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A site-directed mutagenesis study on the conserved alanine residue in the distal third intracellular loops of cholecystokinin$_B$ and neurotensin receptors

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1 An alanine residue at the C-terminal tail of the third intracellular loop is highly conserved among various G_q protein-coupled receptors including rat cholecystokinin$_B$ (CCK_B) and neurotensin receptors. To investigate the functional significance of the conserved alanine in the activation of G_q proteins and phospholipase C (PLC) by CCK_B and neurotensin receptors, the alanine residue was mutated in the present study. Subsequently, the ability of resulting mutant receptors to activate PLC was investigated by measuring the formation of inositol phosphates (IP) in COS-7 cells and recording Ca^{2+}-activated chloride currents from *Xenopus* oocytes.

2 Site-directed mutagenesis was performed to mutate alanine at position 332 of rat CCK_B receptor to glutamate. When the (A332E) mutant receptor was expressed in COS-7 cells and *Xenopus* oocytes, the efficacy and the potency of sulphated cholecystokinin octapeptide (CCK-8) to stimulate polyphosphoinositide hydrolysis in COS-7 cells and evoke calcium-dependent Cl^- currents in oocytes were not significantly affected.

3 Alanine residue at position 302 of rat neurotensin receptor was also mutated to glutamate. When expressed in COS-7 cells and *Xenopus* oocytes, the resulting (A302E) mutant receptor was strongly defective in stimulating phosphatidylinositol turnover in COS-7 cells and evoking Ca^{2+}-dependent chloride currents in oocytes.

4 In summary, the present study demonstrates that alanine residue at the C-terminus of third cytoplasmic domain is required for the full activation of G_q proteins and PLC by neurotensin receptors. However, in contrast to other G_q protein-coupled receptors, alanine at the distal third intracellular loop does not play a significant role in CCK_B receptor activation of PLC.

Keywords: CCK_B receptors; neurotensin receptors; sulphated cholecystokinin octapeptide; neurotensin; phospholipase C; inositol phosphates; G_q proteins; Ca^{2+}-activated chloride currents

Introduction

Our recent whole-cell patch-clamp studies demonstrated that sulphated cholecystokinin octapeptide (CCK-8) and neurotensin excite rat neostriatal neurones and substantia nigra dopaminergic neurones, respectively. CCK-8, by activating cholecystokinin$_B$ (CCK_B) receptors, depolarizes GABAergic medium spiny neurones of the rat neostriatum through the enhancement of a nonselective cationic conductance (Wu & Wang, 1996). Neurotensin depolarizes substantia nigra dopaminergic neurones by opening nonselective cation channels and closing inwardly rectifying K^+ channels (Wu *et al*., 1995). Further investigations indicated that pertussis toxin-insensitive G proteins, which belong to the G_q subfamily (Helper & Gilman, 1992; Berridge, 1993), mediate CCK-8- and neurotensin-induced modulations of ionic conductances (Wang & Wu, 1996; Wu & Wang, 1996). G_q proteins could couple CCK_B and neurotensin receptors to cation and inward rectifier K^+ channels either indirectly through second messengers or directly in a membrane-delimited way. The first transduction mechanism is supported by our previous findings that inositol 1,4,5-trisphosphate (IP_3)-induced Ca^{2+} mobilization is responsible for CCK-8 and neurotensin enhancement of cationic conductance and that protein kinase C mediates neurotensin reduction of inwardly rectifying K^+ conductance (Wu & Wang, 1995; Wu *et al*., 1995). These findings suggest that activation of G_q proteins-phospholipase C (PLC) signalling pathway could be responsible for most of the physiological effects induced by cholecystokinin (CCK) peptides and neurotensin in the brain. Therefore, it is very important to investigate how CCK_B and neurotensin receptors couple to G_q proteins and PLC activation at the molecular level.

Both CCK_B and neurotensin receptors belong to the superfamily of G protein-coupled receptors and contain seven transmembrane domains joined by extracellular and cytoplasmic loops (Tanaka *et al*., 1990; Wank *et al*., 1992b; Lee *et al*., 1993; Vita *et al*., 1993). Previous studies with chimera receptors and deletion mutants indicated that the third intracellular loop of G protein-coupled receptor contains the site for G protein-binding and determines the specificity of G protein coupling (Cotecchia *et al*., 1990; Lechleiter *et al*., 1990; Wong *et al*., 1990; Savarese & Fraser, 1992; Ostrowski *et al*., 1992; Kunkel & Peralta, 1993; Wang *et al*., 1995). Within the third cytoplasmic domains of G protein-coupled receptors, amino acid sequence alignment revealed that an alanine residue is almost ubiquitously found in the C-terminal end of the third intracellular loop, suggesting that this conserved alanine plays an important role in receptor-G protein coupling (Savarese & Fraser, 1992; Strader *et al*., 1994). Consistent with this hypothesis, mutation of alanine residue at the C-terminus of the third cytoplasmic domain prevents thyrotropin and gastrin-releasing peptide receptors from activating G_q proteins and PLC (Kosugi *et al*., 1992; Benya *et al*., 1994). Point mutation of conserved alanine at the distal third intracellular loop led to a significant decrease in the efficiency and the potency of M_3- and M_5-subtype cholinoceptors to stimulate phosphatidylinositol turnover (Blin *et al*., 1995; Burstein *et al*., 1995). Whereas, mutation of alanine at the corresponding site of the α_{1B}-adrenoceptor resulted in a persistent activation of PLC-inositol triphosphate (IP_3) signalling pathway and a defect in agonist-stimulated phosphatidylinositol hydrolysis (Kjelsberg *et al*., 1992).

