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Attenuation of agonist-induced desensitization of the rat substance P receptor by progressive truncation of the C-terminus

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Abstract

We have investigated the C-terminal tail of the rat substance P receptor (SPR) as a domain essential for agonist-induced desensitization. Four progressively shorter mutants, using premature termination in the C-terminus, were constructed and compared with the unaltered SPR using ectopic expression of wild-type and mutant receptors in *Xenopus* oocytes. These mutants were designated D16, D47, D70 and D96 with 16, 47, 70 and 96 amino acids residues deleted from the tail, respectively. Wild type SPR, D16 and D47 exhibited normal current responses when challenged with substance P, but D70 and D96 had reduced maximal current responses (70% and 5% of wild type SPR, respectively). D70, however, exhibited substantial resistance to substance P-induced desensitization in that 55%, versus 8% for wild type SPR, of the peak current of the first response was preserved on second challenge with substance P. Therefore, a domain from residues 338 to 360 of the rat SPR, though not necessary for the functional activity of the receptor, plays an essential role in agonist-induced desensitization.

Key words: Substance P receptor; Desensitization; Truncation mutation; C-terminal tail, Ca^{2+}-dependent Cl^- current; *Xenopus* oocyte

1. Introduction

Substance P receptor (SPR) is a widely distributed neuropeptide receptor in nervous and peripheral tissues, and plays roles in a number of biological processes [1,2]. Molecular cloning has revealed that SPR is a member of the superfamily of G protein-coupled seven transmembrane receptors [3,4]. Functional analysis of the cloned receptor in heterologous cells has confirmed that activation of the receptor leads to intracellular calcium mobilization, which can be conveniently monitored in *Xenopus* oocytes by current responses under voltage clamp [3]. Although the SPR shows rapid and long-lived desensitization in response to repeated application of substance P (SP) when expressed in *Xenopus* oocytes, the mechanisms of desensitization remain to be clarified [2]. Recently, the SPR has been shown to be multiply phosphorylated in vitro by the regulatory enzymes, β-adrenergic receptor kinase (βARK) 1 and 2 , only in the presence of agonist [5]. This suggests that the agonist-dependent regulation of SPR may be mediated in part through βARK enzymes, which are known to phosphorylate β_2-adrenergic receptors at the C-terminal tail [6]. We have investigated the predicted importance of the C-terminus for agonist-induced desensitization using ectopic expression of SPR and truncated mutants in *Xenopus* oocytes.

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Abbreviations: SPR, substance P receptor; SP, substance P; βARK, β-adrenergic receptor kinase; SEAP, secreted alkaline phosphatase.

2. Materials and methods

2.1. SPR and SPR mutant expression vectors

The coding regions for the SPR was amplified by polymerase chain reaction (PCR) using rat SPR cDNA (prTKR2 ; [3]). The amplified SPR was subcloned into the pRc/CMV vector (Invitrogen, San Diego, CA) which allowed readily detectable receptor expression in *Xenopus* oocytes. PCR reactions were as follows: 100 ng of SPR cDNA plasmid was used in 30 PCR cycles in the presence of 200 mM each of the dNTP, 1.5 mM of $MgCl_2$, 2.5 Units of AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, CT) and using two receptor-specific primers. Each cycle involved a denaturation step (95°C, 1 min), an annealing step (49°C, 1.5 min) and a extension step (72°C, 1.5 min). The upstream primers contained a *Hin*dIII restriction site, a translational consensus sequence, and the receptor specific sequence. The down stream primers contained an *Xba*I restriction site, a translation termination codon and the receptor specific sequence as indicated: **SPR-Full** (SPR 5-END = 5′-ACTC AAGCTT CGCCACC ATGGATAACGTCCTTCCTATGGAC-3′, and SPR 3-F = 5′-TTTT GAATTC TCTAGA CTAGGCCAGCATGTT AGAGTA-3′) (underlined nucleotides are receptor-specific). A similar PCR procedure was used to generate tail truncation mutants in rat SPR. Length of tail deletion was selected to include clusters of serine and threonine residues; possible phosphorylation sites. In addition to the full length SPR (designated Full), we made deletions D16, D47, D70 and D96 (the number represents amino acids residues deleted from the C-terminal end of the receptor, see Fig. 1). Substance P receptor downstream PCR deletion primers were: 1. **D16** = (SPR 3-D16 5′-TTTT TCTAGA CTAGTTGCTGCGAGAGGAGCC-3′). 2. **D47** (SPR 3-D47 = 5′-TTTT TCTAGA CTAAGTGGAG ATGGTGGTCTC-3′). 3. **D70** = (SPR 3-D70 = 5′-TTTT TCTAGA CTATTTCATTTCCAGCCCCTC-3′). 4. **D96** (SPR 3-D96 = 5′-TTTT TCTAGA CTACCTGTCG TTGAGGCAACA-3′). The amplified receptor DNA fragments were purified and concentrated using Amicon columns (Amicon, Beverly, MA), restricted with *Hin*dIII and *Xba*I and cloned into the pRc/CMV plasmid using the unique *Hin*dIII–*Xba*I sites. Two different plasmid constructs were generated and analyzed in the *Xenopus* oocytes for Full SPR and for each of the mutants to confirm the results obtained.

