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Coexistence of Glutamate and Acetylcholine in the Developing Motoneurons

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Abstract

Glutamate receptors mediate excitatory neurotransmission in the central nervous systems and are important in neuronal development, aquisition of memory, and are related to some neurodegenerative disorders. In the current study, co-cultures of spinal neurons and myotomal muscle cells were prepared from 1-day-old *Xenopus* embryo. Spontaneous synaptic currents were recorded from innervated myocytes using whole-cell recording. Local perfusion of glutamate receptor agonists, N-methyl-D-aspartate (NMDA) and non-NMDA, at synaptic regions with another micropipette all increased the frequency of spontaneous synaptic currents. Whole-cell recording was also made in the nerve growth cone of cultured spinal neurons. Local application of glutamate, NMDA or kainate all induced an inward current, indicating the coexistence of NMDA and non-NMDA receptors in the nerve terminals of developing motoneurons. Some innervated myocytes contracted spontaneously in the cultures, which were processed for glutamate immunocytochemistry after the recording of spontaneous synaptic currents. Glutamate immunoreactivity appeared in neuronal varicosities and neuromuscular junctions, indicating that glutamate is co-stored with acetylcholine in motoneurons. Double staining for glutamate and choline acetyltransferase further provides the evidence of the colocalization of glutamate and acetylcholine in developing motoneurons. These results suggest that both NMDA and non-NMDA receptors exist in the nerve terminals of developing motoneurons. Furthermore, glutamate and acetylcholine coexist in the motoneurons. The presynaptic glutamate receptors may thus have a physiological role in neuromuscular synaptogenesis in early embryonic stages.

Key Words: glutamate, acetylcholine, motoneuron, *Xenopus* cell culture

Introduction

There is considerable evidence that glutamate is the principal neurotransmitter mediating fast excitatory synaptic transmission in the vertebrate central nervous system (7, 26). The family of glutamate-gated ion channels includes receptors activated by N-methyl-D-aspartate (NMDA), kainic acid and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) (13). A large body of evidence indicates that most of these glutamate receptors are localized postsynaptically. Recently, it has been reported that there is presynaptic NMDA receptor (21), and presynaptic glutamate receptors are upregulated during long-term potentiation (20). Presynaptic glutamate receptors regulating excitatory monosynaptic transmission in central neurons have been demonstrated (6, 12, 13). Previously, we have reported that glutamate greatly potentiates the spontaneous acetylcholine (ACh) release by the activation of presynaptic glutamate receptors at developing neuromuscular synapses in *Xenopus* cell cultures (8). There are developmental changes in the modulation of ACh release by glutamate receptor activation; the potentiating action of glutamate declined or disappeared in older *Xenopus* tadpoles (14). Here we further give the direct evidence that glutamate receptor agonists are able to induce cationic currents in nerve terminals of developing motoneurons. Furthermore, the coexistence of glutamate and ACh in the cultured motoneurons was also demonstrated.

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Materials and Methods

Cell Culture

Xenopus nerve-muscle cultures were prepared as reported previously (22). Briefly, the neural tube and the associated myotomal tissues of 1-day-old *Xenopus laevis* embryos (stages 20-22) were dissociated in Ca^{2+}- and Mg^{2+}-free Ringer's solution supplemented with EDTA. The cells were plated on clean glass coverslips and were used for experiments after 24 h at room temperature (20-22°C). The culture medium consisted of 50% (v/v) Ringer's solution (115 mM NaCl, 2 mM $CaCl_2$, 1.5 mM KCl, 10 mM HEPES, pH 7.6), 49% L-15 Leibovitz medium (Sigma), 1% fetal bovine serum (Gibco) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin).

Electrophysiology

Whole-cell patch-clamp recording methods followed those described by Hamill *et al.* (11). Patch pipettes were pulled with a 2-stage electrode puller (pp-83, Narishige) and the tips were polished immediately before the experiment using a microforge (MF-83, Narishige). Synaptic currents were recorded from innervated myocytes by whole-cell recording in the voltage-clamp mode. Recordings were made at room temperature in Ringer's solution. For whole-cell recording of myocytes, the solution inside the recording pipette contained 150 mM KCl, 1 mM NaCl, 1 mM $MgCl_2$ and 10 mM HEPES (pH 7.2). The extent of the potentiation was measured by the frequency ratio of spontaneous synaptic currents (SSCs), which is defined as the ratio of SSC frequency at peak level observed during application of drugs compared to the mean frequency observed before drug treatment. For whole-cell recordings of nerve growth cone, all KCl of internal solution was replaced by the equimolar of K gluconate, and 1 mM amphotericin B was added to internal solution to get perforated patch. The membrane currents passing through the patch pipette were recorded with a patch-clamp amplifier (Axopatch 200A). The data were digitized using Neuro-corder (Neuro Data DR 390) and stored on a videotape for later playback onto a storage oscilloscope (Tektronix 5113) or an oscillographic recorder (Gould RS3200). The Data 6100 waveform analyser (Data Precision, Danvers, MA, USA) was used to analyse the frequency of SSCs. For analysis of spontaneous events at high frequency when overlaps of current events occurred, the events were counted visually from chart records of an oscillographic recorder driven at high chart speeds (25-100 mm/s). Data are expressed as means±S.E.M. Statistical significance was evaluated by Student's *t* test.