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To understand better the molecular mechanisms underlying receptor-G protein coupling, specific amino acids of CCK_B and neurotensin receptors involved in G_q protein recognition and activation need to be identified. Both rat CCK_B receptor (position 332) and neurotensin receptor (position 302) contain an alanine residue at the carboxyl end of the third intracellular loop. (Tanaka *et al*., 1990; Wank *et al*., 1992b). To investigate the functional significance of this conserved alanine in the activation of G_q proteins and PLC by CCK_B and neurotensin receptors, in the present study this alanine residue was mutated. Subsequently, the resulting mutant CCK_B or neurotensin receptor was studied for its ability to activate PLC and stimulate the formation of inositol phosphates (IP).

Methods

Molecular cloning of rat CCK_B and neurotensin receptors

Forward and reverse primers were designed according to the conserved amino acid sequences of CCK_A and gastrin receptors (Wank *et al*., 1992a; Kopin *et al*., 1992). A 693-bp-long polymerase chain reaction (PCR) product was obtained by use of cDNA prepared from mRNA of rat neocortical tissues. DNA sequencing indicated that this 693-bp PCR product encodes a partial fragment of rat CCK_B receptors (Wank *et al*., 1992b). mRNA of the rat neocortex was used for the construction of cDNA library in the Uni-ZAP XR λ vector (Stratagene). Full-length cDNA encoding rat CCK_B receptors was obtained by screening cDNA library with ^{32}P-random-prime-labelled 693-bp PCR DNA. One positive plaque containing a 2.2 kb DNA insert was identified, and cDNA clone was excised *in vivo* with the helper phage according to Stratagene protocol. The resulting pBluescript vector was used for the DNA sequencing from both ends by using the chain-termination method (Sequenase 2.0, United States Biochemicals). DNA sequencing indicated that this cDNA insert contained the full-length coding and untranslated regions of rat CCK_B receptors (Wank *et al*., 1992b). Subsequently, cDNA clone of CCK_B receptors was excised from pBluescript vector and ligated into the mammalian expression vector, pBK-CMV (Stratagene).

The full-length cDNA clone of rat neurotensin (NT) receptor was obtained by performing PCR amplification with the single-stranded rat brainstem cDNA as the template. PCR was carried out in a programmable thermal controller (Minicycler, NJ Research Inc.) with the following oligonucleotide primers. (a) Forward primer was 5′ATGCACCTCAACAGCTCCGTGCCG3′ and corresponds to nucleotides 1–24 of rat neurotensin receptors (Tanaka *et al*., 1990). (b) Reverse primer was 5′CTAGTACAGGGTCTCCCGGGTGGC3′ and corresponds to nucleotides 1252–1275 of rat neurotensin receptors (Tanaka *et al*., 1990). 1275-bp PCR DNA product containing the full-length coding region of rat neurotensin receptors was purified and ligated into the pBK-CMV vector. The DNA sequence of cDNA clone encoding neurotensin receptor was verified by dideoxy DNA sequencing.

Construction of mutant CCK_B and neurotensin receptors

pBK-CMV vector containing the cDNA of rat CCK_B or neurotensin receptor was used as the DNA template for the oligonucleotide-directed mutagenesis with PCR amplification (Ito *et al*., 1991). According to previous studies (Kosugi *et al*., 1992; Benya *et al*., 1994), a 33-mer oligonucleotide (5′CACCACCCGCTTCTTTTCCAGCAGCTTGGCCTG3′) was designed to convert the codon for alanine (GCT) at position 332 of CCK_B receptor to a codon for glutamate (GAA). Another oligonucleotide (5′GACTCCGTGGCGCAGTTCCTGGACACGACCCGG3′) was used to replace the codon for alanine (GCC) at position 302 of the neurotensin receptor with a codon for glutamate (GAA). The mutations were confirmed by performing dideoxy DNA sequencing.

Transfection of COS-7 cells and radioligand-binding assays

The mammalian expression vector, pBK-CMV, containing the cDNA clone of wild-type or mutant peptide receptor was transfected to COS-7 cells by using the DEAE-dextran method (Cullen, 1987). COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, 1% glutamine, 100 u ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin.

