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Regulation of Acetylcholine Release by Presynaptic Nicotinic Receptors at Developing Neuromuscular Synapses

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SUMMARY

Autoregulation of synaptic transmission in the nervous system is one of the homeostatic processes by which the transmission can be regulated according to varied physiological conditions. The neuromuscular cocultures of *Xenopus laevis* embryos were used to investigate the role of presynaptic nicotinic receptors in the autoregulation of developing motoneurons. The bath application of 2 μM nicotine had no significant effect on the frequency of spontaneous synaptic currents (SSCs). However, nicotine markedly increased the SSC frequency in the presence of low concentrations of glutamate (2 μM) or ATP (0.15 mM) or high K^+ (8 mM), which only slightly increased the frequency of spontaneous acetylcholine (ACh) secretion. Carbachol but not oxotremorine was similar to nicotine in the positive regulation of spontaneous ACh release. Treatment with α-bungarotoxin, hexamethonium, *d*-tubocurarine, or mecamylamine, which only slightly inhibited the SSC amplitude, effectively antagonized the increasing effect of nicotine plus glutamate on SSC frequency. Local perfusion of isolated neurons with nicotine induced an inward current at nerve terminal but not at soma, suggesting that nicotinic receptors localize at nerve terminals. Both *d*-tubocurarine and hexamethonium, which produced tetanic fade in adult neuromuscular preparations, did not show tetanic fade at embryonic neuromuscular junction. The bath application of α-bungarotoxin or hexamethonium but not 6-cyano-2,3-dihydroxy-7-nitroquinoxaline inhibited the frequency of SSCs at high-activity (>3 Hz) synapses. A P_2-purinoceptor antagonist, suramin, or desensitizing P_2-purinoceptor with α, β-methylene ATP also reduced the frequency of SSCs at these high-activity synapses. These results suggest that nicotinic receptors, P_2-purinoceptors and glutamate, receptors coexist at nerve terminals of developing motoneurons. The activation of presynaptic nicotinic receptors, which cooperates with either P_2-purinoceptors or glutamate receptors, may greatly increase the spontaneous ACh secretion. Endogenously released ACh and ATP are both involved in the positive regulation of spontaneous transmitter secretion at developing neuromuscular synapses.

Autoregulation of synaptic transmission in the nervous system is one of the homeostatic processes by which the transmission can be adjusted according to varied physiological conditions. The regulation in central and autonomic nervous systems has been well studied (1, 2). In sympathetic neurons, presynaptic inhibitory α- and β-adrenoceptors are involved in the negative and positive regulation of norepinephrine release, respectively (3). On the other hand, activation of presynaptic muscarinic receptors inhibited ACh release in parasympthetic nerves (4). However, it is less generally accepted for the junction in skeletal muscle, although motor nerve endings possessing cholinoceptors have also been reported (5–7). It is well known that *d*-tubocurarine and related antagonists to nicotinic AChRs produce two effects on tetanic contractions of skeletal muscle evoked by stimulating the motoneurons: they depress peak tension, and they cause a rapid waning of tension to the resting level (i.e., tetanic fade) despite continuing stimulation (7). The electrical counterpart of tetanic fade is the well known rundown, to a plateau, in trains of end-plate potentials or end-plate currents (8, 9).

Development of synaptic connections depends on electrical and trophic interactions between presynaptic and postsynaptic cells. One possible trophic factor at peripheral synapses is ATP, which is known to be costored and coreleased with ACh in the vertebrate peripheral nervous system (10, 11). Muscle cells are also known to secrete substantial amount of ATP in response to electrical activity (12). We have recently shown that ATP potentiates spontaneous ACh release during the early phase of synaptogenesis (13–15). In addition, glutamate, which is also reported to be coreleased from some cholinergic nerve terminals (16, 17), markedly increased the frequency of SSCs at embryonic neuromuscular synapses (18). In this work, we further investigated the autoregulation of motoneurons by presynaptic nicotinic receptors and the

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ABBREVIATIONS: ACh, acetylcholine; AChR, acetylcholine receptor; α-BuTx, α-bungarotoxin; CNQX, 6-cyano-2,3-dihydroxy-7-nitroquinoxaline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NMDA, *N*-methyl-D-aspartic acid; SSC, spontaneous synaptic current.

interactions between ACh with either ATP or glutamate at developing neuromuscular junction. The physiological role of presynaptic nicotinic receptors activated by endogenously released ACh is also discussed.

Experimental Procedures

Chemicals and solutions. ATP, α-BuTx, carbachol, glutamate, hexamethonium, kainate, mecamylamine, α,β-methylene ATP, neostigmine, nicotine, oxotremorine, *d*-tubocurarine, and verapamil were obtained from Sigma Chemical, St. Louis, MO. Suramin was obtained from Kogyo Co. (Seikagaka, Japan). CNQX and NMDA were obtained from Research Biochemicals (Natick, MA).

Culture preparation. *X. laevis* neuromuscular cultures were prepared as previously reported (19). Briefly, the neural tube and the associated myotomal tissue of 1-day-old *X. laevis* embryos (stage 20–22) (20) were dissociated in the Ca^{2+}- and Mg^{2+}-free saline supplemented with EDTA. The cells were plated onto clean glass coverslips and were used for experiments after 24 hr at room temperature (20–22°). The culture medium consisted of 50% (v/v) Ringer's solution (115 mM NaCl, 2 mM $CaCl_2$, 2.5 mM KCl, 10 mM HEPES, pH 7.6), 49% L-15 Leibovitz medium (Sigma), and 1% fetal bovine serum (GIBCO, Grand Island, NY).