Immunocytochemistry

For the immunocytochemistry, cultured cells fixed with 3% glutaraldehyde (phosphate buffered saline; PBS) were treated with $NaBH_4$ (1 mg/ml) for 15 min. The cells were then treated with 0.15% Triton X-100 and 10% bovine serum albumin in PBS for another 10 min. They were further incubated with diluted rabbit anti-glutamate antibody (1:300, Sigma) at 4°C overnight. After washes with PBS the cells were further incubated with rhodamine-conjugated goat anti-rabbit IgG (1:50, Sigma) for 1 hr and washed with PBS. In some experiments, double staining of glutamate and choline acetyltransferase was performed; the latter was shown by staining with mouse anti-choline acetyltransferase (1:100, Chemicon) as primary antibody and FITC-conjugated goat anti-mouse IgG (1:50, Sigma) as secondary antibody.

Results

Potentiation of SSC Frequency by Local Perfusion of Glutamate Receptor Agonists at Synaptic Regions

In nerve-muscle cultures prepared from 1-day-old *Xenopus* embryos, synaptic contacts are established between dissociated spinal neurones and myocytes within the first day of culture. Spontaneous synaptic currents (SSCs) are readily detectable from the innervated myocytes by whole-cell voltage-clamp recording. These currents have been shown to be caused by spontaneous ACh secretion from the neuron, since they were abolished by bath application of d-tubocurarine and unaffected by tetrodotoxin (4). Recently, we have shown that glutamate receptor agonists, both NMDA and non-NMDA, potentiate the spontaneous secretion of ACh at developing neuromuscular synapses of *Xenopus* (8, 14). In the current study, we further investigated the effect of local perfusion of glutamate receptor agonists at synaptic regions. Drugs were locally applied by pressure at synaptic regions by using another glass micropipette (tip opening: 2 μm). As shown in Fig. 1A, local perfusion of glutamate (pipette concentration at 50 μM) greatly increased SSC frequency (SSC frequency ratio was 461.0±262.0, n=5). Local perfusion of either kainate (Fig. 1B, pipette concentration at 15 μM)or NMDA (Fig. 1C, pipette concentration at 100 μM) at synaptic region aiso increased the SSC frequency (SSC frequency ratio was 353.7±173.0, n=4 and 401.5±101.3, n=3, respectively). Bath application of DNQX (6,7-dinitroquinoxaline-2,3-dione; 20 μM) or APV [D(-)-

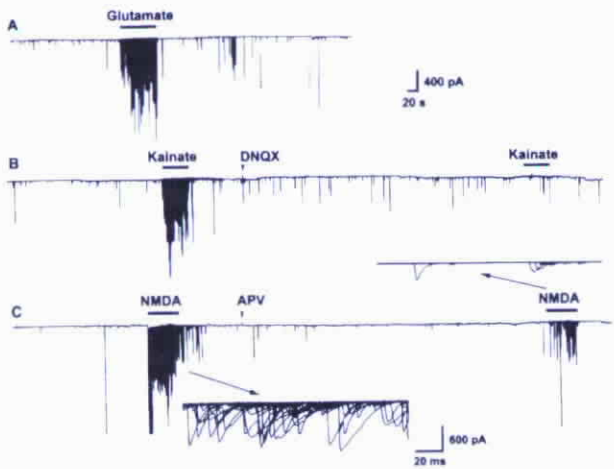

Fig. 1. Potentiation of SSCs by the local perfusion of glutamate receptor agonists at synaptic region
(A) The continuous trace depicts the membrane currents recorded from an innervated myocyte before and after local application of glutamate (pipette concentration at 50 μM). Downward deflections are SSCs (Vh=-60 mV, filtered at 150 Hz). Local perfusion of kainate (B, pipette concentration at 15 μM) or NMDA (C, pipette concentration at 100 μM) at synaptic region also increased SSC frequency and bath application of DNQX (20 μM) or APV (30 μM) inhibited this potentiating action, respectively. Samples of superimposed SSCs in 4 sec during the application of NMDA before and after APV treatment are shown at higher time resolution (filtered at 10 kHz).

