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Substance P modulates the sensitivity of the nicotinic receptor in amphibian cholinergic transmission

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1 The effect of substance P on the sensitivity of nicotinic acetylcholine (ACh) receptors of bullfrog sympathetic ganglion cells and frog skeletal muscle endplate was examined electrophysiologically.

2 The amplitude of ACh-induced postsynaptic potential (ACh potential) and current (ACh current) were reversibly and dose-dependently reduced by substance P at low concentrations (0.42–42 μM).

3 The mean amplitude of the miniature endplate potential (m.e.p.p.) was also reduced by substance P (4.2 μM).

4 Substance P (4.2 μM) shifted the S-shaped dose-response curve of the ACh current downward. A Lineweaver-Burk plot constructed from the dose-response curve revealed that substance P depressed the maximum response ($\dot{V}_{max}$) without changing the apparent affinity (K_m) of ACh for the receptor.

5 Substance P (0.42–42 μM) did not alter the reversal potential of the ACh current of the endplate. The half-decay time of endplate current (e.p.c.) and its voltage-dependency were not altered by substance P in these concentrations.

6 The depression of the ACh current by substance P may not be due to a blockade of the opened channel which has been activated by the preceding combination of ACh with the receptor.

7 These results suggest that substance P suppresses the sensitivity of nicotinic ACh-receptors of the sympathetic ganglion cell and skeletal muscle endplate, acting on a certain allosteric site but not the recognition site of ACh in the receptor-ionic channel complex.

Introduction

During cholinergic transmission, combination of acetylcholine (ACh) with the nicotinic receptor leads to the transient opening of receptor-coupled channels (Katz & Miledi, 1972; Anderson & Stevens, 1973; Neher & Sakmann, 1976). It is well known that a number of chemical substances are able to block this action of ACh on the nicotinic receptor-channel complex in a variety of ways (Steinbach, 1968a, b; Katz & Miledi, 1972; 1973; 1978; Adler & Albquerque, 1976; Feltz, Large & Trautman, 1977; Neher & Steinbach, 1978).

We have recently proposed a hypothesis that the sensitivity of the nicotinic ACh-receptor is modulated by neurotransmitters and some biogenic substances, such as catecholamine, 5-hydroxytryptamine and adenosine triphosphate (Akasu, Hirai & Koketsu, 1981a, b; Koketsu, Akasu, Miyagawa & Hirai, 1982a, b; Koketsu, Miyagawa & Akasu, 1982c). Substance P, an endogenous polypeptide, is considered to be a possible neurotransmitter of slow non-cholinergic e.p.s.p. in the mammalian myenteric plexus (Katayama & North, 1978; Johnson, Katayama, Morita & North, 1981), inferior mesenteric ganglia (Konishi, Tsunoo & Otsuka, 1979; Dun & Jiang, 1982) and bullfrog sympathetic ganglia (Nishi, Katayama, Nakamura & Ushijima, 1980). Ryall & Belcher (1977) have reported that substance P may inhibit excitatory transmission from cholinergic motor axon collaterals to Renshaw cells by preventing interaction of ACh with the nicotinic receptor. The present experiments were carried out to clarify whether or not substance P actually modulates the sensitivity of the nicotinic ACh-receptor in bullfrog sympathetic ganglion cells and skeletal muscle endplate. It was suggested that substance P decreased the sensitivity of the nicotinic ACh-receptor, acting on a certain allosteric site, but not the recognition site of ACh in the receptor-ionic channel complex.

Methods

The 8th or 9th paravertebral sympathetic ganglia isolated from bullfrogs (*Rana catesbeiana*) and sartorius muscle isolated from frogs (*Rana nigromaculata*) with the associated sciatic nerve, were used for the experiments. Microelectrodes filled with 3 M KCl with tip resistances of 25–70 MΩ were selected for recording the membrane potential. To nullify the substance P-induced depolarization, a Wheatstone bridge circuit (Koketsu, Cerf & Nishi, 1959) was employed for injection of constant anodal current through a recording electrode. The ACh-induced postsynaptic potential (ACh potential) and current (ACh current) were produced by iontophoretic application of ACh to the ganglion cells and endplate. ACh was applied every 10 s (0.1 Hz) by rectangular current-pulses (20 nA for 50 ms) which passed through a microelectrode filled with ACh (1 M) and having a tip resistance of about 100 MΩ.

