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Short communication

Direct evidence for the interaction of neurokinin A with the tachykinin NK_1 receptor in tissue

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Abstract

Neurokinin A (NKA) is a tachykinin peptide that binds with high affinity to the tachykinin NK_2 receptor. Recent homologous binding studies, however, have shown that neurokinin A is also a high-affinity ligand for the tachykinin NK_1 receptor. In this report, we demonstrate that a photoreactive neurokinin A analogue specifically labels the NK_1 receptor in rat submandibular gland membranes and show via bioassay that neurokinin A is a potent stimulator of salivary secretion. Through the use of specific non-peptide antagonists in both photolabeling and functional assays, we unequivocally demonstrate that neurokinin A can specifically interact with the NK_1 receptor in vivo and elicit NK_1 receptor-mediated physiological responses. © 2001 Published by Elsevier Science B.V.

Keywords: Neurokinin A; Tachykinin NK_1 receptor; Photoaffinity labeling; Bioassay

1. Introduction

The tachykinins comprise a family of bioactive neurotransmitter/neuromodulatory peptides (Maggio, 1988). Neurokinin A and substance P are two mammalian tachykinins in particular that have been extensively studied (Otsuka and Yoshioka, 1993). The tachykinin receptor with the highest affinity for neurokinin A has been termed the tachykinin NK_2 receptor, and the tachykinin receptor with the highest affinity for substance P has been termed the tachykinin NK_1 receptor (Maggi et al., 1993). Both of these receptors are hepta-helical integral membrane proteins and belong to the rhodopsin-type family of cell-surface G-protein coupled receptors (Nakanishi, 1991).

Initial heterologous binding assays suggested that neurokinin A is a weak-affinity ligand for the NK_1 receptor due to its low efficiency in displacing radiolabeled substance P binding to the NK_1 receptor (Ingi et al., 1991). However, recent homologous binding assays using radiolabeled neurokinin A have shown that neurokinin A, despite its weak ability to compete with substance P binding, is nevertheless a high-affinity ligand for the NK_1 receptor expressed in transfected cell lines (Hastrup and Schwartz, 1996). Previous results from our laboratory have also shown that photoactivatable derivatives of substance P (SP) and neurokinin A (NKA), ^{125}I-Bolton Hunter (BH)-[*p*-benzoylphenylalanine (Bpa)^8]SP and ^{125}I-[Bpa^7]NKA, respectively, each specifically and efficiently photolabel the rat NK_1 receptor when expressed in chinese hamster ovary (CHO) cells (Bremer et al., 2000).

Having demonstrated the usefulness of photoaffinity labeling for the direct biochemical characterization of the rat NK_1 receptor in transfected cell lines, we turned our attention to examining whether this same approach could be used to biochemically characterize the binding site(s) of neurokinin A in tissue. We decided to first study the target of neurokinin A binding in the rat submandibular gland since earlier studies reported that neurokinin A as well as N-terminally extended neurokinin A derivatives stimulated salivary secretion (Takeda and Krause, 1989). Both neurokinin A and substance P are found in the rat submandibular gland (Otsuka and Yoshioka, 1993), and binding sites in this tissue for both radiolabeled neurokinin A and radiolabeled substance P have been documented by autoradiography (Buck and Burcher, 1985). The biochemical nature of the binding protein for substance P in submandibular gland tissue has been extensively characterized in our laboratory and known to be the NK_1 receptor (Boyd et al., 1991); however, the biochemical nature of the binding protein for neurokinin A in this tissue has not been established. Since mRNA encoding the NK_2 receptor has

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not been reported in the rat submandibular gland (Takeda and Krause, 1991), we hypothesized that the NK_1 receptor was the site of neurokinin A binding. In this report, we unequivocally confirm this hypothesis by demonstrating through photoaffinity labeling techniques, biochemical analysis, and bioassay that neurokinin A can directly and functionally interact with the NK_1 receptor in vivo.

2. Materials and methods

2.1. Materials

Purified substance P and neurokinin A were purchased from Sigma, and ^{125}I-Bolton Hunter (BH) reagent (*N*-succinimidyl-3[4-hydroxyphenyl]propionate) and ^{125}I (each with specific activities of 2200 Ci/mmol) were obtained from NEN Life Sciences. ^{125}I-BH-[Bpa^8]SP and ^{125}I-iodohistidyl^1-[Bpa^7]NKA (^{125}I-[Bpa^7]NKA) were prepared in our laboratory as described previously (Boyd et al., 1991; Bremer et al., 2000).

