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The β Subunit of Neuronal Nicotinic Acetylcholine Receptors Is a Determinant of the Affinity for Substance P Inhibition

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SUMMARY

Substance P is known to inhibit nicotinic acetylcholine receptors from neuronal tissue, skeletal muscle, and electroplaque. The interaction of substance P with specific combinations of neuronal nicotinic acetylcholine receptor subunits was studied by expressing various combinations of subunits in *Xenopus* oocytes. The response to acetylcholine was inhibited by substance P with all subunit combinations tested; however, the apparent affinity for substance P varied by 20–30-fold. The affinity seemed to be dependent on the β subtype expressed (β4 or β2). This suggests that the β subunit may contribute, at least partially, to the substance P binding site. In the case of the α7 subtype, which forms a homooligomeric receptor, the apparent affinity for substance P was intermediate between those of the two β subtypes coexpressed with either α3 or α4. As previously found, the inhibition was noncompetitive. Furthermore, the inhibition was not voltage dependent and, therefore, is unlikely to be due to substance P blocking the channel within the transmembrane portion of the pore.

SP is an 11-amino acid peptide that is released from neurons and functions as a neurotransmitter and neuromodulator in the central and peripheral nervous systems (1). As a neurotransmitter, SP interacts with the G protein-coupled neurokinin type 1 receptor, causing elevation of the inositol trisphosphate concentration and release of calcium from intracellular stores (2). Steinacker and Highstein (3) first described a modulatory action of SP on the nAChR at the cholinergic Mauther fiber-giant fiber synapse. Since that report, inhibition by SP of nAChRs from neuronal tissue (4–8), skeletal muscle (7), and *Torpedo* electroplaque (9, 10) has been described. These studies indicate that modulation of nAChRs by SP is a general characteristic and probably reflects a direct interaction with the receptor at a unique binding site, with a structural specificity distinct from that of the neurokinin type 1 receptor. Several lines of evidence suggest that this action of SP on nAChRs is of physiological importance. In the adrenal gland, for example, SP modulates nAChR-mediated catecholamine secretion (11), and the innervation of chromaffin cells by SP-containing neurons has been demonstrated (12). In addition, SP-like immunoreactivity has been observed at the frog neuromuscular junction (13).

With the cloning of neuronal nAChR subunit subtypes (e.g., Refs. 14–17), a wealth of new information about neuronal nAChRs has been obtained. A subset of neuronal subunits have been classified as α subunits due to specific sequence homologies with the α subunits from skeletal muscle. Of these, α2, α3, and α4 require additional subunits (e.g., β2 or β4) to produce a functional receptor (reviewed in Ref. 18), α5 has not been expressed as a functional nAChR either alone or in combination with other subunits (14, 17), and α7 (16) and α8[1] can produce functional homooligomeric receptors. In addition, α7 and α8 form part or all of the α-bungarotoxin binding sites in mammalian and avian central nervous systems (19). The sensitivity to ACh, cytisine, nicotine, and nBGT seems to be dictated by the type of both α and β subunits present in the nAChR heteroligomer (20–22). In the case of the β subunit, these differences were shown to reside in the amino-terminal half of the extracellular domain (21, 22) and, more recently, specific amino acids responsible for β subunit-dependent differences in cytisine and nicotine affinity have been identified (23). Additionally, the single-channel kinetics of neuronal nAChRs were found to be largely determined by the β subunit (24).

The purpose of this study was to examine the role of various neuronal nAChR subunit compositions on the modulation of the nAChR by SP. The results indicate that the IC_{50} for SP differs by approximately 20–30-fold, depending upon whether β2 or β4 is expressed with an α subunit, and therefore the β

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[1] R. Papke, personal communication.

ABBREVIATIONS: SP, substance P; ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor; nBGT, neuronal bungarotoxin; TMA, tetramethylammonium; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

subunit plays a significant role in determining the apparent affinity for SP.