Two-days after the transfection, cells were harvested in 50 mM Tris-HCl buffer (pH = 7.4) containing 1 mM EDTA and 0.2 mg ml^{-1} bacitracin (buffer 1) and pelleted by a centrifugation at 24,000 × *g* for 15 min at 4°C. The pellet was homogenized in buffer 1 with a Polytron homogenizer. The homogenate was then recentrifuged at 48,000 × *g* for 25 min at 4°C. The pellet was resuspended in the binding buffer and used as the membrane preparation for the radioligand binding assay. Protein concentration in prepared membrane was measured by the Bradford method (Bio-Rad protein assay kit).

For the saturation binding assay of CCK_B receptors (Chang & Lotti, 1991), the binding buffer had the following composition (mM): NaCl 130, $MgCl_2$ 5, EGTA 1, HEPES 10 and bacitracin 0.2 mg ml^{-1}. Membrane preparation was incubated for 1 h at 25°C with varying concentrations (0.03 nM to 10 nM) of [^{3}H]-CCK-8 (Amersham). Specific CCK_B receptor binding was defined as that displaced by 5 μM CCK-8. The binding assay was terminated by vacuum filtration through GF/C (Whatman) filters. Filters were washed with ice-cold binding buffer (without bacitracin), and bound radioactivity was measured with a liquid scintillation counter.

For the saturation-binding analysis of neurotensin (NT) receptors (Tanaka *et al*., 1990), buffer 1 was used as the binding buffer. Cell membranes were incubated with various concentrations (0.01 nM to 8 nM) of [^{3}H]-NT (NEN-DuPont) for 45 min at 25°C. Non-specific binding was determined by adding 5 μM neurotensin to the reaction mixture. The binding reaction was terminated by rapid filtration through GF/B (Whatman) filters. Filters were washed with ice-cold buffer 1 (without bacitracin), and bound radioactivity was counted.

InPlot programme (GraphPad Software) was used to analyse the data derived from the saturation-binding assays and obtain B_{max} (the maximum number of binding sites) and K_D (the equilibrium dissociation constant) values.

Measurement of inositol phosphate production

The assay of polyphosphoinositide hydrolysis was performed as described by Godfrey (1992). Briefly, one day after the transfection, COS-7 cells were labelled with *myo*-[2-^{3}H]-inositol (2 μCi ml^{-1}) and grown for a further 24 h. Cells were then washed with phosphate-buffered saline and incubated with Krebs-Ringer-HEPES buffer containing 10 mM LiCl for 30 min at 37°C. Subsequently, cells were incubated with various concentrations of CCK-8 or neurotensin for 30 min at 37°C. To terminate the reaction, the medium was removed, and ice-cold methanol containing 1% HCl was added to cells. After the extraction with chloroform, the aqueous phase solution was loaded onto AG1-X8 Dowex (Bio-Rad) anion exchange columns. Total [^{3}H]-inositol phosphates were eluted with the solution containing 1 mM ammonium formate/0.1 mM formic acid and counted.

In vitro *transcription*

To synthesize sense cRNA of CCK_B or NT receptor, pBK-CMV vector containing the cDNA encoding wild-type or mutant peptide receptor was linearized with Hind III and used as the DNA template. The transcription reaction mixture contained 5 μg DNA, 40 mM Tris-HCl (pH = 7.5), 6 mM

$MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol (DTT), 0.5 mM adenosine 5′-triphosphate (ATP), cytidine 5′-triphosphate (CTP), uridine 5′-triphosphate (UTP), 0.3 mM guanosine 5′-triphosphate (GTP), 1 mM P^1-5′-(7-methyl)-guanosine-P^3-5′-guanosine triphosphate (m^7GpppG), 40 units of RNAsin, and 80 units of T_3 RNA polymerase (Promega). The reaction was carried out at 37°C for 2 h and then the DNA template was removed by adding RQ1DNAse (Promega). cRNA was precipitated and dissolved in RNAse-free water.

Oocyte injection and electrophysiological recordings

Ovarian lobes were removed from *Xenopus laevis* anaesthetized with 0.2% Tricaine solution. The lobes were washed with Ca^{2+}-free OR-2 medium (NaCl 82.5 mM, KCl 2.5 mM, $MgCl_2$ 1 mM, HEPES 5 mM, pH = 7.6) and then incubated for 2 h with collagenase (4 mg ml^{-1}) at 19°C to obtain oocytes. After the incubation, oocytes were kept at 19°C in the modified Barth's solution (composition in mM: NaCl 88, KCl 1, $NaHCO_3$ 2.4, HEPES 10, $Ca(NO_3)_2$ 0,4, $CaCl_2$ 0.4 and $MgSO_4$ 0.8, pH = 7.6). One day after the isolation, oocytes were injected with 20 nl of cRNA solution (1 μg μl^{-1}) with the Drummond digital microdispenser 510X.

Three days after the injection, agonist-evoked whole-cell membrane currents were recorded with the aid of a two electrode voltage clamp amplifier (Oocyte Clamp OC-725A, Warner Instrument Corp.). Holding potentials, data acquisition and analysis were controlled by an on-line IBM-PC compatible computer programmed with AxoTape 2.0 (Axon Instruments). Membrane currents were filtered at 100 Hz, digitized (Digidata 1200 interface, Axon Instruments) and stored for later analysis. The external solution had the following composition (in mM): NaCl 120, KCl 2.5, $CaCl_2$ 2, $MgCl_2$ 2, HEPES 10, pH = 7.2. When filled with 3 M KCl, recording electrodes had a resistance of 1 to 2 M. Experiments were performed at room temperature (23–25°C).