2-amino-5-phosphonovalerate; 30 μM] significantly inhibited the potentiating action of kainate and NMDA, respectively (SSC frequency ratio was 0.7±0.2, n=4 and 58.7±44.7, n=3, respectively). These results indicated the existence of both NMDA and non-NMDA receptors in the nerve terminals of developing motoneurons.

Inward Current Caused by Glutamate Receptor Agonists at Nerve Growth Cone

We further investigated whether both NMDA and non-NMDA receptors exist in the nerve terminals by making a whole-cell recording in the nerve growth cone. As shown in Fig. 2A, the nerve growth cone of cultured spinal neuron was whole-cell voltage-clamped at -60 mV. Drugs were locally applied at nerve growth cone by using another glass micropipette (tip opening: 2 μm). Local perfusion with glutamate (pipette concentration at 100 μM) rapidly induced an inward current, and the current became more noisy at peak level (Fig. 2B). The size of current was about 840.0±28.3 pA (n=3). Several types of glutamate receptors appeared to be involved in the observed effects. As shown in Fig. 2C and 2D, NMDA (30 μM) and kainate (30 μM) all induced an inward current in nerve terminals. The current amplitude was 73.7±10.2 pA and 470±131 pA (n=3 for each), respectively. These experiments were done in nerve terminals of both naive neuron (without contact with myocyte) and myocyte-contact neuron. Some myocytes contracted spontaneously, indicating the innervating neurons are actually motoneurons. These results suggest that both NMDA and non-NMDA receptors coexist in the nerve terminals of developing motoneurons.

Coexistence of Glutamate and ACh at Developing Motoneurons

There is now biochemical evidence for the co-release of glutamate and ACh from some cholinergic nerve terminals (17, 25). We further investigated whether glutamate is co-stored with ACh in the developing motoneurons. Because there are at least 20% of the cultured spinal neurons are not motoneurons, we therefore make a whole-cell recording in the innervated myocyte and record synaptic currents. Once SSCs appear, the innervating neuron is really a motoneuron. As shown in Fig. 3A, the innervated myocyte was whole-cell voltage-clamped at -60 mV. The downward deflections were SSCs resulting from spontaneous ACh secretion from the

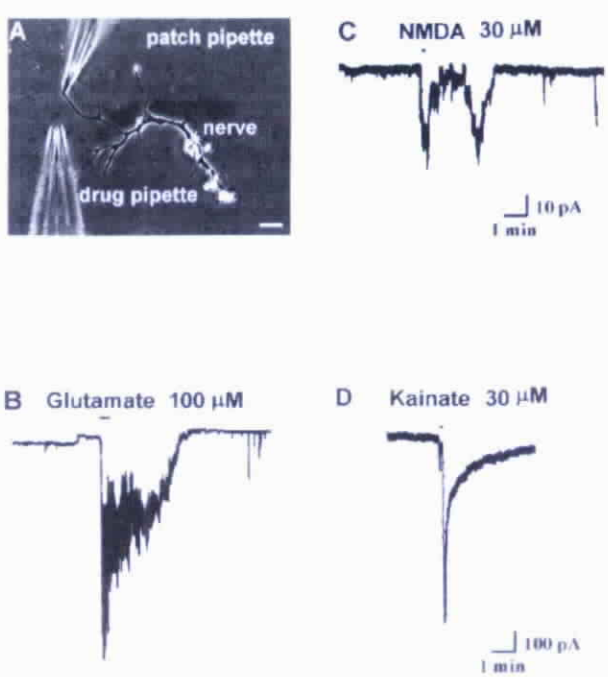

Fig. 2. Membrane currents induced by glutamate receptor agonists at nerve growth cone.
(A) Phase-contrast micrograph showing the arrangement of patch pipette (upper) and drug pipette at nerve growth cone. Scale bar, 10 μm. Nerve growth cone of cultured *Xenopus* spinal neuron was whole-cell voltage-clamped at -60 mV. Local perfusion with glutamate (B), NMDA (C) or kainate (D) from drug pipette induced an inward current at nerve growth cone.