The method for voltage-clamp measurements was essentially similar to those described by Takeuchi & Takeuchi (1959) (see also Kuba & Nishi, 1979; Akasu & Koketsu, 1981). Microelectrodes filled with 1 M K-citrate to be used for injecting feed-back current were inserted into the ganglion cells or endplate region, separately. The feed-back amplifier was a Nihon Kohden CEZ-1100 with maximum gain of 10,000. The feed-back current was monitored by a current-to-voltage converter (mounted in CEZ-1100) connected to a Ag-AgCl electrode in the bath. The small residual fraction of the ACh potential which remained during voltage clamping, amounted to less than 1% of the amplitude of the original ACh potentials. The endplate currents (e.p.cs) were recorded during stimulation of the sciatic nerve in the presence of (+)-tubocurarine (1.4 μM). To eliminate the muscle contraction during recording of the e.p.c., the muscle preparation was treated with 400 mM glycerol for 1 h.

The ionic composition of the Ringer solution used in the present experiment was as follows (mM): NaCl 112, KCl 2, $CaCl_2$ 1.8 and $NaHCO_3$ 2.4. All experiments were carried out at room temperature (22–24°C). Drugs used were (+)-tubocurarine chloride (SIGMA), atropine sulphate (Merck) and substance P (Protein Research, Osaka).

Results

ACh potentials and ACh currents in sympathetic ganglion cell

Figure 1 shows the effect of substance P (4.2 μM) on the membrane potential and ACh-induced postsynaptic potential (ACh potential) produced by iontophoretic application of ACh pulses (20 nA for 50 ms) to the ganglion cells at a rate of 0.1 Hz in Ringer solution containing 10 μM atropine which eliminates the slow e.p.s.p. (Kuba & Koketsu, 1978). When substance P (4.2 μM) was added to the superfusing solution, the sympathetic ganglion cells were slowly depolarized by approximately 5–15 mV at a membrane potential of − 55 mV. During application of substance P in this concentration, the amplitude of ACh potentials was markedly reduced. The substance P-induced depolarization was nullified by constant anodal current, applied to the ganglion cell through a recording microelectrode (Koketsu *et al.*,

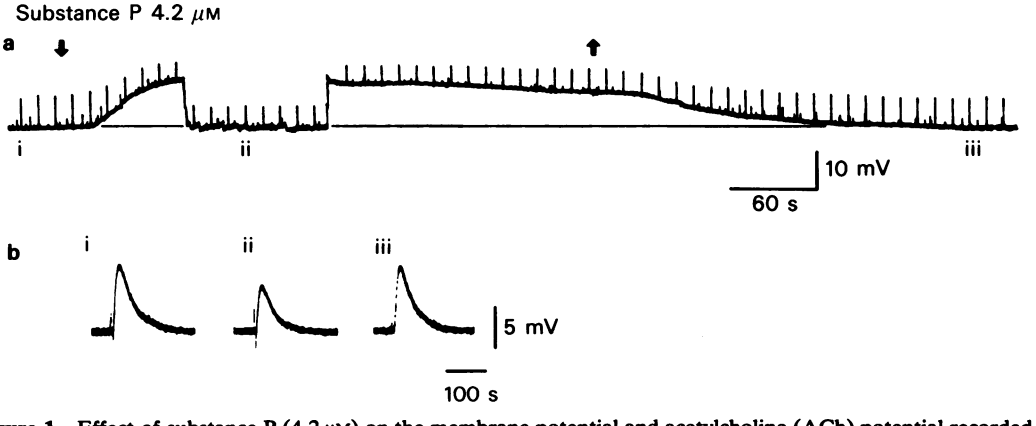

Figure 1 Effect of substance P (4.2 μM) on the membrane potential and acetylcholine (ACh) potential recorded from bullfrog sympathetic ganglion cells. ACh potentials were produced by iontophoretic application of ACh pulses (20 nA for 50 ms) at a rate of 0.1 Hz. Substance P was applied to the ganglion cell between downward and upward arrows. Records (i), (ii) and (iii) were taken before, during and after bath-application of substance P. The substance P-induced depolarization was nullified to obtain record (ii).