Statistics

All results are expressed as the mean ± s.e.mean of n experiments or oocytes. Mann-Whitney test (two-tailed) was used to determine whether the difference was statistically significant ($P < 0.05$).

Peptides and drugs

Neurotensin and CCK-8 were purchased from Peninsula. SR48692 (2-(1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxyphenyl)pyrazol-3yl)carbonylamino-tricyclo(3.3.1.1.^{3,7})decan-2-carboxylic acid) was kindly provided by Dr Danielle Gully, Sanofi Recherches. PD135,158 (4-{[2-[[3-(1H-indol-3yl)-2-methyl-1-oxo-2-[[[1.7.7.-tri-methyl-bicyclo[2.2.1]hept-2-yl) oxy] carbonyl]amino] propyl] amino] -1-phenylethyl]amino -4 - oxo-[1S-1α,2-β[S*(S*)]4α]}-butanoate N-methyl-D-glucamine) was obtained from RBI.

Results

Mutation of alanine in the distal third cytoplasmic loop did not affect PLC activation by CCK_B receptors

A 2.2 kb cDNA clone containing the full-length coding region of rat CCK_B receptor was isolated by screening the rat neocortical cDNA library (Wank *et al.*, 1992b). Two days after the COS-7 cells had been transfected with the mammalian expression vector pBK-CMV containing the cDNA of CCK_B receptors, saturable and specific binding sites for [^{3}H]-CCK-8 were detected in these cells. Scatchard analysis of [^{3}H]-CCK-8 binding revealed the expression of single population of high-affinity binding sites (Table 1). No specific [^{3}H]-CCK-8 binding was observed in non-transfected COS-7 cells (data not shown).

Two days after COS-7 cells had been transfected with the cDNA encoding rat CCK_B receptors, CCK-8 stimulated the formation of IP in a concentration-dependent manner (Figure 1a). The maximal CCK-8-stimulated IP formation was 4.2 ± 0.3 ($n = 5$ experiments) fold increase over the basal value, and pEC_{50}(M) value was 7.90 ± 0.06 ($n = 5$ experiments). Three days after *Xenopus* oocytes had been microinjected with cRNA of wild-type CCK_B receptors, CCK-8 dose-dependently evoked oscillating inward currents at the holding potential (V_H) of −60 mV (Figure 2). CCK-8-induced currents reversed the direction of the Cl^- equilibrium potential (about −20 mV), suggesting that activation of CCK_B receptors in oocytes leads to the opening of Ca^{2+}-dependent chloride channels through the IP_3-Ca^{2+} second messenger pathway (Oosawa & Yamagishi, 1989; Sigel, 1990; Kunkel & Peralta, 1993). The maximal magnitude of Ca^{2+}-activated chloride current induced by CCK-8 (3 μM) was 970 ± 98 nA ($n = 15$

Table 1 Ligand binding characteristics of wild-type and (A332E) CCK_B receptors expressed in COS-7 cells

Receptor	B_{max} (fmol mg^{-1} protein)	pK_D(M)
Wild-type	522 ± 43	8.96 ± 0.09
(A332E)	470 ± 38	9.01 ± 0.05

Saturation binding assays were performed with [^{3}H]-CCK-8. All experiments were performed in triplicate. Each value represents the mean ± s.e.mean of 4 experiments.

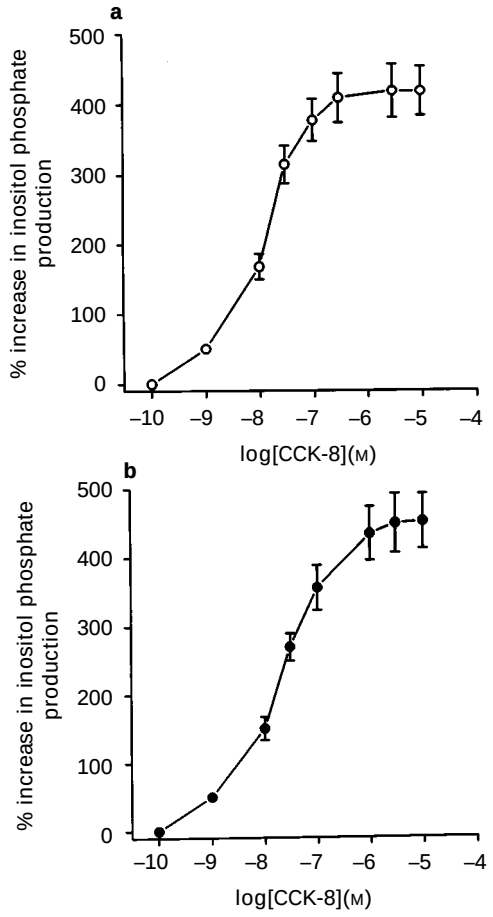

Figure 1 Alanine at the distal third intracellular loop is not required for CCK_B receptor activation of PLC in COS-7 cells. In COS-7 cells transfected with the cDNA encoding wild-type (a) or (A332E) mutant (b) CCK_B receptors, CCK-8 stimulated the formation of inositol phosphate with a similar potency and efficacy. Each point represents the mean value of 5 experiments performed in duplicate; vertical lines show s.e.mean.