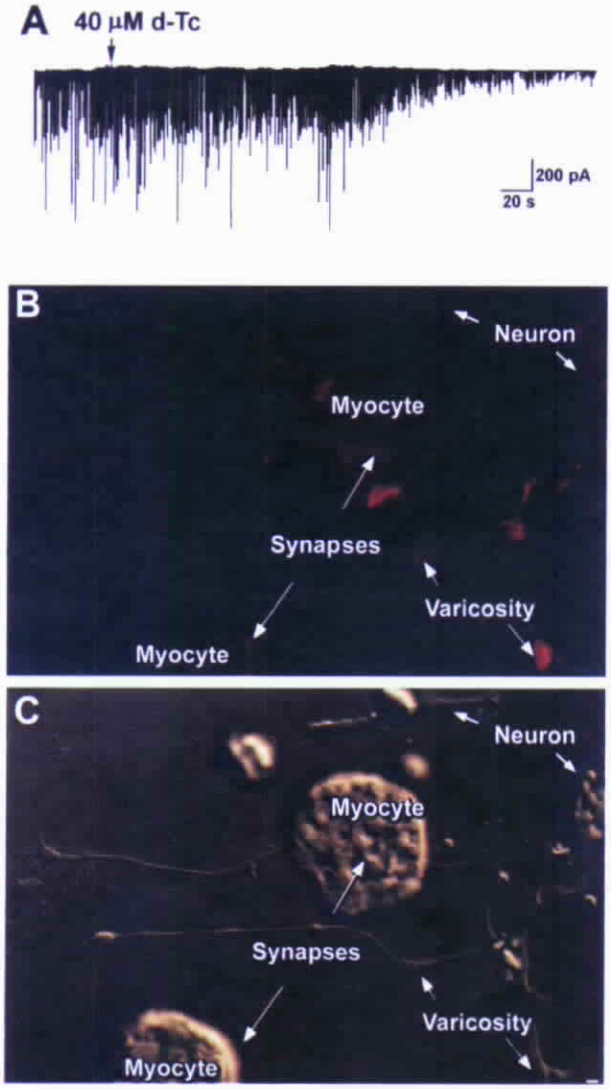

Fig.3. Glutamate immunoreactivity in developing motoneurons. (A) The continuous trace depicts the membrane currents recorded from an innervated myocyte (Vh=-60 mV). Application of d-tubocurarine inhibited the membrane currents, indicating that the myocyte was innervated by motoneuron. (B) Glutamate immunoreactivity was seen in the varicosities and neuromuscular junctions of the same neuron in (A). (C) Normaski photograph of (B). bar=2 μm.

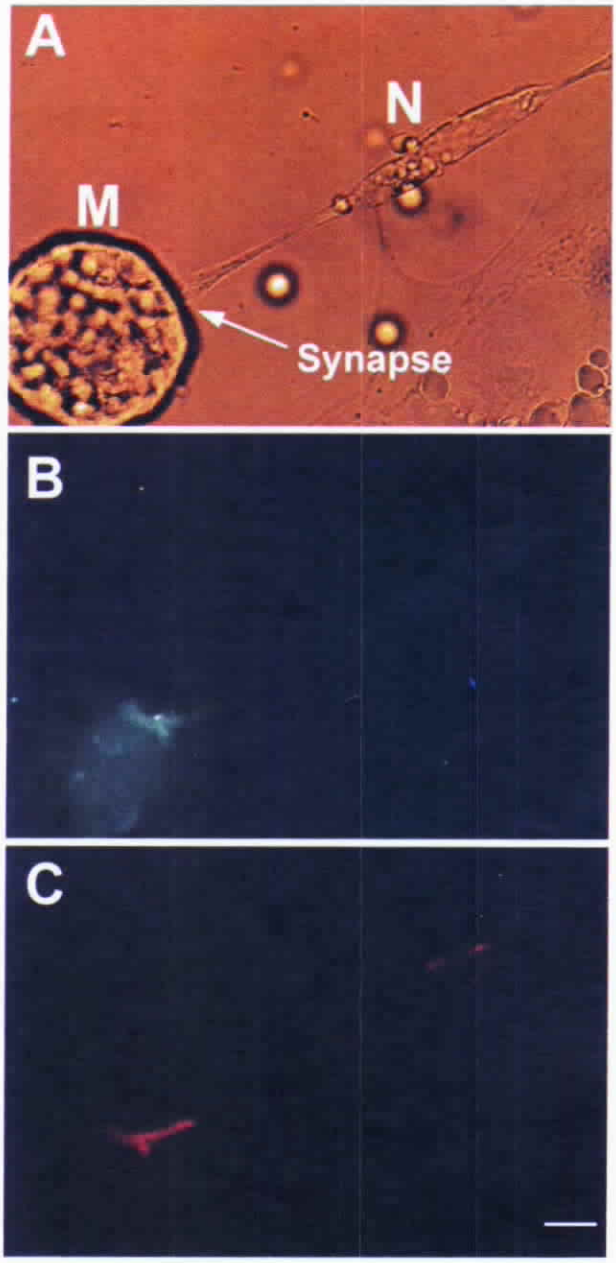

Fig. 4. Coexistence of glutamate and choline acetyltransferase (ChAT) immunoreactivity in the same cultured *Xenopus* spinal neuron. (A) Phase-contrast micrograph showing the relative position of neuron (N), myocyte (M) and synapse. (B) Immunoreactivity of ChAT shown by green (FITC) fluorescence. (C) Immunoreactivity of glutamate shown by red (rhodamine) fluorescence. Bar=10 μm.