Electrophysiology. Synaptic currents were recorded from innervated myocytes by whole-cell recording in the voltage-clamp mode as described previously (21). Recordings were made at room temperature in culture medium. For whole-cell recordings of the myocyte, the solution inside the recording pipette contained 150 mM KCl, 1 mM NaCl, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.2. Evoked synaptic currents were elicited by stimulating presynaptic neurons at the soma with a heat-polished glass microelectrode (tip opening, 2–3 mm). The pipette was filled with Ringer's solution. For suprathreshold stimulation of the neuron, a square current pulse of 0.05-msec duration and 0.2–2-μA amplitude was applied through the pipette. Such currents usually induced twitch contraction of the muscle cell when applied to the soma of the innervating neuron. In all recordings, the membrane currents were monitored by a patch-clamp amplifier (Dagan 8900; Dagan, Minneapolis, MN). ACh was iontophoretically applied to the surface of the myocyte by the use of a glass micropipette filled with 3 M ACh chloride (resistance, 100–200 MΩ). The iontophoretic current was supplied by a Grass stimulator (model SD9, Quincy, MA) through a microelectrode amplifier (model KS-700; WPI, Sarasota, FL), which provided a braking current of 2–10 nA. The nicotine-induced inward current in isolated neurons was recorded at either soma or growth cone by local perfusion with another micropipette. The solution inside the recording pipette contained 150 mM K gluconate, 1 mM NaCl, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.2. The data were stored on a videotape recorder for later playback onto a storage oscilloscope (model 5113, Tektronix, Beaverton, OR) or an oscillographic recorder (model RS3200, Gould, Cleveland, OH).

Results

Potentiation of spontaneous ACh release by nicotine. Cultures of embryonic spinal neurons and myotomal muscle cells were prepared from 1-day-old *X. laevis* embryos. Functional synaptic contacts between the nerve and muscle cells are established within 24 hr after cell plating. SSCs were examined using whole-cell voltage-clamp recording in the innervated myocyte. The bath application of 2 μM nicotine induced an inward current in the myocyte without a significant effect on the SSC frequency (Fig. 1A). On the other hand, glutamate (10 μM) and ATP (1 mM), which are reported to be costored with ACh in the cholinergic nerve terminals, markedly increased the spontaneous ACh release

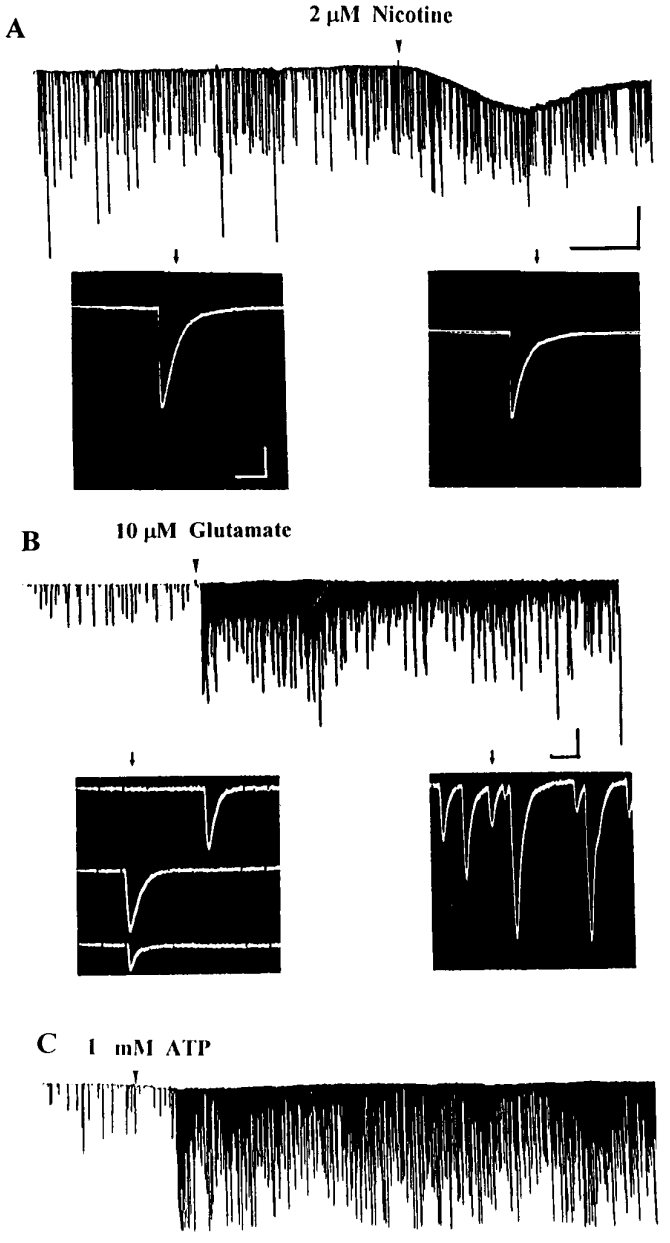

Fig. 1. Effect of nicotine, glutamate, and ATP on the spontaneous ACh release in *X. laevis* neuromuscular cultures. *Continuous tracing*, SSCs recorded from an innervated muscle cell in 1-day-old *X. laevis* culture. The myocyte was voltage-clamped at a potential of −60 mV. Downward deflections are SSCs (filtered at 150 Hz). A, Bath application of 2 μM nicotine induced an inward current in myocytes without affecting the SSC frequency. Samples of SSCs before and after nicotine treatment are shown below at higher time resolution (filtered at 10 kHz). Bath application of 10 μM glutamate (B) or 1 mM ATP (C) markedly increased the SSC frequency. *Scale bar*: 400 pA/100 sec and 200 pA/20 msec for the slow and fast traces, respectively.

(Fig. 1, B and C). The frequency of SSCs increased by a few hundred-fold within minutes after glutamate or ATP treatment. The marked potentiation of spontaneous ACh release was also evident from an increase in spontaneous contractions of other innervated muscle cells in the same culture. Because nicotine at higher concentrations caused the contraction of myocytes and lysis, the concentrations that we used were limited. However, nicotine (2 μM) markedly increased the SSC frequency in the presence of low concentra-

tions of glutamate (2 μM) or ATP (0.15 mM), which only slightly increased the frequency of spontaneous ACh release (Fig. 2, A and B). We previously reported that L-type Ca^{2+} channels are responsible for the positive regulation of spontaneous ACh release by the activation of ATP and non-NMDA receptors at developing neuromuscular synapses (18, 22). The bath application of 8 mM K^+ to slightly depolarize nerve terminals also only weakly increased SSC frequency. However, the SSC frequency increased markedly after the addition of 2 μM nicotine (Fig. 2C). The time course-response curves are shown in Fig. 3. The SSC frequency increased by ~50–100-fold within 5 min after nicotine treatment, and the potentiation gradually subsided to a lower level over a period of 10–20 min. The involvement of L-type Ca^{2+} channels in the potentiation of spontaneous ACh release was tested in the following experiments. Treatment with low concentrations of kainate (10 μM) or NMDA (30 μM) slightly increased SSC frequency, as did glutamate (2 μM). However, only kainate (and not NMDA) enhanced the SSC frequency after the further addition of 2 μM nicotine (SSC frequency ratio: 48.7 ± 13.8, three synapses; 1.09 ± 0.05, four synapses; respectively). Verapamil (10 μM), an L-type Ca^{2+} channel blocker, abolished the SSC frequency potentiating action of kainate plus nicotine (SSC frequency ratio: 1.69 ± 0.80, three synapses).