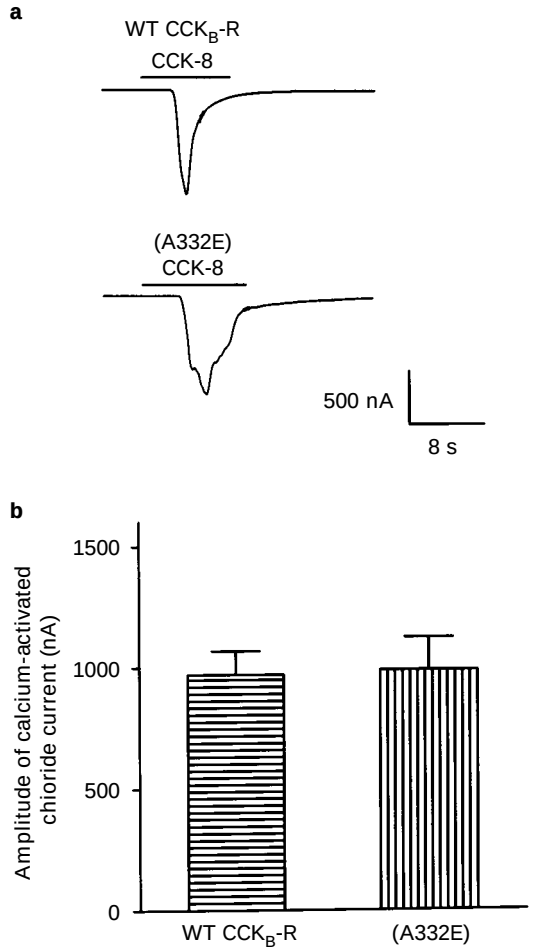

Figure 2 Alanine at the C-terminal end of the third cytoplasmic domain of the CCK_B receptor is not required for the activation of PLC in oocytes. (a) Three days after the microinjection of cRNA, CCK-8 (3 μM) evoked a similar peak amplitude of calcium-dependent Cl^- currents in oocytes expressing wild-type (WT, upper trace) or (A332E) mutant (lower trace) CCK_B receptors (CCK_B-R). Membrane potential was held at -60 mV. (b) The summary of CCK-8-induced Ca^{2+}-activated chloride currents in oocytes microinjected with the cRNA of wild-type or (A332E) CCK_B receptors. Each column shows the mean $\pm$ s.e.mean value of 15 oocytes.

Table 2 Ligand binding parameters of wild-type and (A302E) neurotensin receptors expressed in COS-7 cells

Receptor	B_{max} (fmol mg^{-1} protein)	pK_D(M)
Wild-type	930 ± 89	9.57 ± 0.06
(A302E)	1006 ± 30	9.48 ± 0.05

Saturation binding assays were performed with [^{3}H]-NT. Each value represents the mean $\pm$ s.e.mean of 6 experiments performed in triplicate.

oocytes), and pEC_{50} value was 7.81 ± 0.05 ($n = 5$ oocytes). Both CCK-8-induced IP formation in transfected COS-7 cells and CCK-8-evoked Ca^{2+}-dependent chloride currents in oocytes were blocked by PD135,158 (150 nM), a highly potent and specific antagonist of CCK_B receptors (data not shown) (Hughes *et al*., 1990).

The alanine residue in the distal third cytoplasmic domain of the CCK_B receptor was mutated to glutamate with the aid of site-directed mutagenesis, and a pBK-CMV vector containing (A332E) mutant receptor was transfected to COS-7 cells. In contrast to previous studies on α_{1B}-adrenoceptors (Cotecchia *et al*., 1990; Kjelsberg *et al*., 1992), the basal IP level of COS-7 cells expressing (A332E) CCK_B receptors was similar to that of COS-7 cells transfected with the cDNA encoding wild-type CCK_B receptors (data not shown). As shown in Figure 1, a similar maximal CCK-8-induced IP formation was observed for COS-7 cells transfected with cDNA encoding wild-type or (A332E) CCK_B receptors. pEC_{50} values for IP formation by (A332E) mutant ($pEC_{50} = 7.78 \pm 0.05$, $n = 5$ experiments) and wild-type CCK_B receptors ($pEC_{50} = 7.90 \pm 0.06$, $n = 5$ experiments) were not significantly different. In *Xenopus* oocytes injected with cRNA of (A332E) mutant receptors, the maximal amplitude (990 ± 135 nA, $n = 15$ oocytes, $V_H = -60$ mV) and pEC_{50} value (7.75 ± 0.04, $n = 5$ oocytes) of CCK-8-induced Ca^{2+}-dependent Cl^- currents were similar to those of CCK-8-evoked chloride currents in oocytes expressing wild-type CCK_B receptors (Figure 2). Radioligand binding studies indicated that both B_{max} and K_D values of [^{3}H]-CCK-8 binding in COS-7 cells transfected with cDNA of (A332E) mutant receptors were not significantly different from those measured in COS-7 cells expressing wild-type CCK_B receptors (Table 1). These results suggest that alanine in the distal third intracellular loop of CCK_B receptors is not required for G_q protein activation and stimulation of PLC.