2.2. Rat submandibular gland tissue preparation

Rapidly frozen submandibular gland tissue was obtained from adult Sprague–Dawley rats (Zivic–Miller Laboratories). Tissues were homogenized in TE buffer (50 mM Tris, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4) containing 30 μg/ml bacitracin at 4 °C. Nuclear and cellular debris were removed by centrifugation at $500 \times g$ for 10 min, and membranes were obtained by centrifugation of the supernatant at $30{,}000 \times g$ for 30 min. Following resuspension in TE buffer and recentrifugation, the final membrane pellet was resuspended in 12% sucrose-containing TE buffer, rapidly frozen in liquid nitrogen, and stored at −80 °C. Membrane protein concentration was determined using the bicinchoninic acid (BCA) method (Pierce).

2.3. Photoaffinity labeling of submandibular gland membranes and identification of photolabeled proteins

Rat submandibular gland membranes were prepared and photolabeled as described previously (Boyd et al., 1991). Membranes, containing 3–5 mg of protein/ml, were incubated in the dark with 0.5–1.0 nM ^{125}I-BH-[Bpa^8]SP or 3.0–3.5 nM ^{125}I-[Bpa^7]NKA for 1 h at room temperature in TME buffer (50 mM Tris, 10 mM $MgCl_2$, 1 mM EGTA, pH 7.4) supplemented with 30 μg/ml bacitracin. Competing peptides or non-peptide antagonists were added at the concentrations indicated. Following incubation, the samples were diluted 10-fold with ice-cold TME buffer and centrifuged at $38{,}000 \times g$ for 15 min at 4 °C. The pellets were resuspended in ice-cold TME buffer and recentrifuged twice. Membranes were then resuspended in ice-cold buffer at 1–2 mg of membrane protein/ml and irradiated at 365 nm by exposure to a 100-W long-wave UV lamp for 15 min at a distance of 6 cm. Following photolysis, any ligand that remained reversibly bound to the membranes was dissociated by incubation of the membranes in TME buffer for 30 min at room temperature in the presence of 10 μM 5′-guanylylimidodiphosphate (Gpp[NH]p). ^{125}I-BH-[Bpa^8]SP- or ^{125}I-[Bpa^7]NKA-labeled membranes were then solubilized in sample buffer (0.125 M Tris, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.01% bromophenol blue, pH 6.8), heated at 55 °C for 10 min, and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970).

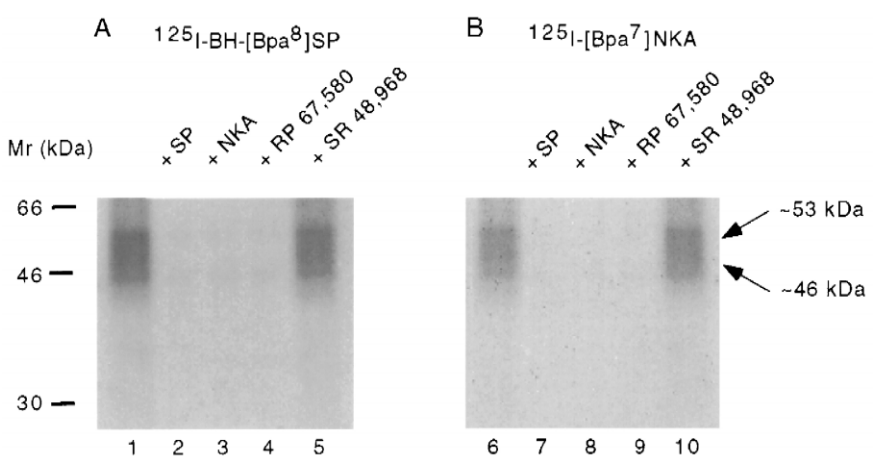

Fig. 1. Photolabeling of rat submandibular gland membranes with (A) ^{125}I-BH-[Bpa^8]SP and (B) ^{125}I-[Bpa^7]NKA. Rat submandibular gland membranes were equilibrated with each photoligand in the presence and absence of indicated competitors and exposed to UV light. The photolabeled proteins were subsequently analyzed by SDS-PAGE/autoradiography. Lanes 1 and 6: no competitor added. Lanes 2 and 7: 1 μM substance P added. Lanes 3 and 8: 1 μM neurokinin A added. Lanes 4 and 9: 1 μM RP 67,580 added. Lanes 5 and 10: 1 μM SR 48,968 added. Arrows denote the full-length (~ 53 kDa) and truncated (~ 46 kDa) versions of the rat NK_1 receptor.