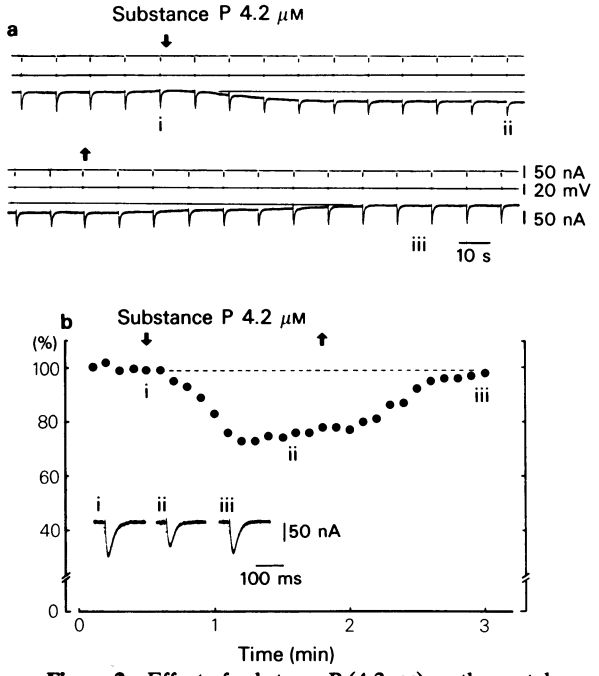

Figure 2 Effect of substance P (4.2 μM) on the acetylcholine (ACh) current recorded by voltage-clamp technique from bullfrog sympathetic ganglion cells. (a) Top traces and second traces indicate the electrical currents used for iontophoretic application of ACh and membrane potential change, respectively. Third traces show ACh currents obtained under the voltage-clamp condition. The membrane potential of the ganglion cell was held at −55 mV. Substance P was applied between downward and upward arrows. (b) Depression of the ACh current by substance P (4.2 μM). Each point was obtained from record (a). Abscissa and ordinate scales indicate time and percentage amplitude of the ACh current, respectively. Control amplitude of the ACh current was expressed as 100%. Records (i), (ii) and (iii) correspond to those indicated in record (a).

1959), to avoid the effect of membrane potential on the amplitude of ACh potentials. The mean amplitude of ACh potentials measured when membrane potential was fixed at the original resting potential level was decreased to 64% of the control.

It has been reported that substance P-induced depolarization of these ganglion cells was accompanied with increase in the membrane resistance (Nishi *et al.*, 1980). Changes in the membrane potential and resistance would modify the amplitude of ACh potentials. Therefore, voltage-clamp experiments were carried out to examine the inhibitory effect of substance P on nicotinic ACh-receptor sensitivity of the ganglion cell.

The resting membrane potential of the ganglion cell was held at −55 mV. When ganglion cells were superfused with Ringer solution containing substance P (4.2–42 μM), the inward membrane current (10–50 nA) which corresponds to the substance P-induced depolarization developed (Figure 2a). The ACh current produced by iontophoretic application of ACh to the ganglion cell at the rate of 0.1 Hz was depressed to 73 ± 8.0% (mean ± s.e.mean, $n = 8$) of the control value obtained with substance P at a concentration of 4.2 μM (Figure 2). These actions of substance P were reversible, the inward current and the amplitude of ACh current recovering to the control level within 3 min of wash out.

ACh potentials and ACh currents in the endplate

The inhibitory effect of substance P on the nicotinic ACh-receptor sensitivity was analysed on the skeletal muscle endplate, because a reliable voltage-clamp method for analysing the kinetics of the receptor-ionic channel complex was established at the endplate. Furthermore, it is well accepted that no detectable muscarinic receptor exists at the amphibian skeletal muscle endplate. The ACh potential was produced by iontophoretic application of ACh pulses (20 nA for 50 ms) to the endplate at a rate of 0.1 Hz. When substance P (0.42–42 μM) was applied to the endplate, membrane potential and resistance showed no detectable changes. The ACh potential gradually decreased in amplitude and reached a maximum level within 2 min after starting the application of substance P (4.2 μM). The depression of ACh potential was sustained almost unchanged throughout the application of substance P. As shown in Figure 3a, the amplitude of the ACh potential was decreased to 63 ± 7.1% ($n = 8$) of the control by application of substance P (4.2 μM). When substance P was removed from the superfusing solution, ACh potential regained its original amplitude within 3 min. Figure 3b shows the effect of substance P (4.2 μM) on the ACh current recorded from a voltage-clamped endplate. The resting membrane potential was held at −90 mV. The amplitude of the ACh current was reduced to 65 ± 6.2% ($n = 11$) of the control by substance P for 2 min application. This effect was dose-dependent. Substance P at concentrations of 10 μM and 42 μM reduced the amplitude of ACh current to 52% and 41% of the control values, respectively. The minimum effective concentration of substance P was 0.42 μM; it reduced the ACh current to 93% of the control.

Figure 4 illustrates the effect of substance P (4.2 μM) on the ACh currents obtained by repetitive iontophoresis of ACh at a rate of 0.1 Hz and those obtained at an interval of 1.5 min, after the beginning of the application of substance P. In the latter case, no repetitive iontophoresis of ACh was applied in this