2.2. Preparation of oocytes

Xenopus laevis were injected with pregnant mares serum (50 IU) 24–48 h before ovarectomy. The ovaries were washed with OR-2 solution: 82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1.0 mM $CaCl_2$,

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2.5 mM $NaHCO_3$ and 5 mM HEPES (pH 7.4). Oocytes were isolated by treating the ovaries with collagenase type I (2 mg/ml) made in Ca^{2+}-free OR-2 solution for 100–110 min. Stage VI oocytes were selected and rolled on plastic culture dishes to insure complete removal of the follicle cells [7]. The defolliculated oocytes were cultured for 16–18 h at 18°C in Modified Barths solution: 88 mM NaCl, 1.0 mM KCl, 0.82 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 2.42 mM $NaHCO_3$, 10 mM HEPES, 300 μg/ml Na-pyruvate, 10 μg/ml penicillin, 10 μg/ml streptomycin, 10 μg/ml gentamycin and 0.5% chicken ovalbumin (pH 7.6).

2.3. Microinjection of SPR expression vector

The oocytes were placed on a bed of 35% of Ficoll with OR2 solution in 50 ml plastic conical tubes and centrifuged 750 × *g* for 10 min. This makes the nucleus visible as a white spot. The oocytes were transferred to an injection dish which has a layer nylon meshes (grid size, 1.5 mm) in the bottom in order to hold the oocytes. The oocytes were microinjected by pressure using a picospritzer (General Valve Corp, Fairfield, NJ). The amount injected was determined by injecting a droplet in the air and measuring the volume of the droplet. A 5 nl of plasmid in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) was injected into the nucleus. In order to confirm that the nucleus has been successfully microinjected, pMT2-secreted alkaline phosphatase (SEAP) plasmid (100 pg/oocyte) was coinjected with SPR (100 pg/oocyte) as described previously [8,9]. The oocytes were cultured 4–5 h in Modified Barths solution at 18°C. The oocytes were then transferred individually to 96-well plates containing 200 μl of Modified Barths solution and cultured for 48 h. SEAP activity in the media was assayed colorimetrically as previously described [8,9].

2.4. Electrophysiology

SEAP expressing oocytes were selected and were voltage-clamped at −60 mV with a two-electrode voltage clamp (Dagan Model TEV-200, Minneapolis, MN) [7]. The oocytes were continually perfused (flow rate: 3.4–3.6 ml/min) with OR-2 solution in chamber. Stimulant (SP solution) was applied to the cell chamber from another reservoir. The oocytes were exposed to stimulant for 18 s. The electrophysiology experiments were performed at 23–26 °C. The results were collected with Axotape software (Axon Instruments, Foster City, CA) and were plotted with Axoplot software.

2.5. Materials

Materials were obtained from: SP peptide from Peninsula laboratories (Belmont, CA); the pRc/CMV plasmid from Invitrogen, (San Diego, CA). the cDNA clone for rat SPR (prTKR2) was a gift from Dr. Shigetada Nakanishi (Kyoto university, Kyoto, Japan); pMT2 plasmid containing SEAP cDNA was kindly provided by the Genetics Institute (Cambridge, MA).