Materials and Methods

Sources and preparation of plasmids. Plasmids coding for rat neuronal nAChR subunits $\alpha 4$, $\beta 2$, and $\beta 4$ were gifts from Dr. Jim Boulter (Salk Institute), rat $\alpha 3$ cDNA was a gift from Dr. Roger Papke (Salk Institute), and chick $\alpha 7$ cDNA was provided by Dr. Marc Ballivet (University of Geneva). The $\alpha 7$ cDNA was obtained in the flip DNA expression vector, which contains a simian virus 40 promoter and terminator (25). The cDNAs coding for the $\alpha 3$, $\alpha 4$, $\beta 2$, and $\beta 4$ subunits were subcloned into the oocyte DNA expression vector pOEV (a gift from Dr. William L. Taylor, Vanderbilt University), at the polylinker sequence that is located between the *Xenopus* transcription factor (TFIIIA) promoter and the simian virus 40 transcription terminator (26). Plasmids were propagated in the *Escherichia coli* host (HB101 strain) and purified using a Plasmid Quik kit (Stratagene, La Jolla, CA). mRNA was transcribed using the SP6 and T3 Pac-Kits (Epicentre, Madison, WI) and capped by the inclusion of diguanosine triphosphate (Pharmacia, Piscataway, NJ). In most cases, equivalent results were obtained by injection of the corresponding cDNA or mRNA. The only exceptions were $\alpha 3$ combinations, for which only mRNA-injected oocytes produced measurable currents.

Preparation and injection of *Xenopus* oocytes. Oocytes were harvested from adult *Xenopus laevis* (Nasco, Fort Atkinson, WI) under anesthesia (0.15% MS222) and were manually dissected into groups of several dozen. The follicle layers were removed by incubation in Ca^{2+}-free oocyte saline solution (82.5 mM NaCl, 2.5 mM KCl, 1 mM Na_2HPO_4, 15 mM HEPES, 1 mM $MgCl_2$, pH 7.4) containing collagenase type I (1–2 mg/ml). Oocytes were maintained at 18° in oocyte saline solution (with 1 mM $CaCl_2$) containing 5% horse serum, penicillin, and amikacin, and the medium was changed daily. A 10-nl volume of DNA (~2 ng of plasmid DNA) was injected into the nucleus 4–5 days before recording. Alternatively, 50 nl of RNA (~20 ng/subunit) were injected into the cytoplasm 3–5 days before recording.

Voltage-clamp measurements and analysis. Two-electrode voltage-clamp measurements were made at room temperature using a Turbo Tec 01C amplifier (Adams & List, Westbury, NY). The voltage electrode was filled with 3 M KCl and had a resistance of 0.4–2 MΩ. The current electrode was filled with 250 mM CsCl, 250 mM CsF, 100 mM EGTA, pH 7.3. The resistance of the current electrode was between 0.5 and 2 MΩ. Cells were held at −70 mV. Bath solution (oocyte saline solution with 1 μM atropine) was delivered at ~6 ml/min, through a linear perfusion system, to oocytes placed in a Delrin chamber with a total volume of 0.45 ml. ACh/peptide solutions were delivered by preloading 2 ml in a loop at the terminus of the perfusion system, using a Monoject or SMI syringe. A Mariotte flask filled with oocyte saline solution was used to maintain constant hydrostatic pressure, and the ACh/peptide application was initiated by a computer-triggered stream-switching valve (Rainin, Emeryville, CA). The time between applications was 8–10 min, to allow recovery from desensitization. In some experiments, to generate an I/V curve a 1-sec computer-generated voltage ramp was applied at peak current. Data were collected on-line using an IBM AT computer and software developed in this laboratory. Concurrently, the current traces were recorded on a chart recorder. The digitized records were transferred to a VAXStation II computer for further analysis using PLOT (Gradient Software, Ithaca, NY). ACh activation curves and SP inhibition curves were fitted by nonlinear least squares analysis using KaleidaGraph (Synergy Software, Reading, PA) on a Macintosh computer. The EC_{50}, apparent Hill coefficient (n_H), and maximum current (I_{max}) for ACh were determined using a form of the Hill equation,

$$I = \frac{I_{max}[\mathrm{ACh}]^{n_H}}{[\mathrm{ACh}]^{n_H} + EC_{50}^{n_H}} \quad (1)$$

where I is the peak current measured in the presence of [ACh]. The IC_{50} for SP was determined using the equation

$$I = \frac{I_{max}IC_{50}}{[\mathrm{SP}] + IC_{50}} \quad (2)$$

where I is the peak current in the presence of [SP] and I_{max} is the peak current in the absence of SP. When a Hill coefficient was incorporated into eq. 2, the values determined from the nonlinear fits were not significantly different from 1. Statistical significance was evaluated by Student's t test, using the logarithms of the EC_{50} and IC_{50} values.