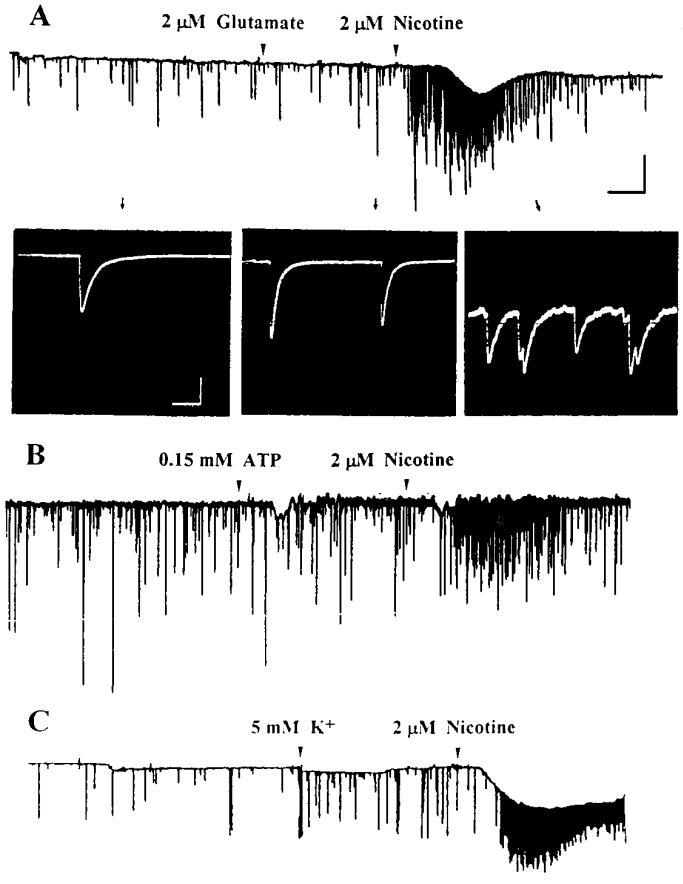

Fig. 2. Potentiation of spontaneous ACh release by nicotine in the presence of low concentrations of glutamate or ATP or high K^+. *Continuous tracing*, inward membrane current recorded from an innervated muscle cell in 1-day-old *X. laevis* culture. The myocyte was voltage-clamped at a potential of −60 mV. Downward deflections are SSCs. Glutamate (A), ATP (B), or high K^+ (C) only slightly increased the frequency of spontaneous ACh release at low concentration, and the SSC frequency was markedly increased after the further addition of 2 μM nicotine. *Scale bar*, 400 pA/100 sec and 200 pA/20 msec for the slow and fast traces, respectively.

Characteristics of presynaptic nicotinic receptors. To determine the site of action of nicotine, either nerve terminal (growth cone) or soma of isolated spinal neurons was whole-cell voltage-clamped at −60 mV (Fig. 4A). Local perfusion of nicotine with another micropipette (intrapipette concentration: 20 μM) induced an inward current in nerve terminals but not in soma (Fig. 4, B and C), suggesting that a higher density and/or higher sensitivity of nicotinic receptors exists at nerve terminals.

ACh is known to act on both nicotinic and muscarinic receptors. To further characterize the presynaptic AChRs at developing motoneurons, we tested the effects of carbachol and oxotremorine on SSC frequency. Carbachol (2 μM) alone had no significant effect on spontaneous ACh release. In the presence of low concentration of glutamate (2 μM), the subsequent application of 2 μM carbachol markedly increased SSC frequency (SSC frequency ratio: 92.4 ± 30.8, eight synapses). Atropine (5 μM) treatment did not affect the frequency-increasing action of glutamate plus carbachol, suggesting that nicotinic receptors are involved in the action of carbachol. On the other hand, the muscarinic receptor agonist oxotremorine (10 μM) had no effect on SSC frequency alone or in the presence of a low concentration of glutamate (2 μM) (SSC frequency ratio: 1.81 ± 0.14, five synapses), indicating that muscarinic receptors are not involved in the regulation of spontaneous ACh release at the developing neuromuscular junction.

There are three branches of the AChR gene family (23) [(a) muscle AChRs, which bind α-BuTx; (b) neuronal AChRs that, unlike those of muscle, do not bind α-BuTx, and (c) neuronal AChRs that do bind α-BuTx]. To further characterize the subtypes of presynaptic nicotinic receptor, we investigated the inhibitory effect of several nicotinic antagonists. As shown in Fig. 5A, pretreatment with 10 nM α-BuTx for 10 min, which only slightly inhibited the amplitude of SSCs, completely abolished the SSC frequency-increasing effect of glutamate plus nicotine. Treatment with 10 μM hexamethonium had an antagonizing action similar to that of α-BuTx (Fig. 5B). The time course-inhibitory response curves are shown in Fig. 5, C and D, respectively. In addition, 0.1 μM *d*-tubocurarine and 10 μM mecamylamine had an inhibitory effect against nicotinic-potentiating action (Table 1). The iontophoretic ACh-induced currents were inhibited only ~10–20% by these nicotinic antagonists at the concentrations that we used, suggesting that the SSC frequency-inhibitory effect is not the result of postsynaptic inhibition.