3. Results and discussion

The predicted intracellular C-terminus has the greatest sequence divergence between the three closely related tachykinin receptors [2]. In addition, naturally occurring C-terminal truncations of transcripts [10] or SPR protein [11] have been reported, suggesting alternative transcription may produce a variant SPR lacking a significant intracellular tail. These observations imply a physiological importance for this region. Truncation mutations in the C-terminal tail were constructed based on the clustering of serine/threonine amino acid residues. Our rationale was that the SPR was shown to be phosphorylated in vitro by the G-protein coupled receptor kinases; βARK 1 and 2 [5] suggesting phosphorylation of serine/threonine residues, localized to the C-terminal tail in the case of the β_2-adrenergic receptor [6], may play a role in agonist-induced desensitization. Accordingly, four different truncation mutants in the tail of SPR were constructed; designated 'D16', 'D47', 'D70' and 'D96' with 16, 47, 70 and 96 amino acids residues deleted from the tail, respectively (Fig. 1). Their functional activities and sensitivity to agonist-induced desensitization were compared with the unaltered SPR ('Full'). The D96 corresponded to the naturally truncated, potentially alternatively spliced variant, described earlier [10]. The functional expression of SPR and mutants was examined by voltage-clamp analysis of SP-induced currents.

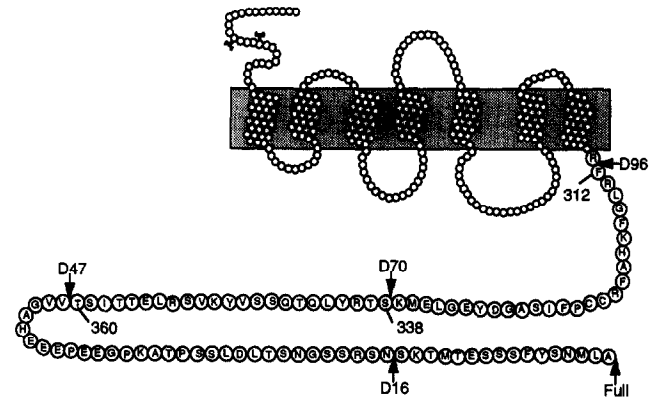

Fig. 1. A schematic presentation of the predicted structure of rat SP receptor showing the exact amino acid residues of the C-terminal tail. The wild type of SPR (Full) and the positions of the constructed truncation mutants (D16, D47, D70 and D96) are indicated. The designation of each mutant represents the number of residues deleted from the C-terminal. The numbers (312, 338 and 360) refer to amino acid positions.

Fig. 2 (left panels, 'First') shows typical first application of SP-induced current responses for Control, Full-, D16-, D70- and D96-injected oocytes. The control (pRc/CMV plasmid) injected oocytes had no detectable response, while Full, D16 and D47 injeced oocytes had very similar SP-induced current responses (Fig. 2, left panels and Fig. 3A,C). In contrast to Full SPR, D70 had a slight reduction (approximately 30% of peak current) in activity (Fig. 2, left panels and Fig. 3A,C). Fig. 3C shows the concentration–response curves for SP in Full-, D16-, D47- and D70-injected oocytes. SP induced a concentration-dependent increase in current responses, having maximum effects at 30 nM in Full, D16, D47 and D70-injected oocytes. D70, on the other hand, had approximately 30% smaller maximum current responses. The apparent EC_{50} of SP for Full, D16, D47 and D70 were identical (4.5 nM). This suggests that wild type and mutant SPRs have similar affinity to SP.

As previously reported, there is a substantial loss of activity for D96 with 95% reduction in peak currents compared to full-length SP receptor. (Fig. 2, left panels and Fig. 3A) [10]. The D96 results suggest that the domain between 312 to 337 has a crucial role for receptor function, such as G-protein coupling, whereas residues from 338 to the C-terminus are not essential for normal

receptor function. This finding is consistent with recent studies on the comparable regions of adrenergic receptors [12,13].