2.4. Salivation bioassay

Female rats weighing 100–200 g were anesthetized with pentobarbital (45 mg/kg). After 10 min, test solutions of substance P (3 nmol/kg) or neurokinin A (30 nmol/kg) were injected via the tail vein. Saliva was collected from the mouth with a Pasteur pipette, put into tared microfuge tubes, and weighed. In rats pre-treated with either the non-peptide rat NK_1 receptor antagonist RP 67,580 (7,7-diphenyl-2-[1-imino-2-(2-methoxyphenyl)ethyl]perhydroisoindol-4-one(3aR,7aR)) (Garret et al., 1991) or the non-peptide rat NK_2 receptor antagonist SR 48,968 ((*S*)-*N*-methyl-*N*-[4-(4-acetylamino-4-phenyl piperidino-*z*-(3,4-dichloro-phenyl)-butyl] benzamide) (Emonds-Alt et al., 1992), 5 mg/kg of the respective receptor antagonist dissolved in acidified saline was administered intraperitoneally 30 min prior to intravenous administration of substance P or neurokinin A.

3. Results

3.1. ^{125}I-[Bpa^7]NKA specifically photolabels the NK_1 receptor in rat submandibular gland membranes

In a comparative analysis (Fig. 1, lanes 1 and 6), we show that the same diffuse photolabeling pattern is observed when submandibular gland membranes are photolabeled with ^{125}I-BH-[Bpa^8]SP and ^{125}I-[Bpa^7]NKA. Since we have previously shown using pharmacological, biochemical, and immunological methods that ^{125}I-BH-[Bpa^8]SP specifically photolabels the full-length (~53 kDa) and C-terminal truncated versions (~46 kDa) of the glycosylated rat NK_1 receptor in rat submandibular gland membranes (Kage et al., 1993), these results demonstrate that ^{125}I-[Bpa^7]NKA specifically photolabels both of the rat NK_1 receptor populations photolabeled by ^{125}I-BH-[Bpa^8]SP. Pharmacological evidence of the specificity of the interactions of these two photoligands with the rat NK_1 receptor is provided by results showing that the addition of 1 μM substance P (Fig. 1, lanes 2 and 7), 1 μM neurokinin A (Fig. 1, lanes 3 and 8), or 1 μM RP 67,580 (a specific non-peptide rat NK_1 receptor antagonist) (Fig. 1, lanes 4 and 9) prevents photolabeling of the rat NK_1 receptor by both photoligands. In marked contrast, the addition of 1 μM SR 48,968 (a specific non-peptide rat NK_2 receptor antagonist) (Fig. 1, lanes 5 and 10) has no effect on the photolabeling of the rat NK_1 receptor by each photoreactive peptide.

3.2. Neurokinin A specifically stimulates NK_1 receptor-mediated salivation in the rat

To assess the functionality of the neurokinin A/NK_1 receptor interaction, a rat salivation bioassay similar to the one used to document the functionality of substance P in stimulating salivation (Leeman and Hammerschlag, 1967) was used. As shown, neurokinin A is a potent stimulator of salivary secretion in rats (Fig. 2A, column 3), albeit a weaker stimulator of salivary secretion than substance P (Fig. 2A, column 1). Pharmacological evidence that each ligand stimulates salivation via a functional interaction with the NK_1 receptor is provided by data showing that both the substance P- and the neurokinin A-stimulated