Results

Activation of subunit combinations by ACh. The activation of inward cation currents by ACh in oocytes injected with various neuronal nAChR subunit combinations is shown in Fig. 1. Although some variability resulted from the slow perfusion system, $\alpha 7$ produced a rapidly activating and rapidly desensitizing current and α/β combinations produced more slowly desensitizing currents. The peak current for ACh activation was concentration dependent; the EC_{50} values and apparent Hill coefficients for each subunit combination are shown in Table 1. Both the α and β subunits seem to contribute to the apparent affinity for ACh, with the following rank order: $\alpha 4\beta 4 > \alpha 3\beta 2 = \alpha 4\beta 2 > \alpha 3\beta 4 \approx \alpha 7$. With this slow perfusion system, the current responses are composites of drug diffusion,

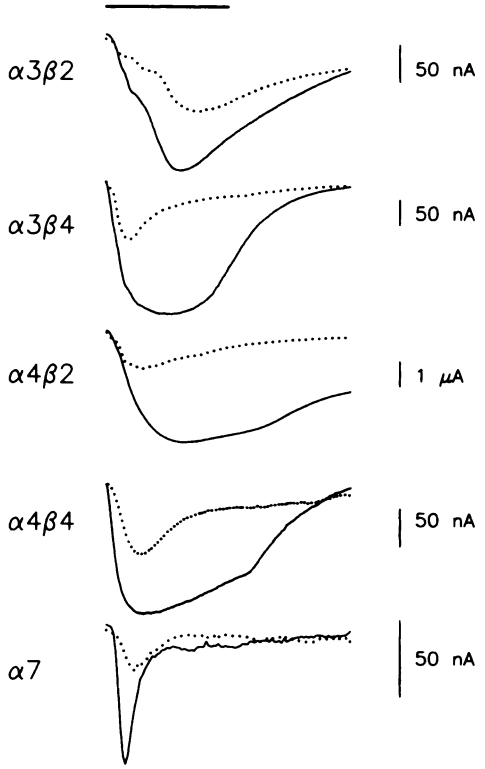

Fig. 1. Time courses of cation currents with application of ACh or ACh plus SP to oocytes expressing different neuronal nAChR subunit combinations. Inward cation currents are indicated by downward deflections. ACh or ACh plus SP was applied for 20 sec (*solid bar at the top of the plot*) and the current response was filtered at 60 Hz and recorded at 125 Hz (in some cases, 250 Hz). For each subunit combination, the two traces (with and without SP) were recorded from the same oocyte. *Solid traces*, ACh alone; *dotted traces*, ACh plus SP. The concentrations of ACh and SP applied were as follows: $\alpha 3\beta 2$, 50 μM ACh, 50 μM SP; $\alpha 3\beta 4$, 100 μM ACh, 5 μM SP; $\alpha 4\beta 2$, 100 μM ACh, 100 μM SP; $\alpha 4\beta 4$, 10 μM ACh, 6 μM SP; and $\alpha 7$, 100 μM ACh, 63 μM SP.

TABLE 1

Concentration dependence of ACh-induced currents and SP inhibition for various receptor subunit combinations

To determine EC_{50} and n_H values for ACh, peak current was measured in the presence of increasing concentrations of ACh and normalized to the response to 100 μM ACh. The parameters of ACh activation were determined by fitting data with eq. 1 (see Materials and Methods). IC_{50} values for SP were determined from eq. 2, as described for Fig. 2 (for α7, [ACh] = 100 μM). Data for each subunit combination are from 4–15 oocytes.

Subunit combination	ACh EC_{50}	ACh n_H	SP IC_{50}
	μM		μM
α3β2	43 ± 9.3	0.71 ± 0.084	67 ± 18[a]
α3β4	86 ± 18	2.4 ± 0.87	3.3 ± 0.48[b]
α4β2	43 ± 9.1	0.81 ± 0.099	74 ± 17[c]
α4β4	10 ± 3.9	1.1 ± 0.40	2.8 ± 0.36
α7	95 ± 20	1.8 ± 0.54	27 ± 7.4

[a] Differs from α3β4 at $p < 0.001$; not significantly different from α4β2.
[b] Not significantly different from α3β4.
[c] Differs from α4β4 at $p < 0.001$.