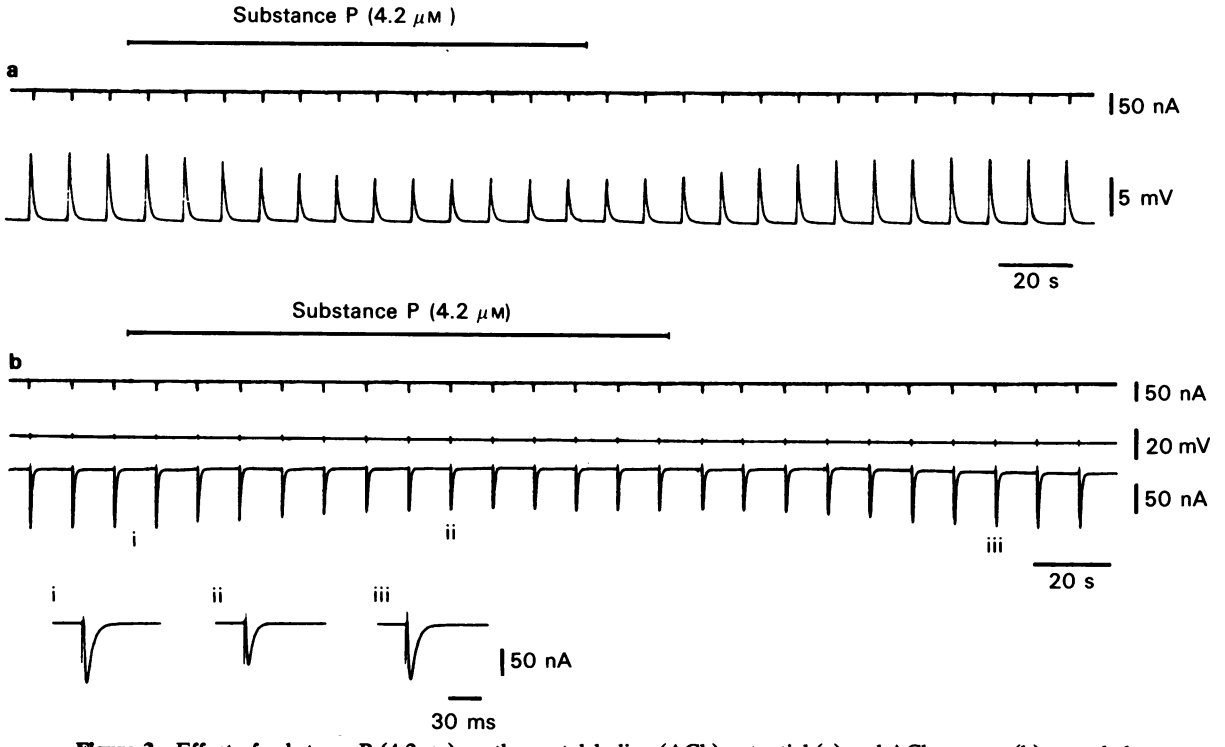

Figure 3 Effect of substance P (4.2 μM) on the acetylcholine (ACh) potential (a) and ACh current (b) recorded from skeletal muscle endplate. ACh potentials and ACh currents were produced by repetitive iontophoresis of ACh pulses (20 nA for 50 ms) at a rate of 0.1 Hz. The application of substance P is indicated by horizontal bar. (a) Upper traces indicate the electrical current used for iontophoresis of ACh and lower traces indicate the ACh potentials. (b) Top and the second traces indicate the electrical current used for iontophoresis of ACh and membrane potential, respectively. The third traces show the ACh current obtained by voltage-clamp condition. The resting membrane potential was held at − 90 mV. Records (i), (ii) and (iii) were taken before, during and after a bath-application of substance P.

