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Functional Expression of Muscle-Type Nicotinic Acetylcholine Receptors in Rat Forebrain Neurons *in vitro*

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SUMMARY

Molecular studies aimed at modifying ligand-gated ion channel function in primary neuronal cultures have been hampered by the limited efficacy of gene transfer techniques. Although viruses can effectively deliver genes to neurons, their preparation requires a substantial effort and their usefulness in delivering multiple subunits of a single protein has not been well established. In this study, we have successfully cotransfected mammalian expression plasmids for all subunits of the muscle nicotinic acetylcholine receptor (nAChR), along with a plasmid containing green fluorescent protein (GFP), into rat forebrain neurons using a calcium phosphate method. Immunostaining experiments and cell counting showed that the nAChR were successfully expressed in approximately 1% of the neurons in the culture. The functionality of the expressed receptors was demonstrated electrophysiologically as carbachol-induced currents were present in nearly 90% of the GFP-positive neurons tested. These currents could be completely inhibited by the irreversible antagonist α-cobratoxin. Surprisingly, no currents were obtained in any of the GFP-negative cells tested. These results demonstrate that gene delivery of complex, multi-subunit proteins can be successfully performed in cultured neurons, thereby opening new strategies for the pharmacological study of ligand-gated ion channels.

Key words: Calcium phosphate - Central nervous system - Muscle - Neurons - Nicotinic acetylcholine receptor - Tissue culture - Transfection

INTRODUCTION

Rapid chemical synaptic transmission throughout the nervous system is mediated by neurotransmitter receptors functioning as ligand-gated ion channels. The structure of these channels consists of 4 or 5 transmembrane subunits, each with multiple hydrophobic domains and a hydrophilic segment lining the channel pore (1, 2). Neurons are not only able to coexpress a variety of heteromeric neurotransmitter receptors, but also to target them to appropriate locations on the cell surface. Most of our knowledge about the molecular details for assembly, transport and stabilization of ligand-gated ion channels comes from studies performed in heterologous expression systems (3, 4). Although mutational analyses carried out in these systems have revealed critical information about required sequences for receptor assembly and function, it has been difficult to translate this information to a neuronal system due to the inherent problems of gene delivery to postmitotic cells. In fact, gene transfer into primary cortical neurons has been proven to be very inefficient using different approaches other than viral gene transfer. Viruses can indeed effectively deliver genes of interest to neurons (5), but their preparation is substantially more labor-intensive than traditional plasmid construction. Although viral vectors can be constructed to deliver multiple genes (6-8), to our knowledge the use of viruses in delivering multiple subunits of neurotransmitter receptor has not been documented. In the present study we evaluated whether gene delivery to neurons via a calcium phosphate precipitation technique (9) ould be achieved for a multi-subunit complex. We chose to deliver the muscle-type nicotinic receptor, as neurons do not endogenously express it and its functional properties coud be easily evaluated with electrophysiological and pharmacological techniques.

MATERIALS AND METHODS

Forebrain neuronal-enriched cultures were prepared as previously described (10). Briefly, dissociated cells from E17 rat fetuses were plated on poly-L-ornithine-treated tissue culture plates in a growth medium containing 80% Dulbecco's modified minimum essential medium (high glucose with L-glutamine and without sodium pyruvate; Gibco BRL), 10% Ham's F-12 nutrients, and 10% heat-inactivated bovine calf serum, and 1x antimycotic/antibiotic mixture with amphoterin B and streptomycin sulfate (Gibco). Cultures were maintained at 37 ºC in 5% CO_2. Glial cell proliferation was inhibited after

48 h in culture with 1-2 μM cytosine arabinoside. Serum-containing medium was replaced after 3 days *in vitro* with a serum-free medium containing Neurobasal medium (without L-glutamine; Gibco), B27 supplement (Gibco), and antimycotic/antibiotic mixture as above. At 2 weeks *in vitro*, these cultures were composed of > 95% neurons as assessed by Hoechst and glial fibrillary acidic protein staining (10).

For the expression of muscle nAChR in transfection experiments, we used pSM plasmids with an SV40 promoter driving the expression of the α1, β1, δ and ε cDNA subunits (a gift from Dr. Z.Z. Wang, University of Pittsburgh). The subunit ratios used were: 1.32α1: 0.66β1: 0.32δ: 1.00ε (Z.Z. Wang, personal communication). Green fluorescent protein (GFP) was used as a reporter gene (1/4 of total DNA). The transfection method utilized was similar to that described by Namgung and Xia (9). Neurons were transiently transfected 2-3 weeks after plating. DNA in 250 mM $CaCl_2$ was mixed with an equal volume of 2x HEPES-buffered saline (HBS). The precipitates were allowed to form for 25-30 min at room temperature before addition to the cells. Cells were washed twice with Neurobasal media and the conditioned culture medium was removed and saved; 1.5 ml of transfection media was added to each 35 mm dish. The pH of the transfection media was kept high by bubbling with nitrogen gas for 10 min. Calcium phosphate precipitates were added to the cell and mixed gently. Plates were incubated at room temperature for 5 min, and then in a humidified incubator with 5% CO_2 at 37 ºC, for 35-45 min. The incubation was stopped by treating the cells for 2 min with 1x HBS, 1 mM sodium kynurenate, 10 mM $MgCl_2$ in 5 mM HEPES, pH 7.5, and 5% glycerol. Cells were washed twice with Neurobasal media. Saved conditioned medium was added back to each plate, and cells returned to 5% CO_2 and 37 ºC for 48 h. Additional transfection experiments were attempted with FuGENE transfection reagent (Roche), but we found it to be somewhat toxic to the neurons.