The SPR mutants were tested in oocytes for their sensitivity to agonist-induced desensitization. Oocytes were first stimulated with a maximal dose of SP (30 nM), washed for 10 min, and re-stimulated with the same dose of SP. The second current responses of Full, D16 and D47-injected oocyte were diminished to the same extent (Fig. 2, right panels). The magnitudes of the second currents were 8.3 ± 1.3%, 9.2 ± 0.9% and 8.1 ± 1.8% of first response to SP, respectively (Fig. 3B). Strikingly, the D70 mutant exhibited remarkable resistance to desensitization, in that the second response was 54 ± 6.7% of first response (Fig. 2, right panels and Fig. 3B), nearly 7 times greater than normally desensitizing SPR responses.

Because the magnitude of the first current responses might affect the magnitude of the second current responses, we modulated the level of SPR expression using a reduced level of nuclear cDNA injection. Using half the amount of cDNA (e.g. 50 pg) of Full, D16 and D47-injected oocytes, the first stimulation of SP induced similar reduced magnitudes of current responses, comparable to that observed with injection of 100 pg of D70

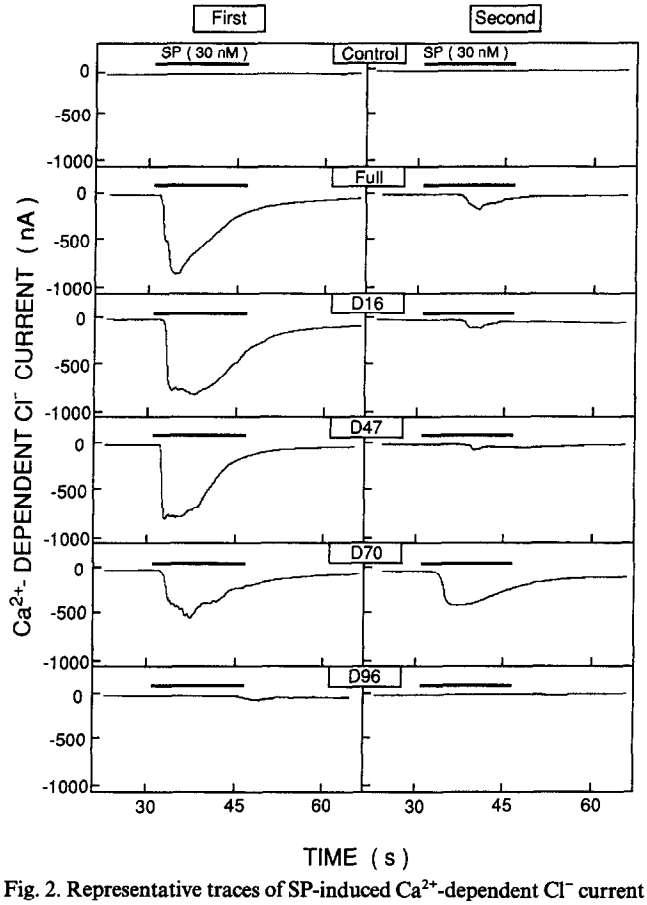

Fig. 2. Representative traces of SP-induced Ca^{2+}-dependent Cl^- current responses in oocytes injected with SPR or SPR mutants expression plasmids. The oocytes were microinjected with pRc/CMV plasmid (Control) and SPR expression plasmids (Full, D16, D47, D70 and D96; 100 pg/oocyte) (from top to bottom panels) into nucleus and cultured for 48 h as described in section 2. The oocytes were stimulated with SP (30 nM, 18 s) (left panels; 'First'). The oocytes were washed for 10 min and re-stimulated with SP (right panels; 'Second'). Typical data obtained from 5–22 different oocyte preparations are presented.