Both *d*-tubocurarine and hexamethonium produced tetanic fade in adult neuromuscular preparations at low concentrations (7–9). We thus further studied the effect of nicotinic receptor antagonists on evoked synaptic currents at high frequency stimulation. Evoked synaptic currents were recorded from the innervated muscle cells in 1-day-old *X. laevis* cultures by the whole-cell voltage-clamp method. The presynaptic neurons were stimulated extracellularly at the soma to initiate action potentials at a train of 100 Hz and 1 sec or 100 Hz and 0.2 sec. As shown in Fig. 6, treatment with either 10 μM hexamethonium or 0.1 μM *d*-tubocurarine did not show tetanic fade at embryonic neuromuscular junctions. Treatment with anticholinesterase drugs coupled with a higher

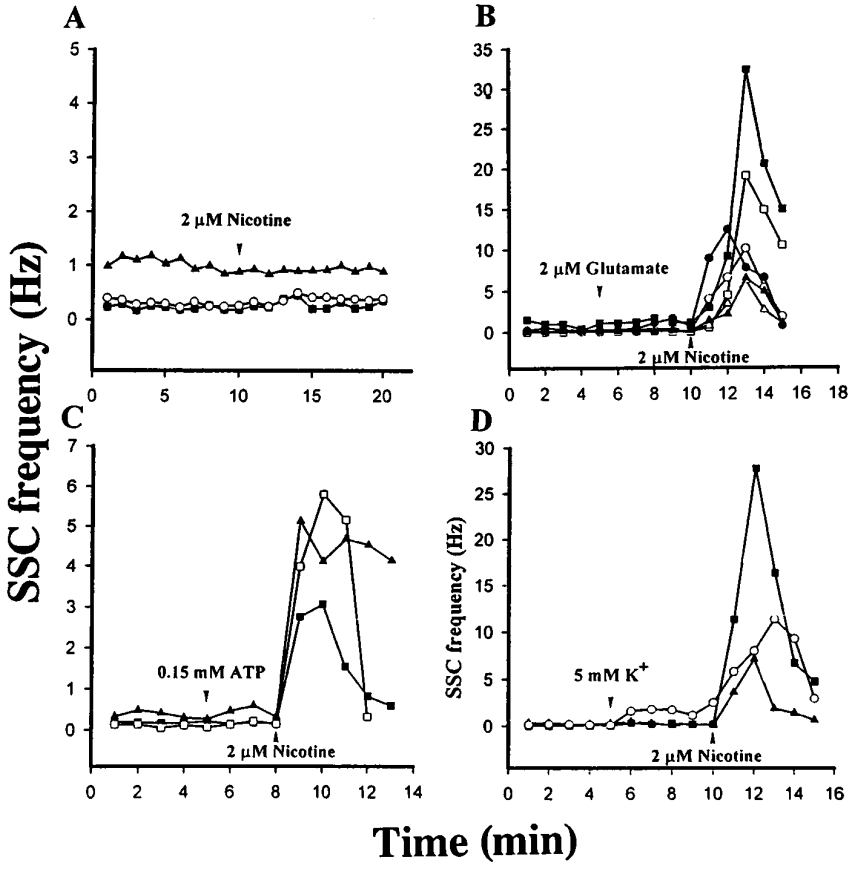

Fig. 3. The time course-response curves of nicotine in the presence of low concentrations of glutamate or ATP or high K^+. Drugs were added to the bath at the time indicated (*arrow*) and remained in the bath throughout the experiment. *Curves* connect data collected from one synapse.

rate of nerve stimulation also resulted in reduced release of ACh in adult preparations. However, treatment with 0.1 μM neostigmine only slightly increased the decay time of SSCs, and there was no tetanic fade during tetanic stimulation (30 Hz, 1 sec) at developing neuromuscular junctions (data not shown).

The physiological role of endogenously released ACh. Spontaneous ACh release at the developing neuromuscular synapse may play a trophic function in regulating synapse maturation and muscle differentiation. In these *X. laevis* cultures, many of the spontaneous synaptic potentials are capable of eliciting action potentials and contractions in the muscle cell, and such contractions accelerate the development of muscle striation (23). The frequency of spontaneous synaptic events was found to vary greatly from cell to cell (by >2 orders of magnitude). In a previous report, we found that synapses with a high frequency of spontaneous events and contractions are under the influence of endogenously released ATP (24). In the current study, we further examined the role of endogenously released ACh on SSC frequency. This notion was tested by examining the effect of nicotinic receptor antagonist on the frequency of spontaneous synaptic events at high-activity synapses (≥3 Hz). We found that α-BuTx (10 nM) and hexamethonium (10 μM) markedly reduced the frequency of SSCs at these high-activity synapses (Fig. 7, A and B). The inhibitory effect is not reversed within a 1-hr experimental recording period after the washout of hexamethonium. As shown in Fig. 7, C and D, the application of a P_2-purinoceptor antagonist, suramin, or desensitizing P_2-purinoceptor with α, β-methylene ATP also reduced the frequency of SSCs at these high-activity synapses. The washout of suramin did not reverse the inhibitory action (Fig. 7C). The initial increase in SSC frequency by the washout of α,β-methylene ATP may result from the agonist action of α,β-methylene ATP (Fig. 7D). Further washout showed the decline of SSC frequency. The time course-inhibitory actions of both nicotinic and purinoceptor antagonists are shown in Fig. 8, A–D. Fig. 8E provides a summary of the results of all experiments involving hexamethonium and α-BuTx. There is a clear correlation of the nicotinic antagonist effect with the frequency of SSCs. The higher SSC frequency, the greater was the inhibitory effect of hexamethonium and α-BuTx. Taken together, these results indicate that tonic activation of presynaptic P_2-purinoceptors and nicotinic receptors, presumably due to endogenously released ATP and ACh, may have synergistic action in the maintenance of high levels of spontaneous ACh release at these developing neuromuscular synapses. In contrast, the application of 10 μM CNQX, a non-NMDA receptor antagonist, did not affect the frequency of SSCs at these high-activity synapses (SSC frequency ratio: 0.96 ± 0.04, four synapses) (Fig. 7E).