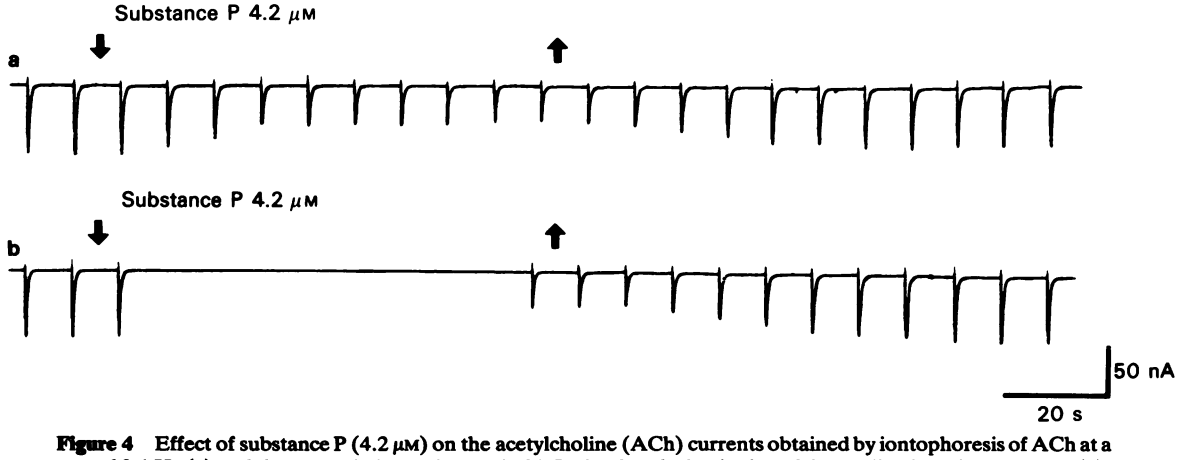

Figure 4 Effect of substance P (4.2 μM) on the acetylcholine (ACh) currents obtained by iontophoresis of ACh at a rate of 0.1 Hz (a) and that recorded at an interval of 1.5 min after the beginning of the application of substance P (b). Substance P was applied to the superfusing solution between downward and upward arrows. Note that the depression of ACh current by substance P clearly occurred without any preceding ACh iontophoresis.

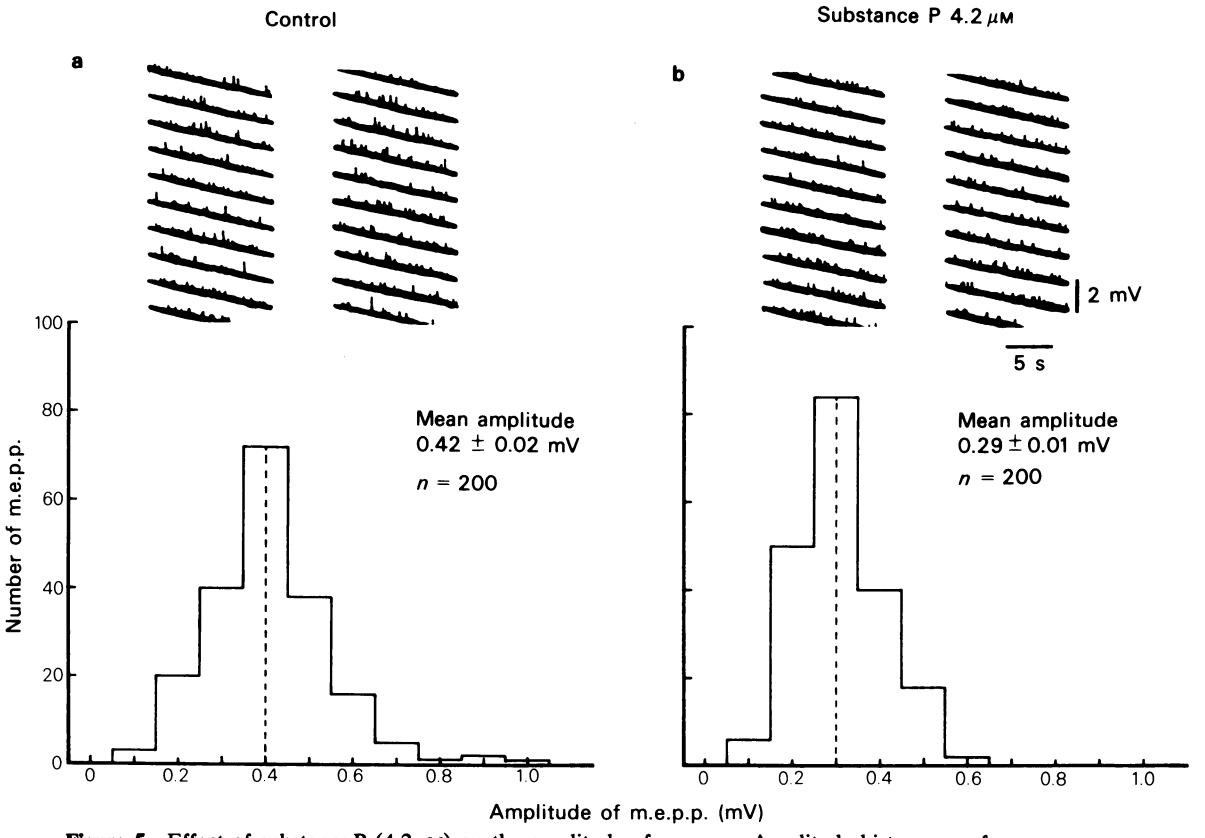

Figure 5 Effect of substance P (4.2 μM) on the amplitude of m.e.p.ps. Amplitude histograms of m.e.p.ps constructed from the oscilloscope recordings (upper columns) are shown. The histograms (a) and (b) were taken before and during an application of substance P. Ordinates indicate numbers of m.e.p.ps and abscissae indicate the amplitude of m.e.p.ps. The mean amplitude of m.e.p.ps in (a) and (b) are shown in each histogram. The broken lines in (a) and (b) indicate the mean amplitude of m.e.p.ps. *n* represents the total number of sample recording of m.e.p.ps.

period. The ACh currents produced at regular intervals of 10 s were reduced to 66% of the control by application of substance P for 1.5 min (Figure 4a). Substance P also reduced the ACh current recorded after application of substance P at an interval of 1.5 min to 61% of the control value (Figure 4b). This indicated that the inhibitory action of substance P is not due to the blockade of the opened endplate channels activated by preceding combination of ACh (Steinbach, 1968a, b; Neher & Steinbach, 1978).