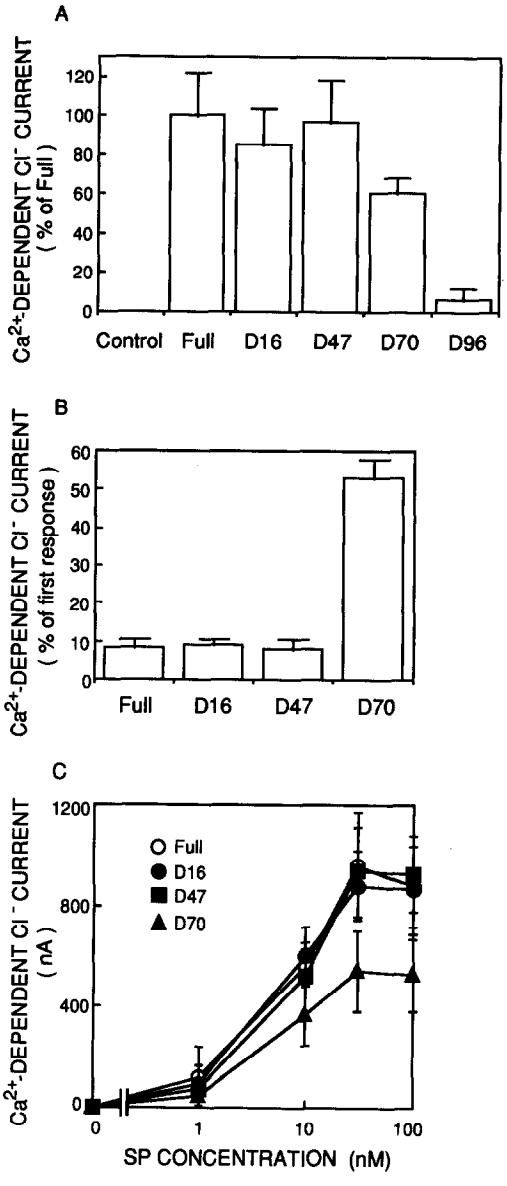

Fig. 3. SP-induced Ca^{2+}-dependent Cl^- current responses in oocytes injected with SPR and SPR mutants expression plasmids. (A) Current responses in pRc/CMV plasmid (control), Full, D16, D47, D70 and D96 cDNA-injected oocytes. Current responses induced by first application of SP (30 nM) are shown as percentage of the first response in wild type (Full) of SPR expressed oocytes. The absolute values of first response of Full was 910 ± 38 (n = 5). (B) Desensitization of SP-induced current responses in oocytes following Full, D16, D47 and D70 nuclear microinjection. The results shown are percentage of the second current response relative to the first current response. The absolute values of first response of Full, D16, D47 and D70 were 861 ± 92, 838 ± 51, 844 ± 66 and 509 ± 38 nA (n = 5–9), respectively. (C) SP concentration–response curves for current responses in Full, D16, D47 and D70 cDNA-injected oocytes. The oocytes were stimulated with different concentration of SP. Maximum values of the currents were plotted. The results shown are mean ± S.E. of 3–5 determinations from same batch of oocytes.

cDNA into oocyte nuclei. The second current responses for Full, D16 and D47-injected oocyte were diminished to the same degree as observed with larger magnitudes of initial responses (currents were 6.1 ± 1.3%, 5.8 ± 1.9% and 6.3 ± 1.8% of first response ($n = 5$), respectively). Thus, it is highly unlikely that the reduced SP-induced desensitization in D70 is due to reduced expression.

The loss of current responses to SP with second application was also reflected in the alterations in latencies of initial responses, in that as the peak current response to SP diminished, the latency of response became greater. The response latency upon second application of SP was 2.5 s in D70-injected oocytes, whereas the latency for second responses was invariably longer for Full (7.7 s), D16 (7.8 s) and D47 (8.1 s) (Fig. 2). Since the first current responses induced by SP were very small, the D96 mutant was not examined for the desensitization.

This identifies a specific region of the C-terminal tail of rat SPR from residues 338 to 360 as playing a crucial role in agonist-induced desensitization. This domain has 5 serine and 5 threonine residues, and thus would be a candidate for the site of phosphorylation by βARK 1 and βARK 2. The stoichiometry of maximum phosphorylation in vitro was calculated to be 9 [5], suggesting that a subset of sites may undergo agonist-induced phosphorylation. In addition, this domain, when phosphorylated, would be predicted to be the site of binding of the regulatory protein, β-arrestin [6,14]. In the β_2-adrenergic receptor and rhodopsin, the functionally equivalent domain lies at a different position in the tail, the extreme C-terminus [14]. This may suggest some potential differences in the mechanisms of βARK/β-arrestin pathway in regulation of SPR.

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