for Promotion of Cancer Research, the Mochida Memorial Foundation for Medical and Pharmaceutical Research, Arima Memorial Foundation for Medical Research, Japan Heart Foundation Research Grant for 1991, and Kudo Science Foundation.

REFERENCES

[1] Stryer, L. and Bourne, H.R. (1986) Annu. Rev. Cell Biol. 2, 391–419.
[2] O'Dowd, B.F. and Lefkowitz, R.J. (1991) Encyclopedia of Human Biology 1, 81–92.
[3] Kobilka, B.K., Kobilka, T.S., Daniel, K., Regan, J.W., Caron, M.G. and Lefkowitz, R.J. (1988) Science 240, 1310–1316.
[4] Strader, C.D., Dixon, R.A.F., Cheung, A.H., Candelore, M.R., Blake, A.D. and Sigal, I.S. (1987) J. Biol. Chem. 262, 16439–16443.
[5] O'Dowd, B.F., Hantowich, M., Regan, J.W., Leader, W.M., Caron, M.G. and Lefkowitz, R.J. (1988) J. Biol. Chem. 263, 15985–15992.
[6] Wong, S.K.-F., Parker, E.M. and Ross, E.M. (1990) J. Biol. Chem. 265, 6219–6224.
[7] Kühn, H. and Hargrave, P.A. (1981) Biochemistry 20, 2410–2417.
[8] Weiss, E.R., Kelleher, D.J. and Johnson, G.L. (1988) J. Biol. Chem. 263, 6150–6154.
[9] König, B., Arendt, A., McDowell, J.H., Kahlert, M., Hargrave, P.A. and Hofmann, K.P. (1989) Proc. Natl. Acad. Sci. USA 86, 6878–6882.
[10] Franke, R.R., König, B., Sakmar, T.P., Khorana, H.G. and Hofmann, K.P. (1990) Science 250, 123–125.
[11] Kubo, T., Bujo, H., Akiba, I., Nakai, J., Mishina, M. and Numa, S. (1988) FEBS Lett. 241, 119–125.
[12] Nishimoto, I., Murayama, Y., Katada, T., Ui, M. and Ogata, E. (1989) J. Biol. Chem. 264, 14029–14038.
[13] Okamoto, T., Nishimoto, I., Murayama, Y., Ohkuni, Y. and Ogata, E. (1990) Biochem. Biophys. Res. Commun. 168, 1201–1210.
[14] Murayama, Y., Okamoto, T., Ogata, E., Asano, T., Iiri, T., Katada, T., Ui, M., Grubb, J.H., Sly, W.S. and Nishimoto, I. (1990) J. Biol. Chem. 265, 17456–17462.
[15] Okamoto, T., Katada, T., Murayama, Y., Ui, M., Ogata, E. and Nishimoto, I. (1990) Cell 62, 709–717.
[16] Okamoto, T. and Nishimoto, I. (1991) Proc. Natl. Acad. Sci. USA 88, 8020–8023.
[17] Okamoto, T. and Nishimoto, I. (1992) J. Biol. Chem. 267, 8342–8346.
[18] Federman, A.D., Conklin, B.R., Schrader, K.A., Reed, R.R. and Bourne, H.R. (1992) Nature 356, 159–161.
[19] Eason, M.G., Kurose, H., Holt, B.D., Raymond, J.R. and Liggett, S.B. (1992) J. Biol. Chem. 267, 15795–15801.
[20] Kobilka, B.K., Matsui, H., Kobilka, T.S., Yang Feng, t.L., Francke, U., Caron, M.G., Lefkowitz, R.J. and Regan, J.W. (1987) Science 238, 650–656.
[21] Guyer, C.A., Horstman, D.A., Wilson, A.L., Clark, J.D., Cragoe Jr., E.J. and Limbird, L.E. (1990) J. Biol. Chem. 265, 17307–17317.
[22] Okamoto, T., Murayama, Y., Hayashi, Y., Inagaki, M., Ogata, E. and Nishimoto, I. (1991) Cell 67, 723–730.
[23] Katada, T., Oinuma, M., Kusakabe, K. and Ui, M. (1987) FEBS Lett. 213, 353–358.
[24] Okamoto, T., Ikezu, T., Murayama, Y., Ogata, E. and Nishimoto, I. (1992) FEBS Lett. 305, 125–128.
[25] Cerione, R.A., Regan, J.W., Nakata, H., Codina, J., Benovic, J.L., Gierschik, P., Somers, R.L., Spiegel, A.M., Birnbaumer, L., Lefkowitz, R.J. and Caron, M.G. (1986) J. Biol. Chem. 261, 3901–3909.
[26] Murayama, T., Kajiyama, Y., Takahashi, A. and Nomura, Y. (1990) Arch. Biochem. Biophys. 276, 146–152.