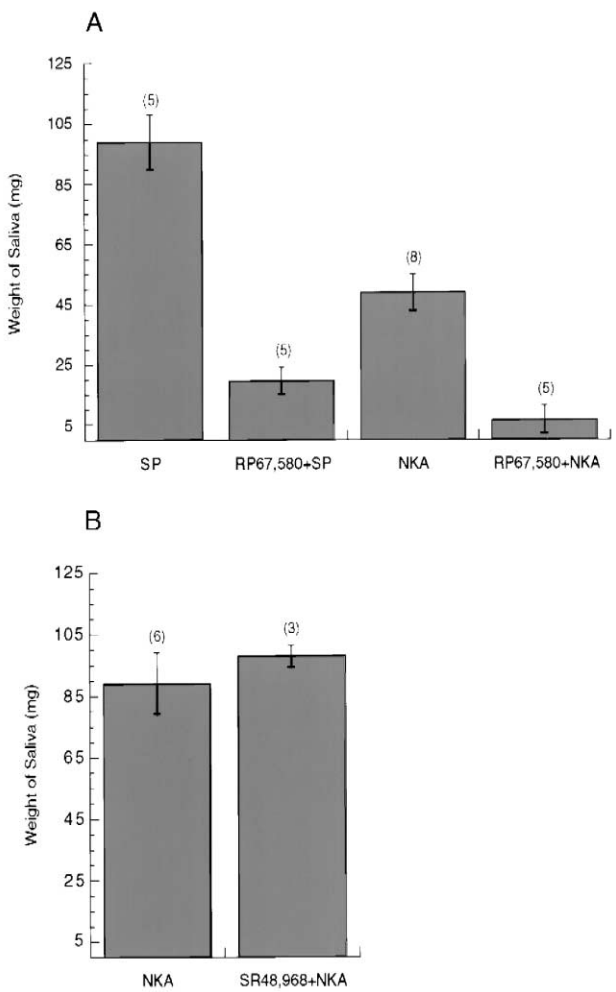

Fig. 2. Pharmacological characterization of substance P- and neurokinin A-stimulated salivary secretion. (A) Inhibitory effects of RP 67,580 on substance P- and neurokinin A-stimulated salivary secretion. Rats (100–120 g) were injected with either substance P (3 nmol/kg) or neurokinin A (30 nmol/kg), and salivary secretion was measured for 2 min following injection of the peptide. Other rats were pre-treated with RP 67,580 (5 mg/kg) 30 min prior to the administration of substance P or neurokinin A, and salivary secretion was measured for 2 min following injection of peptide. Numbers in parentheses correspond to the number of rats in each test group. (B) Lack of an inhibitory effect of SR 48,968 on neurokinin A-stimulated salivary secretion. Rats (180–200 g) were injected with neurokinin A (30 nmol/kg) and salivary secretion was measured for 2 min following injection of peptide. Other rats were pre-treated with SR 48,968 (5 mg/kg) 30 min prior to the administration of neurokinin A, and salivary secretion was measured for 2 min following injection of peptide. Numbers in parentheses correspond to the number of rats in each test group.

salivation is inhibited by pre-treatment with the specific non-peptide rat NK_1 receptor antagonist RP 67,580 (Fig. 2A, columns 2 and 4). In marked contrast, pre-treatment with the specific non-peptide rat NK_2 receptor antagonist SR 48,968 has no demonstrable effect on neurokinin A-stimulated salivation (Fig. 2B).

4. Discussion

In this report, we have used photoaffinity labeling as a method to biochemically characterize the specific target of neurokinin A binding in the rat submandibular gland. Although photoaffinity labeling is also an extremely valuable method for examining peptide/receptor interactions at the molecular level, these experiments demonstrate the power of this technique in biochemically characterizing the specific targets of peptide binding in tissue, information that cannot always be reliably obtained by conventional reversible binding assays.

In the present study, data from photolabeling assays performed in parallel with ^{125}I-BH-[Bpa^8]SP and ^{125}I-[Bpa^7]NKA show that the same proteins are photolabeled by each ligand in rat submandibular gland membranes. We can conclude that the proteins photolabeled by each ligand are the same based on (i) comparable molecular weight as determined by SDS-PAGE and (ii) comparable pharmacological characterization with the use of competitor peptides and non-peptide antagonists (Fig. 1). Bioassay data also show that neurokinin A specifically stimulates salivation in vivo through its functional interaction with the NK_1 receptor (Fig. 2). That neurokinin A is a somewhat less potent stimulator of salivation than substance P may be due to its relative lower affinity for the NK_1 receptor (Hastrup and Schwartz, 1996) or possible differences in signaling mechanisms (Sagan et al., 1996). However, the ability of a specific non-peptide rat NK_1 receptor antagonist but not a specific non-peptide rat NK_2 receptor antagonist to inhibit both photolabeling and salivation demonstrates unequivocally that neurokinin A can specifically interact with the NK_1 receptor in the rat submandibular gland to elicit an NK_1 receptor-mediated physiological response.

Confirming that neurokinin A functionally interacts with the NK_1 receptor in vivo is critical in permitting the use of the ^{125}I-[Bpa^7]NKA photoligand as a probe in subsequent studies to biochemically characterize the target of neurokinin A binding in other tissues. The presence of neurokinin A and NK_2 receptor immunoreactivity has been documented in the spinal cord (Otsuka and Yoshioka, 1993; Zerari et al., 1998); but of interest to many investigators in the tachykinin field is the apparent discrepancy between the amount of neurokinin A present and the relative expression of its 'preferred' NK_2 receptor in several discrete regions of the brain. Autoradiographic data demonstrates the binding of radiolabeled neurokinin A derivatives to proteins in many of these specific brain regions (Dam and Quirion, 1994), and based on the present data, the ^{125}I-[Bpa^7]NKA photoprobe should be useful in future studies aimed at biochemically characterizing the binding site(s) of neurokinin A in these and other tissues.

That both substance P and neurokinin A can functionally interact with the NK_1 receptor may have physiological significance since both peptides arise from the same preprotachykinin A gene (Nawa et al., 1984) are frequently co-localized (Otsuka and Yoshioka, 1993), and potentially initiate different NK_1 receptor-mediated second-messenger signaling pathways (Sagan et al., 1996). The ability of these two peptides to functionally interact with the NK_1 receptor permits a more complex regulation of tachykinin-mediated events; moreover, defects involving this regulation may have significant pathophysiological sequelae (Kraneveld et al., 2000; Quartara and Maggi, 1998).

Acknowledgements

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