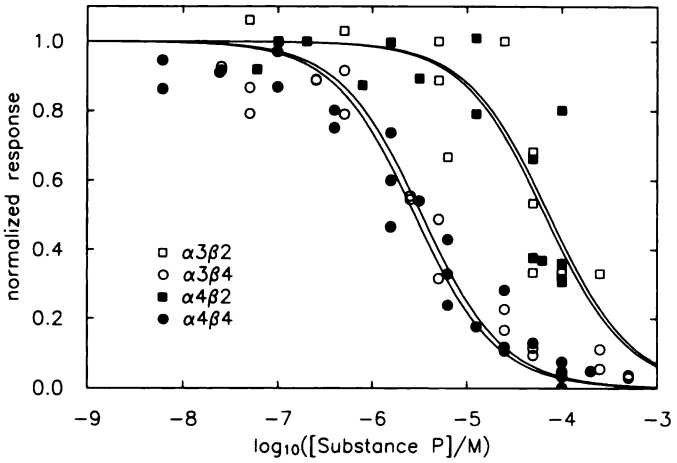

Fig. 2. Concentration dependence of SP inhibition of ACh-induced currents for various subunit combinations. Peak current responses to ACh were measured in the presence of increasing concentrations of SP. For each subunit combination the concentration of ACh used was approximately equal to the EC_{50} (α3β2, 50 μM; α3β4, 100 μM; α4β2, 100 μM; α4β4, 10 μM). Data for each subunit combination are from 4–7 oocytes. For each oocyte the responses were normalized to the current measured in the presence of ACh alone. The *lines* are from nonlinear least squares fits with eq. 2 (see Materials and Methods).

channel activation, desensitization, and, at high agonist concentrations, channel blockade. Therefore, the apparent Hill coefficient cannot be considered an accurate measure of cooperativity. Keeping this in mind, the β2-containing receptors were generally associated with a low Hill coefficient ($n_H < 1$), whereas those with β4 may have Hill coefficients near or greater than 1. The α7 subunit expressed alone exhibited a Hill coefficient significantly greater than 1.

Inhibition of subunit combinations by SP. In the absence of ACh, SP produced little or no detectable current response in uninjected oocytes or oocytes injected with nAChR subunits (data not shown). However, the response of all subunit combinations to ACh was inhibited by SP (Fig. 1). As shown in Fig. 2 and Table 1, the apparent affinity for SP was significantly affected by the subtype of β subunit present. Subunit combinations that included β4 exhibited an approximately 20–30-fold higher affinity for SP than did those containing β2 ($p < 0.001$). The α subtype that was coexpressed had no significant effect on the apparent affinity for SP. The homooligomeric α7 receptor was intermediate in its apparent affinity for SP. Because of the relatively slow perfusion system and the difficulty of applying step increases in agonist and SP concentrations, we were not able to quantitate accurately the time dependence of the current responses. However, as shown in Fig. 1, SP altered the time course of the current response of β4-containing receptors and α4β2 but not α3β2 or α7. Both a decrease in the peak amplitude and an increase in the apparent rate of current decay were observed for β4-containing receptors and α4β2, whereas only an effect on the peak amplitude was observed for α3β2 and α7. Again, because of the relatively slow perfusion system and the rapid desensitization of α7 receptors, changes in the time courses in the presence of SP were difficult to assess but, as shown in Fig. 1, there was no evidence for an increased rate of current decay for this subunit.

Nature of SP inhibition of nAChRs. The nature of the inhibition by SP was examined by determining the effect of the peptide on the concentration dependence of ACh activation. ACh-evoked current was measured in the presence and absence of SP. As shown in Fig. 3 for the α3β4 combination, the inhibition is consistent with a noncompetitive interaction, because increasing concentrations of agonist could not entirely overcome the inhibition. As shown in Table 2, this was clearly the case with four of the five subunit combinations. For the

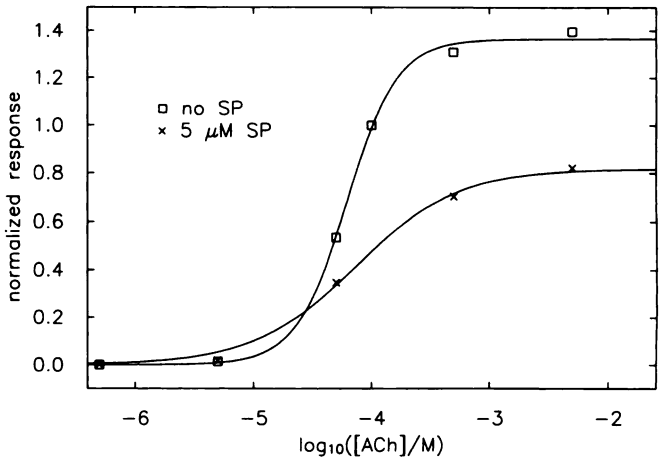

Fig. 3. Effect of SP on the concentration dependence of ACh activation. Peak current for α3β4 was measured as a function of ACh concentration, in the absence and presence of 5 μM SP, as described in Fig. 1. Representative data shown are from a single oocyte and have been normalized to the response to 100 μM ACh alone. The *lines* are from nonlinear least squares fits with eq. 1.