Discussion

It has long been known that in adult neuromuscular preparations paralyzed with the use of *d*-tubocurarine, end-plate potentials run down in amplitude during a train of repetitive stimulation. This has been ascribed to a fall-off in transmitter release during the train (6, 18, 25). In addition to blocking postjunctional AChRs, *d*-tubocurarine and related drugs act on the nerve endings to impair a component of evoked ACh release in a use-dependent manner. It is thus proposed that

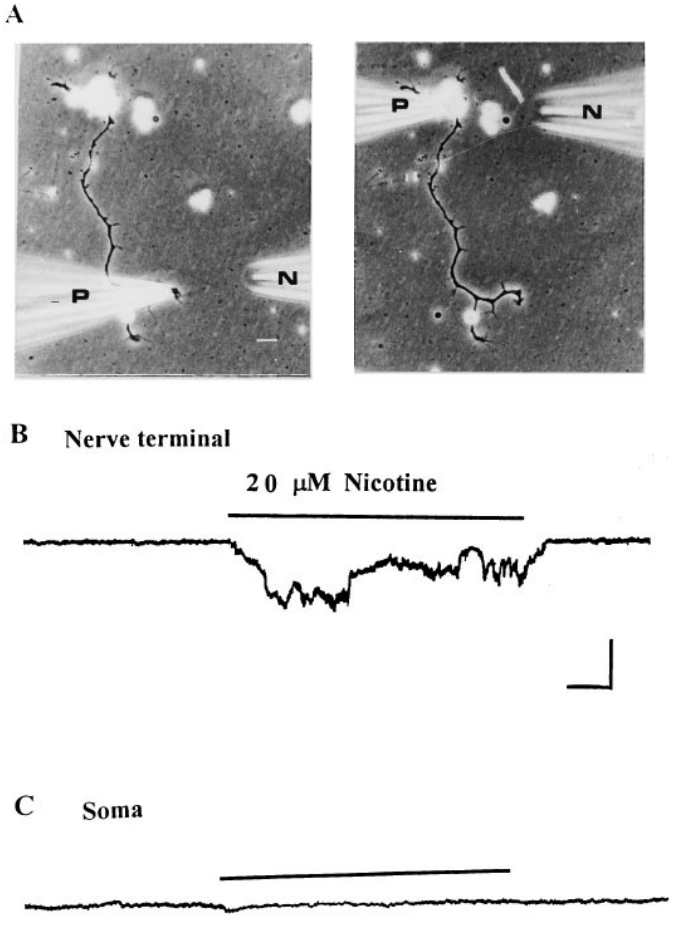

Fig. 4. Depolarizing effect of nicotine at nerve terminals in *X. laevis* cultured spinal neuron. A, Nerve growth cone (*left*) or soma (*right*) of isolated neuron was whole-cell voltage-clamped at −60 mV. *P*, Patch pipette, *N*, Micropipette containing 20 μM nicotine. *Scale*, 10 μm. Local perfusion with nicotine from a glass micropipette (tip opening: 1 μm) induced an inward current at nerve growth cone (B) but not at soma (C). *Scale bar*, 100 pA and 20 sec.

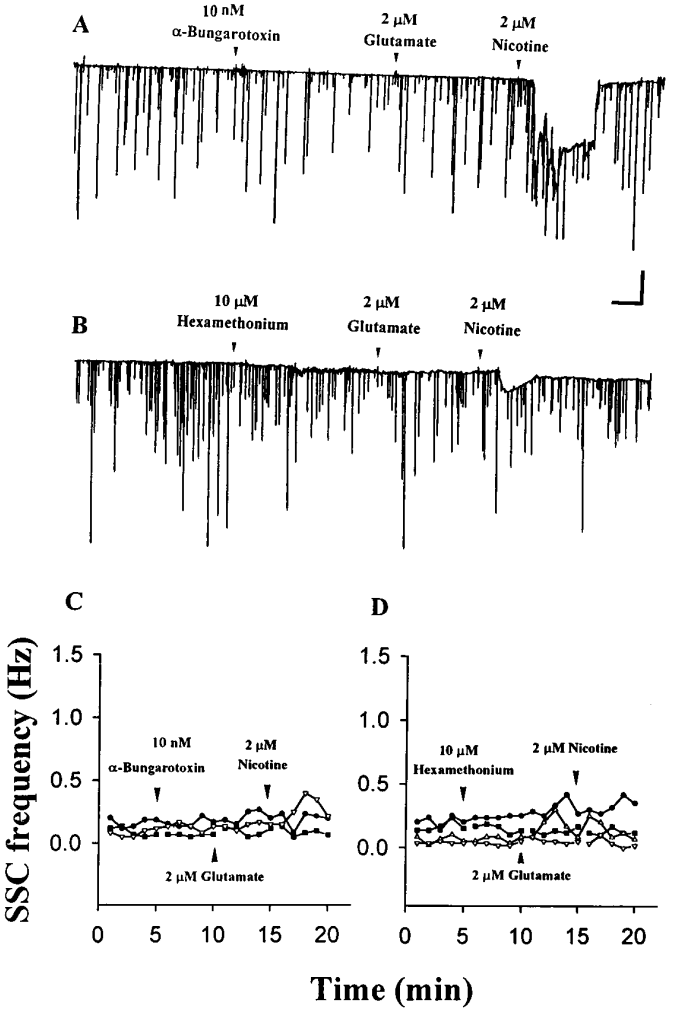

Fig. 5. Inhibition by nicotinic receptor antagonists on the SSC potentiating action of nicotine. Pretreatment with α-BuTx (A) or hexamethonium (B) abolished the SSC frequency-increasing effect of nicotine in combination with 2 μM glutamate. C and D, Time course-response curves, respectively. *Curves* connect data collected from one synapse. *Scale bar*, 400 pA and 100 sec.

nicotinic autoreceptors in motoneuron endings play a physiological role in a positive feedback mechanism that enhances transmitter mobilization. In the current study, we found that there is no tetanic fade at the developing neuromuscular junction in the presence of either neuromuscular blocking agents or anticholinesterase agents, indicating that the regulation of ACh release at embryonic stage may be different from that in adult preparations. However, the application of nicotine in the presence of low concentrations of ATP or glutamate or high K^+ markedly increased the frequency of spontaneous ACh release. Carbachol also has similar potentiating action. Although it was found that carbachol and oxotremorine produced reduction in miniature end-plate potential frequency and in the quantal content of the end-plate potential at the adult frog neuromuscular junction via presynaptic muscarinic receptors (26, 27), the potentiating effect of carbachol on SSC frequency of *X. laevis* cultures was not blocked by atropine, suggesting that nicotinic receptor is involved in the action of carbachol. Furthermore, oxotremorine had no effect on SSCs. The direct effect of nicotine on the nerve terminals of isolated cultured neurons of inducing an inward current further supports the notion that there are presynaptic nicotinic receptors. Glutamate has similar direct depolarizing actions at nerve terminals in *X. laevis* cultured spinal neurons.[1] In addition, when an excised patch of muscle membrane (in the outside-out configuration) was used as

TABLE 1

Inhibition by nicotinic receptor antagonists on the SSC potentiating action of nicotine in combination with glutamate

The extent of the potentiation is measured by the SSC frequency ratio, which is defined as the ratio of maximum frequency (Hz) obtained within 5 min after the bath application of 2 μM nicotine in the presence of 2 μM glutamate to the mean frequency before the application of nicotine. The cultures were pretreated with antagonists for 20 min before the application of nicotine.