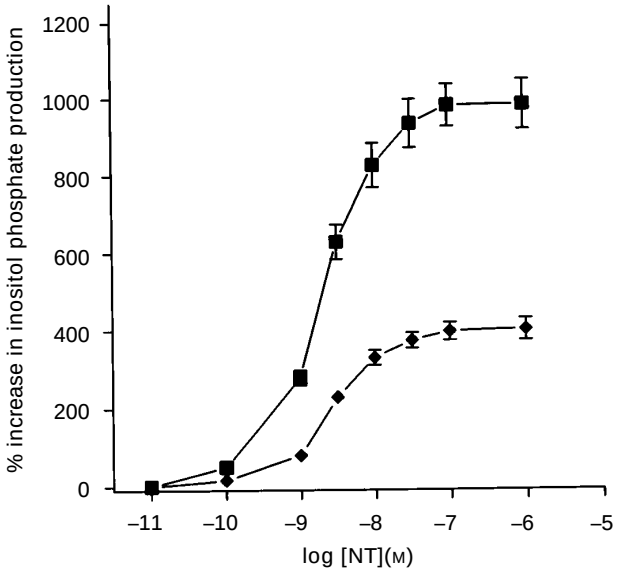

Figure 3 Alanine residue at position 302 of neurotensin receptor (NTR) is required for the full activation of PLC in COS-7 cells. In COS-7 cells transfected with the cDNA encoding (A302E) mutant neurotensin receptors (◆), NT-stimulated inositol phosphate (IP) production was significantly reduced compared with NT-induced IP formation in COS-7 cells expressing wild-type NT receptors (■). Each point shows the mean value of 7 experiments performed in duplicate; vertical lines show s.e.mean.

Mutation of alanine at the distal third intracellular loop of neurotensin (NT) receptor results in a G_q coupling defect

A single population of saturable and high-affinity binding sites for [^{3}H]-NT was observed in COS-7 cells transfected with the cDNA encoding wild-type NT receptors (Table 2). No specific [^{3}H]-NT binding was observed in non-transfected COS-7 cells (data not shown). In COS-7 cells expressing wild-type NT receptors, neurotensin activated PLC and induced the formation of IP dose-dependently (Figure 3). The maximal NT-induced IP production was 9.8 ± 0.6 fold increase over the basal value ($n = 7$ experiments), and pEC_{50} value was 8.81 ± 0.04 ($n = 7$ experiments). Neurotensin also evoked Ca^{2+}-dependent chloride currents in oocytes microinjected with cRNA of wild-type NT receptors (Figure 4). The maximal amplitude of NT

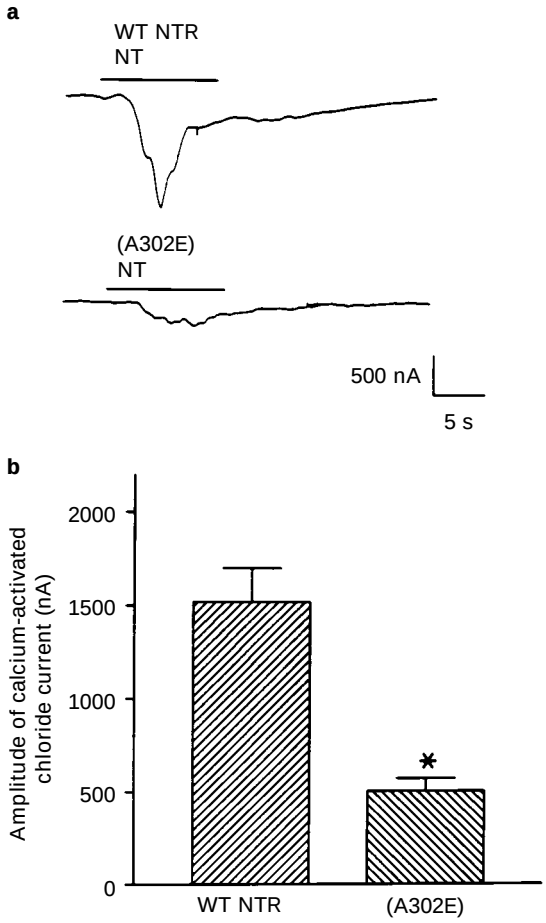

Figure 4 Point mutation of alanine residue in the distal third intracellular loop of neurotensin receptor (NTR) results in a G_q coupling defect in oocytes. (a) Neurotensin (1 μM) evoked Ca^{2+}-dependent Cl^- currents in oocytes expressing wild-type (WT) and (A302E) mutant NT receptors at a holding potential of -60 mV. Note that the peak amplitude of NT-induced chloride current in oocytes injected with cRNA of (A302E) mutant receptor was significantly diminished. (b) A summary of NT-evoked Ca^{2+}-dependent Cl^- currents in oocytes injected with cRNA of wild-type or (A302E) mutant NT receptors. Each column shows the means $\pm$ s.e.mean value of 17 oocytes. $^*P<0.01$.