TABLE 2

Effect of SP on the maximum peak current induced by ACh for various receptor subunit combinations

Peak current was measured in the presence of increasing concentrations of ACh in the absence and presence of SP (5 μM SP for β4-containing combinations and 50 μM SP for others). Data were normalized to the response to 10 μM ACh (α4β4) or 100 μM ACh alone. I_{max} was determined as described for Fig. 3.

Subunit combination	I_{max}	
	Control	+SP
α3β2	1.6 ± 0.07	1.8 ± 0.44
α3β4	2.0 ± 0.10	0.82 ± 0.032[a]
α4β2	1.5 ± 0.09	1.1 ± 0.19[b]
α4β4	2.0 ± 0.16	1.5 ± 0.17[b]
α7	2.1 ± 0.16	1.0 ± 0.08[a]

[a] Differs from control at $p < 0.001$.
[b] Differs from control at $p < 0.05$.

$\alpha 3\beta 2$ combination, the I_{max} was not significantly different from control. This was primarily due to the extremely large variability in peak current at high concentrations of ACh (≥500 μM) in the presence of SP. For all subunit combinations, the EC_{50} values for ACh in the presence of SP were not significantly different from control (data not shown).

To determine whether the inhibition was voltage dependent, 1-sec voltage ramps from −100 to +100 mV were applied at peak current in the presence of 50 μM ACh, with or without 50 μM SP, for the $\alpha 4\beta 2$ subunit combination. Fig. 4 shows that, under both conditions, the currents underwent strong inward rectification, as generally observed for neuronal nAChRs (17). Significantly, below −30 mV both currents were linear with respect to membrane potential, indicating that inhibition by SP is clearly not voltage dependent.

Discussion

Using various combinations of neuronal nAChR subunits, we have shown that, when present, the β subtype is an important determinant of the apparent affinity for SP inhibition of ACh-induced currents. The characteristics of this inhibition are consistent with a noncompetitive mechanism that displays no voltage dependence.

The affinities for ACh measured in these experiments are consistent in most cases with those in the literature. As previously reported (16), the EC_{50} for activation of α7-containing nAChRs is relatively high (~100 μM). The EC_{50} values for activation of $\alpha 3\beta 2$ and $\alpha 3\beta 4$ differ from those reported by Cachelin and Jaggi (27). The reason for this discrepancy is unclear. On the other hand, the apparent affinities for the α/β combinations are very similar to those reported by Couturier *et al.* (17) for the corresponding avian subunit combinations,

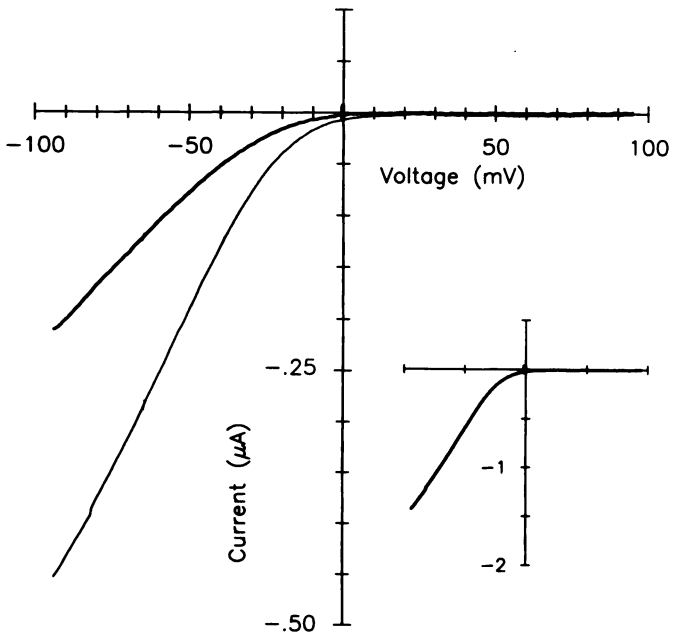

Fig. 4. ACh-induced current as a function of voltage in the absence and presence of SP. The current response to 50 μM ACh was measured in the absence (*thin line*) and the presence (*thick line*) of 50 μM SP during the application of a 1-sec voltage ramp from −100 to +100 mV. Although the current is reduced in the presence of SP, it remains a linear function of the membrane potential. *Inset*, currents normalized to the absolute values of their respective currents at −70 mV.

with the exception of $\alpha 4\beta 2$, which was reported to have a much lower EC_{50} (~0.8 μM) than we observed. The differences may be methodological, because Papke (28) has shown that exposure of perfusion solutions to some of the commonly used types of syringe barrels can result in the release of sufficient Tinuvin 770 (Ciba-Geigy Corp.) to produce significant open channel blockade. This noncompetitive blockade is more effective at high concentrations of ACh and artifactually decreases the EC_{50}. The syringes used to deliver the solutions in the current studies were manufactured without Tinuvin 770.