Values are mean ± standard error ([a] $p < 0.05$, Student's t test).

	Concentration	n	SSC frequency ratio
Control	μM	9	76.10 ± 36.44
α-BuTx	0.01	8	1.87 ± 0.77[a]
Hexamethonium	10	6	1.41 ± 0.33[a]
d-Tubocurarine	0.1	4	1.63 ± 0.25[a]
Mecamylamine	10	6	1.26 ± 0.33[a]

[1] W.-M. Fu and H. C. Liou, unpublished observations.

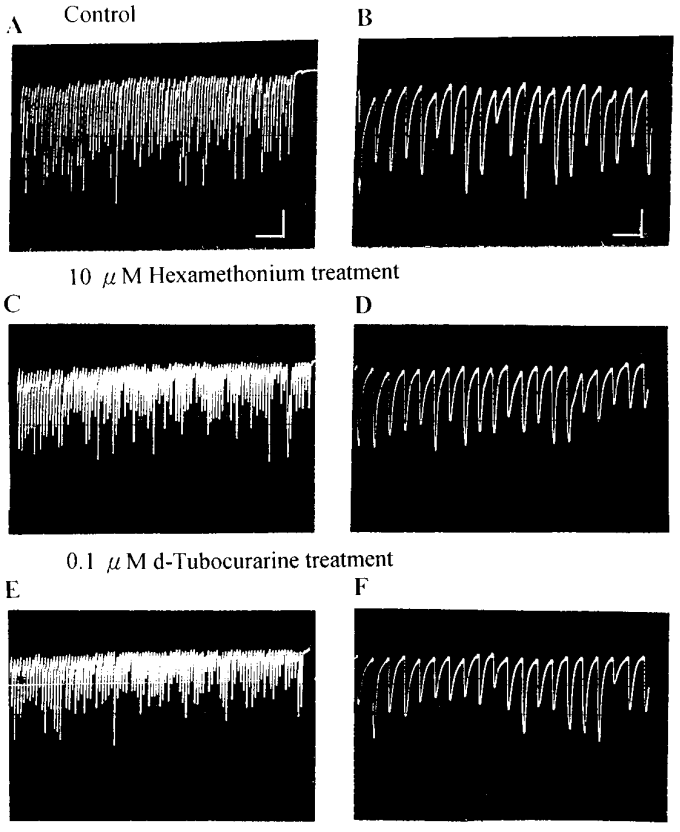

Fig. 6. Effect of nicotinic receptor antagonists on the tetanic stimulated synaptic currents. Presynaptic neurons were stimulated with an extracellular microelectrode at the soma to initiate action potentials, with a train of 100 Hz and 1 sec (A, C, and E) or 100 Hz and 0.2 sec (B, D, and F). The membrane current of the innervated myocyte was monitored to record evoked synaptic currents. Note that compared with control (A and B), treatment with hexamethonium (C and D) or *d*-tubocurarine (E and F) only slightly decreased the amplitude of evoked synaptic currents but had no tetanic fade. *Scale bar*, 1 nA and 0.1 sec.