(1 μM)-induced chloride currents was 1520 ± 175 nA ($n=17$ oocytes, $V_H=-60$ mV), and pEC_{50} value was 8.60 ± 0.06 ($n=5$ oocytes). Both NT-induced IP formation in COS-7 cells and NT-evoked Ca^{2+}-dependent chloride currents in oocytes were blocked by 150 nM SR48692 (data not shown), a specific nonpeptide antagonist for neurotensin receptors (Chabry *et al*., 1994).

The alanine residue at position 302 of the neurotensin receptor was mutated to glutamate. When (A302E) mutant NT receptor was expressed in COS-7 cells, the basal level of inositol phosphates was not significantly affected (data not shown). However, the (A302E) NT receptor was strongly defective in activating G_q proteins and stimulating polyphosphoinositide hydrolysis (Figure 3). Mutation of alanine at position 302 resulted in a decrease in both the maximal response (4.0 ± 0.2 fold increase, $n=7$ experiments) and the potency ($pEC_{50}=8.60\pm0.04$, $n=7$ experiments) of NT-induced phosphatidylinositol turnover. In oocytes expressing (A302E) mutant NT receptors, NT (1 μM)-evoked Ca^{2+}-dependent Cl^- currents were significantly smaller in the peak amplitude, compared with NT-induced chloride currents in oocytes injected with cRNA of wild-type NT receptors (Figure 4). The maximal magnitude of NT (1 μM)-induced chloride current was 504 ± 70 nA ($n=17$ oocytes, $V_H=-60$ mV). The pEC_{50} value of NT-evoked Cl^- currents in oocytes expressing the (A302E) mutant receptor (8.37 ± 0.05, $n=5$ oocytes) was significantly increased compared to that of NT-induced Cl^- currents in oocytes injected with cRNA of the wild-type NT receptor. Saturation binding studies demonstrated that (A302E) mutant receptors bind to [^{3}H]-NT with a similar affinity (K_D) as the wild-type NT receptors and that the expression level (B_{max}) of mutant receptors is comparable to that of wild-type receptors (Table 2). Therefore, the G_q coupling defect exhibited by the (A302E) mutant NT receptor was not due to a lower expression level of mutant receptors or the failure of mutant receptors to bind adequately to neurotensin. These findings indicate that the alanine residue at the C-terminal segment of the third intracellular loop is required for the full activation of G_q proteins and PLC by neurotensin receptors.

Discussion

Different neurotransmitter receptors which couple to G_q proteins-PLC signalling pathway do not exhibit any similarity in amino acid sequence of the third cytoplasmic domain. However, the alanine residue located near the sixth transmembrane segment is highly conserved among a variety of G_q coupled-receptors (Savarese & Fraser, 1992; Strader *et al*., 1994), suggesting that it plays a key role in the activation of G_q proteins. In the present study, site-directed mutagenesis in combination with the expression of CCK_B or neurotensin receptors in COS-7 cells and *Xenopus* oocytes was used to investigate the functional role played by the conserved alanine at the C-terminus of the third intracellular loop in activating G_q proteins and PLC. Consistent with previous findings that mutation of conserved alanine at the C-terminal tail of the third cytoplasmic domain caused a grave defect in the receptor-G_q protein interaction (Kosugi *et al*., 1992; Benya *et al*., 1994; Blin *et al*., 1995; Burstein *et al*., 1995), point mutation of the alanine at position 302 of the neurotensin receptor resulted in a G_q coupling defect and a significant reduction in the maximal neurotensin-induced IP production.

Previous site-directed mutagenesis experiments and the present investigation on the neurotensin receptor indicate that conserved alanine in the distal third cytoplasmic domain is required for receptor-G_q protein coupling. Interestingly, in contrast to other G_q protein-coupled neurotransmitter receptors, replacing the conserved alanine in the distal third intracellular loop of CCK_B receptor with glutamate did not affect the potency and the efficacy of CCK-8-stimulated IP formation in COS-7 cells and *Xenopus* oocytes. It has also been shown that mutation of alanine in the distal third cytoplasmic loop renders α_{1B}-adrenoceptors constitutively active (Kjelsberg *et al*., 1992). However, the present study did not find evidence of constitutive activity of the (A332E) CCK_B receptor or (A302E) neurotensin receptor.