The type of β subunit present clearly affects many of the activation properties of neuronal nAChRs. Papke and Heinemann (24) have shown that the $\alpha 3\beta 4$ receptor subunit combination differs significantly from the $\alpha 3\beta 2$ receptor in the conductance, open times, and burst kinetics for ACh. The most dramatic difference involved the burst kinetics. β4-containing receptors exhibited a high probability of reopening after a closure, leading to prolonged bursts of channel openings, whereas β2-containing receptors had a lower probability of reopening and tended to produce more isolated channel openings. The β subunit also affects the sensitivity of the nAChR to cytisine, nicotine, TMA, and nBGT (20–23). Receptors containing the β4 subunit exhibit larger responses to cytisine, nicotine, and TMA, relative to ACh, than do those containing the β2 subunit (20, 21). Using chimeric β4/β2 subunits, the region responsible for these effects has been localized to the amino-terminal extracellular domain (21) and, more recently, to specific amino acids (β4 residues 105–116) within this domain (23). nBGT can block nAChRs containing either the β2 or β4 subunit, but the blockade exhibits significantly faster onset and reversibility for β4-containing receptors than for β2-containing receptors (22). The domain of the β subunit responsible for this has been shown to be in the first 121 amino-terminal amino acids.

The reduction of ACh-induced current by SP was noncompetitive, as found previously in other systems (10, 29). From the data presented it might be argued that the $\alpha 3\beta 2$ combination may display competitive inhibition, because SP did not significantly reduce I_{max}. Although we cannot rule out competitive inhibition by SP for this subunit combination, we think that it is unlikely to be the major effect. We found substantial scatter in the peak height at 500 μM ACh and above in the presence of SP, but total current, as opposed to peak current (used to estimate I_{max}), was always reduced. It is likely that at higher concentrations of peptide a significant reduction of I_{max} could be observed, as found for all other subunit combinations.

The inhibition observed in these experiments appears to reflect a direct action of SP on the nAChR, for several reasons. G protein-coupled tachykinin receptors are not endogenously expressed in *Xenopus* oocytes (30), and we observed essentially no response to the application of SP in either uninjected or mock-injected oocytes (a slight outward current of ≤5 nA was sometimes seen ~15 sec after application; data not shown). When tachykinin receptors are expressed in *Xenopus* oocytes, a second messenger-mediated Cl^- current (seen as an inward current in voltage-clamp experiments at −70 mV) is observed upon application of SP (30).

The apparent affinity of SP observed for β4-containing receptors is similar to that reported for SP inhibition of sodium flux in PC-12 cells ($IC_{50} \approx 1$ μM) (29). This is consistent with

the expression of the β4 subunit in PC-12 cells, although the β2 subunit may also be expressed in this cell line (14).

The inhibition by SP was not voltage dependent. This argues against SP inhibiting ACh-induced currents by physically blocking the channel within its transmembrane region. If the positively charged peptide entered the channel deeply enough, the inhibition should be sensitive to the potential across the membrane (31), although there are exceptions (32). However, the possibility cannot be ruled out that SP may block at the extracellular mouth of the channel, outside of the potential field of the membrane. The suggestion that SP may not block within the transmembrane region of the pore is consistent with previous studies demonstrating that the peptide does not bind to the same site as local anesthetics (33).

The experiments described here indicate that the β subunit can play an important role in determining the apparent affinity of the nAChR for SP. Preliminary results using chimeras of β2 and β4 subunits indicate that, unlike the regions that determine the affinity for nBGT, cytisine, and TMA, the region that determines the affinity for SP does not reside completely in the amino-terminal extracellular domain of the β subunit.[2] Thus, as indicated by previous work on the interaction of SP with the nAChR (9, 10, 29), the site with which SP interacts may be a unique regulatory site on the nAChR.

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[2] G. Stafford, unpublished observations.

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