Miniature endplate potentials

Since the miniature endplate potential (m.e.p.p.) is the minimum unit of ACh quantity released from the motor nerve terminal (Fatt & Katz, 1952; Boyd & Martin, 1956), the amplitude of m.e.p.p. may be an indicator of receptor-sensitivity at the endplate. Figure 5 shows the amplitude of the histogram of the m.e.p.ps obtained in Ringer solution and in Ringer solution containing substance P (4.2 μM). As shown in this figure, the mean amplitude of m.e.p.p. was decreased to 69 ± 11% ($n = 5$) of the control by substance P, while the frequency of m.e.p.p. showed no significant changes.

The dose-response curve of the ACh current

To understand the mechanism of the inhibitory action of substance P on the ACh-receptor sensitivity, the effect of substance P was analysed on the dose-response relationship between the amplitude of ACh current and ACh concentration. The dose-response curve of ACh current was obtained by plotting the amplitude of the ACh current against the logarithm of the ACh quantity used for iontophoresis (Dreyer, Peper & Sterz, 1978). Substance P (4.2 μM) shifted the S-shaped dose-response curve of the ACh current downward (Figure 6a). A Lineweaver-Burk plot was constructed from the dose-response curve as-

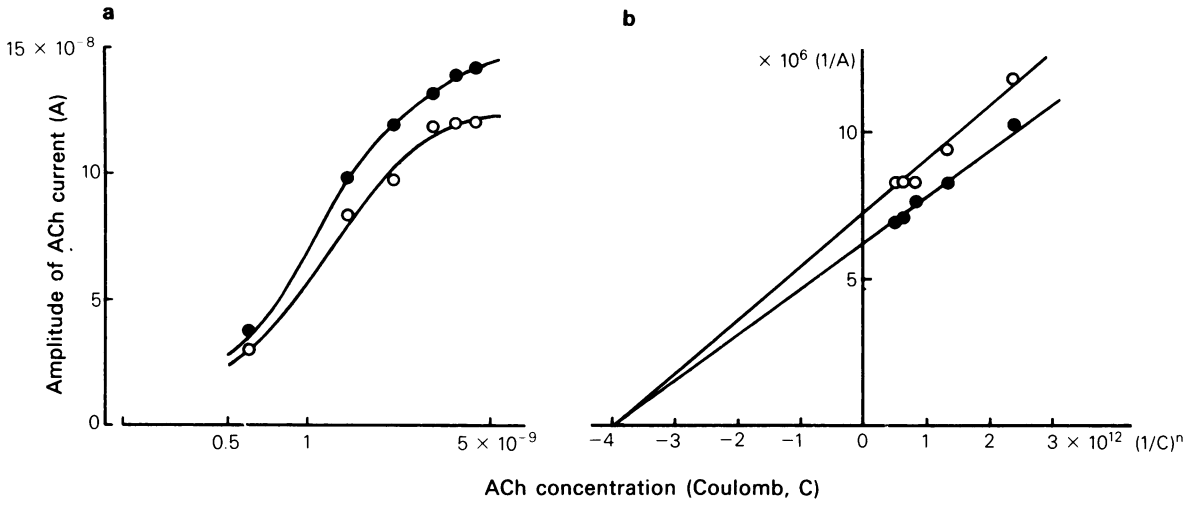

Figure 6 (a) The log-dose relationship between the amount of acetylcholine (ACh) applied iontophoretically to the endplate (abscissa scale) and peak amplitude of ACh currents obtained by voltage-clamp technique (ordinate scale). The amount of ACh applied is expressed as an electrical charge for iontophoresis (coulomb: C). (●) and (○) indicate results obtained before and during a bath-application of substance P (4.2 μM), respectively. (b) The kinetic analysis by double reciprocal plot (Lineweaver-Burk plot) constructed from (a) by assuming Hill number (n_H) = 1.4. Note that substance P (4.2 μM) markedly depressed the maximum response of the dose-response curve ($\dot{V}_{max}$), while it did not affect the apparent affinity (K_m) significantly.