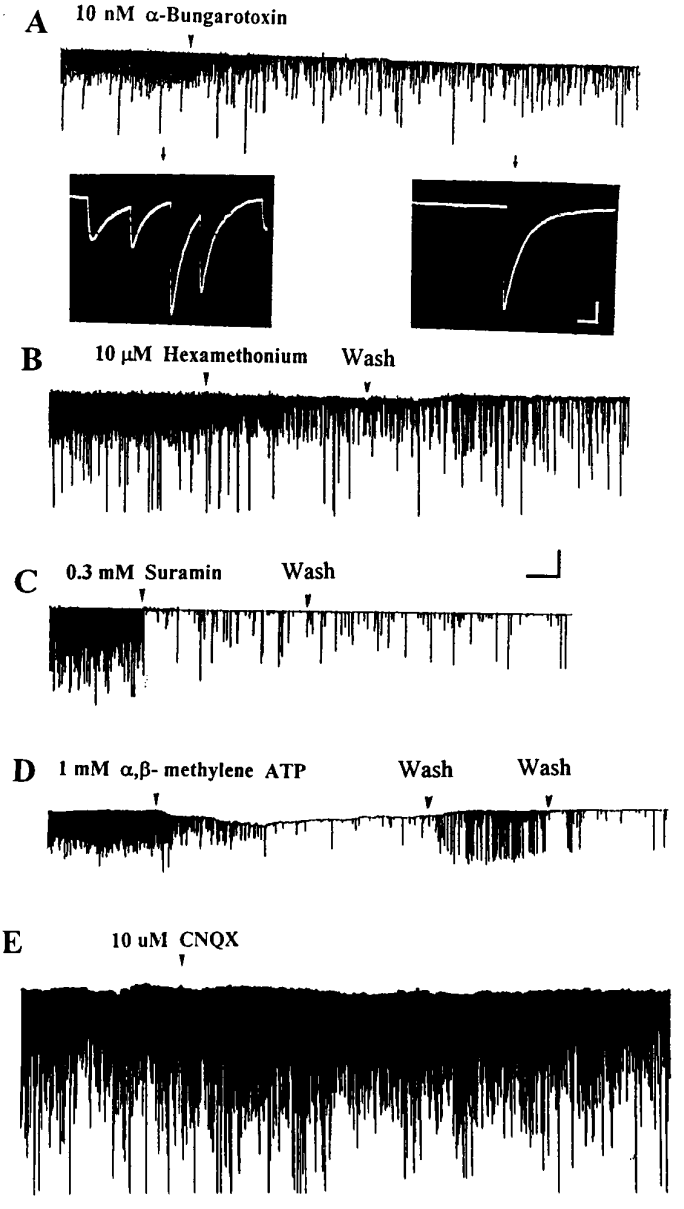

Fig. 7. Inhibitory effect of nicotinic receptor antagonists and purinoceptor antagonists on the spontaneous ACh release at high-activity synapses. The synapses with frequency of spontaneous ACh release of >3 Hz were chosen for these experiments. Application of the nicotinic receptor antagonists α-BuTx (A) and hexamethonium (B) or the purinoceptor antagonist suramin (C) inhibited spontaneous ACh release. α,β-Methylene ATP, which was used to desensitize P_2-purinoceptors, also inhibited the spontaneous secretion of ACh (D). The inhibitory actions was prolonged throughout the experiment, for >1 hr after washout of the antagonists. *Scale bar*, 400 pA/100 sec and 200 pA/20 msec for the slow and fast traces, respectively.

a detector for ACh release from the growth cone of an isolated neuron, an increase in spontaneous secretion of ACh was also detected after ATP or glutamate application (13, 18). These results suggest that there are prejunctional nicotinic receptors but no muscarinic receptors that function in a positive feedback mechanism to facilitate spontaneous transmitter release at developing synapses. Presynaptic ATP and glutamate receptors may act in cooperation with presynaptic nicotinic receptor to depolarize nerve terminals and increase spontaneous ACh release.

Nicotinic AChRs are members of a gene superfamily of ligand-gated ion channels; the three branches of the AChRs are (a) muscle AChRs, (b) neuronal AChRs that, unlike those of muscle, do not bind α-BuTx, and (c) neuronal AChRs that do bind α-BuTx (28, 29). The nicotinic AChR from the vertebrate neuromuscular junction and fish electric organ is one of the most extensively characterized receptors (30). It is composed of four distinct subunits of molecular mass of 50,000–60,000 Da of the stoichiometry $\alpha_2\beta\gamma\delta$, with the ion channel being formed by all of the subunits. The functional and pharmacological diversity of neuronal nicotinic receptors is now seen to result from distinct α as well as distinct β subunits, and variable stoichiometries arising from a given number of subunits may further increase diversity within a neuron (31). α-BuTx did not affect nicotinic receptor-mediated activities in a variety of neuronal tissues in different species that were inhibited by conventional nicotinic antagonists, such as mecamylamine, hexamethonium, and *d*-tubocurarine. These included nicotinic receptor-mediated responses in chick and rat sympathetic neurons, rat sympathetic ganglia, chick ciliary ganglia, cat Renshaw cells, and others (32, 33). In the current study, pretreatment with α-BuTx, hexamethonium, *d*-tubocurarine, or mecamylamine effectively abolished the

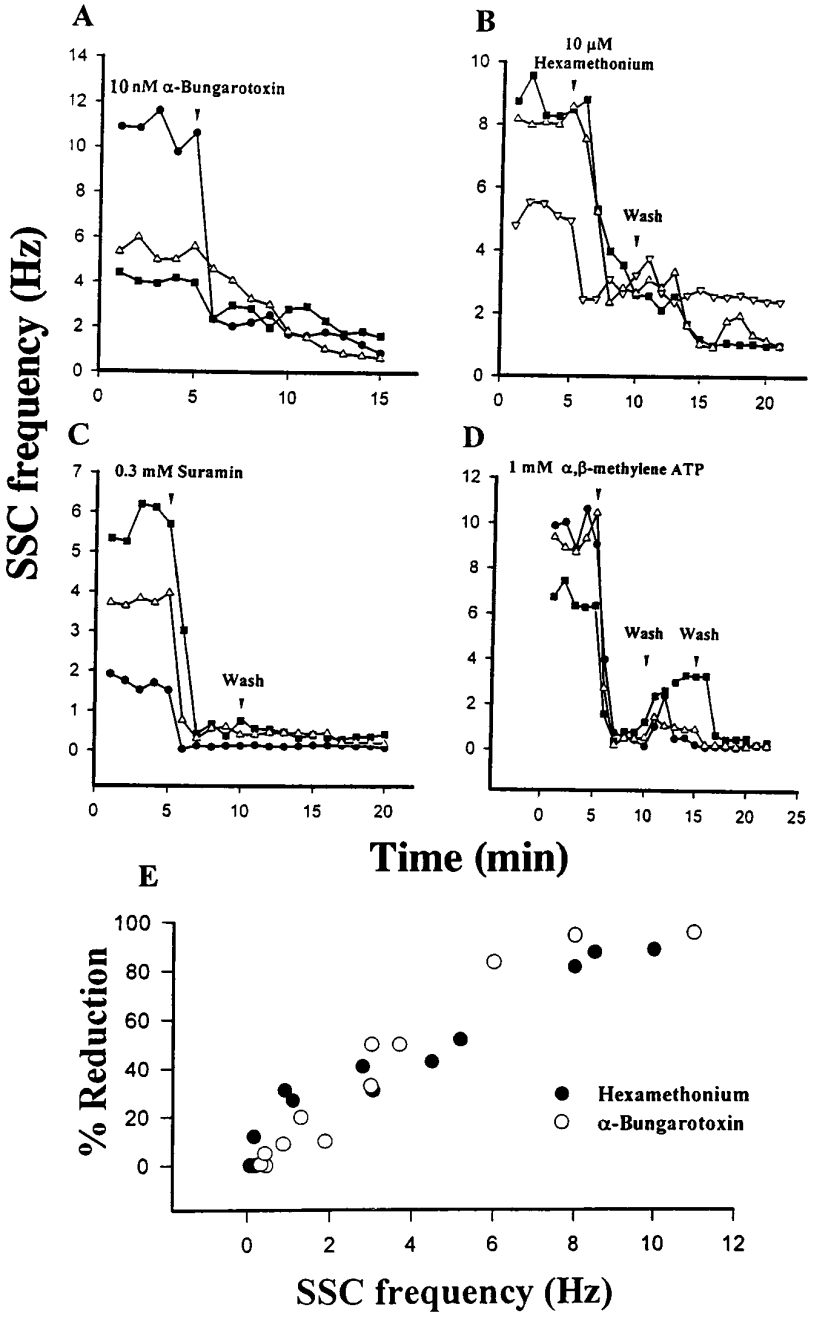

Fig. 8. A–D, The time course-inhibitory response curves of nicotinic receptor antagonists and purinoceptor antagonists at high-activity synapses. The synapses with frequency of spontaneous ACh release of >3 Hz were chosen for these experiments. *Curves* connect data collected from one synapse. E, Correlation of the inhibitory effect of hexamethonium and α-BuTx with the frequency of SSC of the synapse. Synapses with a complete range of frequency of spontaneous ACh release were chosen for these experiments. Note that the higher SSC frequency, the greater was the inhibitory effect of hexamethonium (10 μM) and α-BuTx (10 nM).