neuron. Bath application of d-tubocurarine (d-TC, 40 μM) markedly inhibited the amplitude of SSCs, indicating that the myocyte was really innervated by motoneuron (Fig. 3A). After SSC recordings, this specific nerve-myocyte couple was then fixed and immunostained for glutamate. Glutamate immunoreactivity was found in neuronal varicosities and neuromuscular junctions, indicating that glutamate is co-stored with ACh in motoneurons (Fig. 3, B and C). No fluorescence was seen in the primary antibody blank control. Double staining of glutamate (rhodamine-labelled) and choline acetyltransferase (ChAT, FITC-labelled) in the same nerve-myocye couple of the culture further gave the evidence that both glutamate and ACh coexist in the same motoneuron (Fig. 4). The slight FITC fluorescence of the myocyte in Fig. 4B was due to the presence of ChAT in the myocyte (9).

Discussion

L-Glutamate is widely known as a major

excitatory neurotransmitter in the mammalian central nervous system. It is important in several forms of synaptic plasticity such as long-term potentiation, and in neuronal cell degeneration (3, 5). We previously found that glutamate receptor agonists markedly potentiated the frequency of SSCs (8). In the present work, we further demonstrated that local perfusion of either NMDA or kainate at synaptic regions increased SSC frequency. In addition, we also provide strong evidence for the existence of glutamate receptors located at the presynaptic site of the developing neuromuscular synapses. Both NMDA and kainate induced a cationic current in the growth cone of developing motoneurons. NMDA receptors are known to possess high Ca^{2+} permeability (1). The activation of NMDA receptor alone is sufficient to increase spontaneous ACh release. On the other hand, the opening of voltage-dependent Ca^{2+} channels is involved in the potentiation of neurotransmitter release by the activation of non-NMDA receptors (2). Consistent with this, here we found that a larger cationic current was induced by the activation of presynaptic kainate receptors. Co-release of ACh and glutamate from isolated cholinergic synaptosomes of *Torpedo* electric organ has been reported (25). In addition, glutamate-like immunoreactivity has been shown in the nerve endings of motoneurons of hindlimb muscle (17). The involvement of glutamate and ACh in the plasticity of the nervous system has been documented extensively in recent years. ACh potentiates the response of NMDA receptors in the hippocampus (16, 19), and NMDA increases ACh release in rat striatum and cortex (23). We recently found that glutamate and nicotine also had synergistic action in increasing SSCs at developing neuromuscular junctions (10). The direct evidence was shown here for the coexistence of glutamate and ACh in the motor nerve terminals of developing neuromuscular synapses of *Xenopus*, indicating that glutamate may play some regulatory role in the neuronal development.

Potentiation of the spontaneous ACh release at developing neuromuscular synapses may have a profound developmental significance. Many of spontaneous ACh release in *Xenopus* cultures are capable of eliciting action potentials and contractions in muscle cell (27). This frequent suprathreshold excitation produces a global influence on the development of post-synaptic muscle cell (13). In addition, all spontaneous synaptic potential brings about the localized influx of ions at the subsynaptic site of the muscle, especially Ca^{2+} (24). Local Ca^{2+} accumulation and the consequent Ca^{2+}-dependent enzymatic reactions are likely to play an important role in the development of postsynaptic structure. Thus, the existence of glutamate receptors at motor nerve terminals and the coexistence of glutamate and ACh in developing motoneuron may enhance the maturation of neuromuscular connections. The factors which trigger the release of glutamate in physiological conditions need further investigation.

In conclusion, the present experiment demonstrates the presence of presynaptic NMDA and non-NMDA receptors on cholinergic nerve terminals of developing motoneurons. These receptors may be involved in the positive regulation of spontaneous synaptic transmission. Furthermore, glutamate and ACh coexist in the motoneurons. Since the spontaneous ACh secretion is responsible for the maturation of embryonic myocytes and neuromuscular junction, the activation of presynaptic glutamate receptor by endogenously released glutamate may thus play some roles in synaptogenesis at earlier embryonic stages.

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