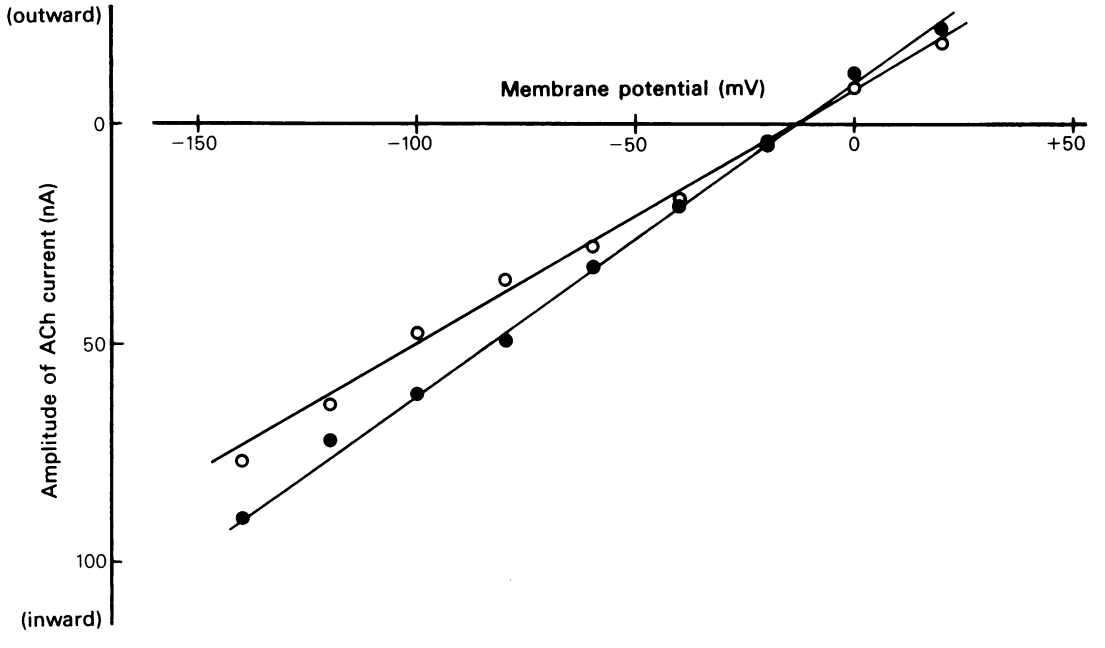

Figure 7 Effect of substance P (4.2 μM) on the equilibrium potential of acetylcholine (ACh) current recorded from voltage-clamped endplate of frog skeletal muscle. Ordinate and abscissa scales indicate the peak amplitude of ACh current and the holding membrane potential, respectively. (●) Before and (○) during bath-application of 4.2 μM substance P.

suming the Hill number (n_H) of 1.4. As shown in Figure 6b, substance P (4.2 μM) decreased the maximum response ($\dot{V}_{max}$) from 16.3×10^{-8} A to 13.9×10^{-8} A, while it did not significantly affect on the apparent affinity (K_m) of ACh to the receptor. These results suggest that substance P decreased the sensitivity of the nicotinic receptor in a non-competitive manner.

The equilibrium potential of the ACh current

The effect of substance P on the equilibrium potential of the ACh current was studied to test whether or not the reduction of ACh current was due to the change in the reversal potential of the ACh-induced endplate current. Figure 7 shows the effect of substance P (4.2 μM) on the relationship between the amplitude of the ACh currents and the holding membrane potentials. The ACh currents were recorded at holding membrane potentials from −140 to +20 mV. The ACh current decreased in amplitude and finally reversed its polarity by conditioning depolarization. The reversed potential of the ACh current obtained in the Ringer solution was -10 ± 5 mV ($n = 6$). Substance P (4.2 μM) did not change the reversal potential. As shown in this figure, the linear relationship of ACh current against membrane potential was unchanged by substance P.