SSC frequency-increasing effect of nicotine plus glutamate in *X. laevis* embryonic neuromuscular cocultures. The SSC amplitude is only slightly inhibited by these nicotinic antagonists at the concentrations that we used, suggesting that the SSC frequency-inhibitory effect is not due to postsynaptic inhibition. It also demonstrates that the presynaptic nicotinic receptors of embryonic motoneurons do not show restrict selectivity toward antagonists. Consistent with this result, α-BuTx has also been shown to have a prejunctional effect at motoneurons (33, 34). Complete blockade of postsynaptic AChRs of *X. laevis* cultures by α-BuTx requires higher concentrations at ~0.5–1 μM, similar to that reported in phrenic nerve/diaphragm preparations (7). The neuronal nicotinic receptor assembled in oocytes from the avian α7 subunit is α-BuTx sensitive (35). It is not yet clear whether α7 forms homo-oligomeric channels or hetero-oligomers in combination with other subunits *in vivo*. Whether the presynaptic nicotinic receptors at embryonic motoneurons belong to muscle-type nicotinic receptors or α-BuTx-sensitive neuronal-type nicotinic receptors needs further investigation. It has been found that the α7 gene is expressed at relatively high levels during embryonic development of the chick optic tectum (36). However, the prejunctional receptors seem to differ from the postjunctional receptors. Hexamethonium is more potent in producing the presynaptic inhibition than it is in blocking postjunctional receptors. In addition, the presynaptic and postsynaptic nicotinic receptors seem to differ in their desensitization characteristics. The enhancement of spontaneous ACh release declines much faster than that inhibition of SSC amplitude after treatment with either nicotine or carbachol. Parallels may be drawn with reports concerning other nicotinic receptor types. Neuronal receptors (ganglionic

and central nervous system) tend to desensitize readily, whereas muscle receptors are less readily desensitized (37).

Ca^{2+} entry into nerve terminals through presynaptic channels is usually accepted as the first step in the sequence of events underlying neurotransmitter release. There are at least four types of voltage-dependent Ca^{2+} channels that have been demonstrated in neurons: L-, N-, T-, and P-channels (38). In *X. laevis* neuromuscular cocultures, the N-type Ca^{2+} channel is also involved in the impulse-evoked transmitter release as that in many vertebrate fast-transmitting synapses.[2] We previously reported that L-type Ca^{2+} channels on the presynaptic nerve terminals of an embryonic cholinergic synapses are involved in the regulation of spontaneous transmitter release (22). The L-type Ca^{2+} channels may open in response to the depolarization caused by glutamate or ATP receptor activation (18, 22). A low concentration of kainate, which may cause a higher degree of depolarization at terminals than NMDA, potentiated the SSC frequency-increasing effect of nicotine. Verapamil, an L-type Ca^{2+} channel blocker, inhibited the potentiating action of kainate plus nicotine, suggesting that the L-type Ca^{2+} channel is also involved in the increasing effect of nicotine on SSC frequency. Previously, we showed that endogenously released ATP is involved in the maintenance of high levels of spontaneous ACh release at high-activity synapses (24). In the current study, we found that nicotinic antagonists such as α-BuTx or hexamethonium also inhibited the frequency of spontaneous ACh secretion at these high-activity synapses. The inhibitory effects of either P_2-purinoceptor antagonists or nicotinic antagonists are not reversed within a 1-hr recording period after washout of the drugs. These results suggest that both endogenously released ATP and ACh are responsible for the induction and maintenance of high-activity synapses. ATP is known to be costored in the synaptic vesicles within presynaptic nerve terminals and coreleased with ACh by vesicular exocytosis (10, 11). ATP and ACh may thus cooperate to positively regulate transmitter release by activation of presynaptic P_2-purinoceptors and nicotinic receptors, respectively. Consistent with these results is that extracellular application of the L-type Ca^{2+} channel blockers (e.g., verapamil, nifedipine, and diltiazem) also reduced SSC frequency of these high-activity synapses (24). On the other hand, CNQX, which is a non-NMDA receptor antagonist, did not affect the frequency of SSCs at these high-activity synapses, indicating that endogenous glutamate may not be involved in the induction of high-activity synapses. The physiological role of glutamate in the potentiation of spontaneous ACh release at developing neuromuscular synapses requires further investigation.

Evidence has accumulated for the modulation of the release of γ-aminobutyric acid, glycine, serotonin, ACh, and glutamate via activation of presynaptic nicotinic AChRs throughout the central nervous system (39). The results of our studies suggest that nicotine exerts positive regulation on neuromuscular transmission by the activation of presynaptic autoreceptors at developing synapses. Spontaneous ACh release at developing neuromuscular synapses may play an important role in synapse maturation. Many of spontaneous synaptic potentials are capable of eliciting action potentials and contractions in the muscle cell (40). The increase of synaptic activity may enhance muscle development and play a role in many aspects of neuromuscular synaptic development, including synaptic competition, elimination of polyneuronal innervation, and regulation of AChR synthesis (15). Activation of presynaptic nicotinic receptors, which cooperates with the activation of P_2-purinoceptors or glutamate receptors, may greatly increase the spontaneous ACh release. Endogenously released ACh and ATP are both involved in the positive regulation of spontaneous transmitter release at developing neuromuscular synapses and may play a physiological role in synaptic maturation.

[2] W.-M. Fu and C. J. Liou, unpublished observations.

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