Mutation of alanine 302 in the third cytoplasmic loop of the neurotensin receptor resulted in a 60% reduction of neurotensin-induced polyphosphoinositide hydrolysis, suggesting that alanine at position 302 is not the sole structural determinant involved in coupling the neurotensin receptor to G_q proteins. In agreement with this hypothesis, a previous study in which a deletion mutant neurotensin receptor was used indicated that a group of amino acids in the third intracellular loop (amino acids 270–282) also play a critical role in the activation of G_q proteins and PLC (Yamada *et al*., 1994). It will be interesting to perform point mutation experiments on this domain of the third cytoplasmic loop (amino acids 270–280) and identify amino acids that are also required for efficient neurotensin receptor-G protein coupling.

When expressed in Chinese hamster ovary (CHO) cells, the neurotensin receptor has been shown to couple to two signal transduction pathways, phosphatidylinositol turnover and adenosine 3′:5′-cyclic monophosphate (cyclic AMP) formation, through different G proteins (Yamada *et al*., 1994). For

thyrotropin receptors that also couple to G_q-PLC and G_s-adenylate cyclase signalling pathways, it has been shown that mutation of conserved alanine in the distal third cytoplasmic domain led to a defect in the receptor-G_q protein interaction and polyphosphoinositide hydrolysis without affecting thyrotropin-induced cyclic AMP formation (Kosugi *et al*., 1992). Recent investigations also indicated that the alanine residue in the corresponding site of M_3- or M_5-subtypes of cholinoceptors is the major determinant of receptor affinity and selectivity for G_q proteins (Blin *et al*., 1995; Burstein *et al*., 1995). Further studies with (A302E) neurotensin receptors expressed in CHO cells are required to investigate whether alanine at position 302 is selectively involved in coupling neurotensin receptor to G_q proteins that stimulate phosphatidylinositol turnover.

The conserved alanine residue in the distal third intracellular loop is likely to play a critical role in receptor-G_q coupling either by directly interacting with G proteins or being involved in forming a proper secondary structure. If the first hypothesis is correct, one would expect mutation of this alanine to lead to a receptor-G protein coupling defect of every G_q-coupled receptor. However, the present study shows that alanine at position 332 of the CCK_B receptor is not involved in the activation of G_q proteins and PLC. The second hypothesis is also supported by the fact that the distal third cytoplasmic domains of G protein-coupled receptors are heterogeneous in amino acid sequence and size. Therefore, a secondary structure, instead of specific amino acids of the distal third intracellular loop, is likely to play a critical role in G protein activation (Savarese & Fraser, 1992; Strader *et al*., 1994).

Secondary structure analysis suggests that the C-terminal tail of the third cytoplasmic segment of G protein-coupled receptor may form the amphipathic α-helical extension of the sixth transmembrane domain (Ostrowski *et al*., 1992; Strader *et al*., 1994; Burstein *et al*., 1995; Wang *et al*., 1995; Hill-Eubanks *et al*., 1996). G protein-activating peptides, which include mastoparan and synthetic peptides corresponding to the N- or C-terminal portion of the third intracellular loop of α_2 adrenoceptors or M_4 cholinoceptors, are also expected to form amphiphilic α-helices (Higashijima *et al*., 1990; Sukumar & Higashijima, 1992; Okamoto & Nishimoto, 1992). Thus, it is reasonable to hypothesize that the C-terminal segments of the third cytoplasmic domains of CCK_B and neurotensin receptors also adopt an amphiphilic α-helical conformation. With the exception of the conserved alanine residue, the carboxyl ends of the third intracellular loops of rat CCK_B and neurotensin receptors do not exhibit any similarity in amino acid sequence (Tanaka *et al*., 1990; Wank *et al*., 1992b). Therefore, it is expected that proper α-helical secondary structures, which are essential for G protein activation, in the distal third cytoplasmic loops of CCK_B and neurotensin receptors require different amino acid residues. The present study shows that mutation of conserved alanine in the distal third intracellular loop impairs neurotensin receptor activation of PLC without affecting CCK_B receptor-G_q coupling. One possible explanation for this finding is that alanine at position 302 of the neurotensin receptor, but not alanine 332 of the CCK_B receptor, is required for the formation of a proper α-helical conformation. Instead of the alanine residue, our recent investigation proposes that three basic amino acids next to alanine, K333/K334/R335, play a critical role in CCK_B receptor activation of G_q proteins by being involved in forming a proper amphiphilic α-helix (Wang *et al*., unpublished results). Further investigations with insertion mutagenesis and molecular modelling (Bluml *et al*., 1994; Jagerschmidt *et al*., 1995) are needed to test the proposed amphipathic α-helix hypothesis.

In conclusion, the present site-directed mutagenesis experiment provides evidence that alanine in the C-terminus of the third intracellular loop of the neurotensin receptor is required for the full activation of G_q proteins and PLC. However, unlike other G_q protein-coupled receptors, the alanine residue in the corresponding site of the CCK_B receptor is not involved in activating G_q proteins and PLC. Future studies in which the approach described here is used should also be useful in identifying other specific amino acids of neurotensin and CCK_B receptors that are essential for G protein activation or agonist-induced desensitization.

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