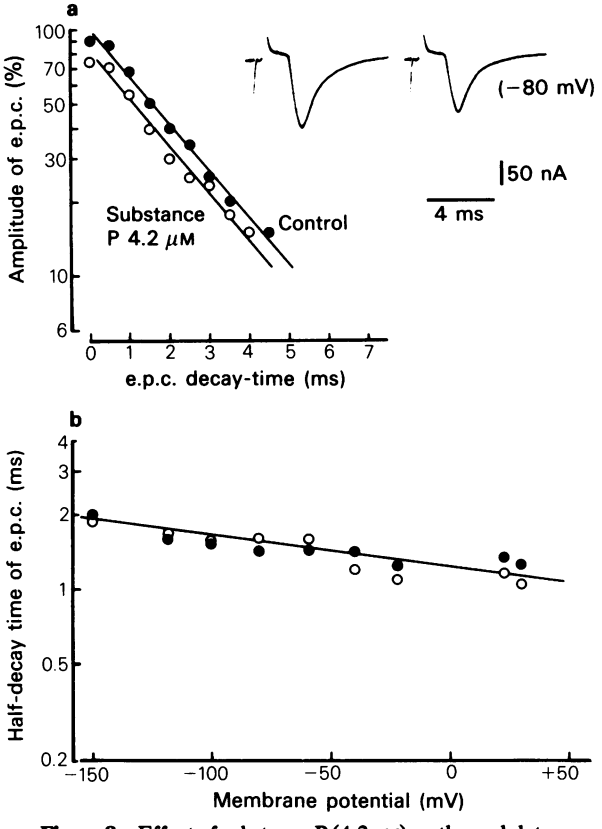

Figure 8 Effect of substance P (4.2 μM) on the endplate current (e.p.c.) produced by stimulation of sciatic nerves. (a) Effect of substance P on the time course of the falling phase of e.p.c. Percentage amplitudes of the falling phase of e.p.c. (ordinate scale) are plotted against time (abscissa scale). The maximum amplitude of e.p.c. is expressed as 100%. (●) Before and (○) during bath-application of substance P (4.2 μM). (b) Effect of substance P on the half-decay times of the falling phase of e.p.c. Abscissa scale indicates the holding membrane potential. Ordinate scale indicates the half-decay times of e.p.c. Closed and open circles were obtained (●) before and (○) during application of substance P (4.2 μM).

Time course of the falling phase of e.p.c.

The effect of substance P (4.2 μM) on the time course of e.p.c. was examined to test a possibility that the decrease in the amplitude of ACh current was due to changes of opening and closing time (the life-time) of the endplate channel. Figure 8 illustrates the effect of substance P on the duration of the falling phase of e.p.c. recorded in the presence of (+)-tubocurarine (1.4 μM). Substance P (4.2 μM) decreased the amplitude of the e.p.c. to approximately 75% of the control e.p.c. recorded at the holding membrane potential of −90 mV. The falling phase of e.p.c. exhibited a single exponential time course in the presence or the absence of substance P (4.2 μM). The half-decay time (1.6 ± 0.3 ms) of the falling phase was not significantly changed by substance P (4.2 μM). It has been known that the time course of the falling phase of e.p.c. was dependent on the membrane potential; the half-decay time of e.p.c. is lengthened and shortened by membrane hyperpolarization and depolarization, respectively (Magleby & Stevens, 1972; Gage, 1976; Dreyer *et al.*, 1978). According to the present experiment (Figure 8b), the potential-dependency of the half-decay time was not altered by the application of substance P (4.2 μM).

Discussion

The present experiments clearly demonstrated that micromolar concentrations of substance P (0.42–42 μM) decreased the sensitivity of the nicotinic ACh-receptor in bullfrog sympathetic ganglion cell and skeletal muscle endplate.

The combination of ACh with the receptors leads to the opening of receptor-coupled channels, with a given life-time and conductance (Katz & Miledi, 1972; Anderson & Stevens, 1973). The depression of ACh sensitivity may be due to changes in either of

these channel properties or the affinity of ACh for the receptor. Substance P shifts the dose-response curve of the ACh current downward. A Lineweaver-Burk plot constructed from the dose-response curve showed that substance P decreased the maximum response ($\dot{V}_{max}$) without affecting the apparent affinity (K_m). These results suggested that substance P decreased the sensitivity of the nicotinic receptor in a non-competitive manner, acting on a certain allosteric site of the receptor-ionic channel complex.

The fact that the duration of the falling phase and the voltage-dependency of half-decay time of e.p.c. were not altered by substance P suggest that the decrease of ACh current may not be due to changes in the kinetics of endplate channels.

Jan & Jan (1982) reported that substance P-like peptide was found to be contained in bundle of axons passing through the sympathetic ganglion of bullfrogs. Substance P produced the slow depolarization which resembles the late slow excitatory postsynaptic potential (Nishi *et al.*, 1980). In mammalian autonomic ganglia, substance P is the most probable neurotransmitter of non-cholinergic slow e.p.s.p. (Katayama & North, 1978; Konishi *et al.*, 1979; Johnson *et al.*, 1981; Dun & Jiang, 1982). Therefore, the present results support a hypothesis that substance P may be a modulator of the sensitivity of the nicotinic receptor in cholinergic transmission. The present paper, together with recent reports on the action of 5-hydroxytryptamine (Akasu *et al.*, 1981a), catecholamine (Koketsu *et al.*, 1982a, b, c) and adenosine triphosphate (Akasu *et al.*, 1981b) provide important experimental evidence showing that the sensitivity of a receptor for its agonist can be modulated by the action of other neurotransmitters or neurohormones.

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