For immunohistochemistry, cultures were fixed in formaldehyde and stored at 4 ºC until use. Cells were washed twice with PBS and permeabilized with 0.1% Triton-X/PBS for 5 min. Nonspecific binding was blocked by incubation with 1% bovine serum albumin in PBS for 5 min. Cells were incubated with a 1/200 dilution of an antibody against α-subunit Mab210 (Covance, Princeton, NJ, USA) for 2 h at 37 ºC. Subsequently, a rhodamine-conjugated secondary antibody was applied to cells for 30 min at room temperature. Finally, nuclei were counterstained with Hoechst, mounted and visualized under a Nikon microscope using IPlab software.

Electrophysiological recordings were performed 48 h after transfection at room temperature (25 ºC) using the whole-cell configuration of the patch-clamp technique. Cells were bathed in external solution containing (in mM): 150 NaCl, 1.0 $CaCl_2$, 2.8 KCl, 10 HEPES, 0.01 glycine, 0.0001 tetrodotoxin (Calbiochem, La Jolla, CA, USA) and pH was adjusted to 7.2 with NaOH. Electrodes were of 1.5-3 Momega resistance when filled with internal solution containing (in mM): 140 CsF, 10 EGTA/CsOH, 1 $CaCl_2$, and 10 HEPES (pH adjusted to 7.2 with CsOH). Signals were amplified using an Axopatch 200B integrating patch-clamp amplifier (Axon Instruments, Foster City, CA, USA), filtered using an 80 dB/decade filter at 1 kHz, and digitized at 2 kHz with a DigiData 1200B (Axon Instruments) computer interface. The reference electrode was an Ag/AgCl wire bridged with 2M KCl/1% agarose. Drugs were applied via a perfusion system with a stepper motor for fast solution changes (Warner Instruments Corp., Hamden, CT, USA). NMDA, carbachol (Sigma Chemical Company, St. Louis, MO, USA), and α-cobratoxin (Calbiochem) were dissolved in external solution. Data were collected and analyzed using commercially available software (pCLAMP 8, Axon Instruments).

RESULTS AND DISCUSSION

A successful transfection of nAChRs in the neuronal enriched cultures would require the functional expression and assembly of 5 receptor subunits (there are 2 α subunits in the pentameric receptor), in addition to expression of the marker vector. The latter is critical for identifying positively transfected cells for electrophysiological recordings. Cell counts of GFP positive cells versus total number of nuclei revealed an overall transfection efficiency of 0.6 ± 0.3% (2029 cells counted in 4 separate coverslips). This level of expression rendered between 30 and 50 GFP-positive cells per 12 mm coverslip. In order to evaluate the number of nAChR-expressing cells that also expressed GFP, we first performed immunostaining against the α subunit of the nAChR in the transfected cultures. We observed that less than 10% of the cells expressing GFP were negative for the α-subunit. Figure 1 shows a cell that was positive for both nAChR and GFP, and another cell that was positive for GFP alone.

In a second set of studies, whole-cell recordings were performed from a total of 16 GFP negative and 25 GFP positive cells in 3 independent transfection experiments. NMDA (30 μM) applications elicited inward currents mediated by endogenously expressed ionotropic glutamatergic receptors in all 41 cells tested. The peak amplitude of these NMDA receptor-mediated currents ranged from –44 to –3000 pA at a holding voltage of –60 mV. Although rat hippocampal and cortical neurons in dissociated tissue culture have been shown to sometimes express endogenous neuronal nicotinic receptors (11-13), we were unable to detect any carbachol-mediated currents in any of the 16 GFP-negative cells tested (Fig. 2A). This could be due to the fact that these endogenous cholinergic receptors are mostly α7 homomers (12, 14), a receptor that desensitizes very rapidly (15), and as

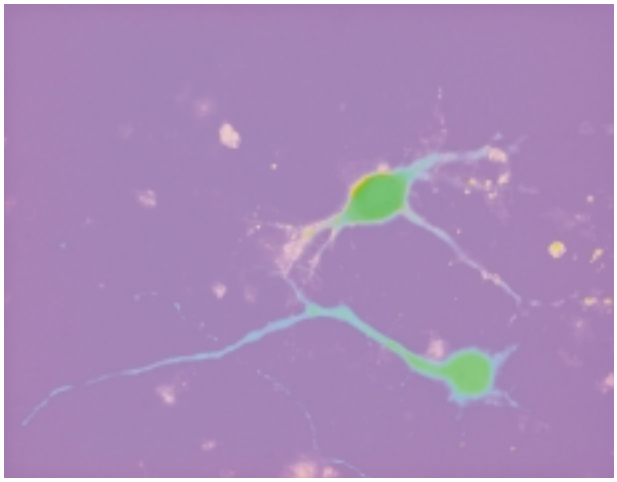

FIG. 1 Staining of transfected neurons with an antibody directed against the α subunit. Neurons expressing muscle-type nAChR and GFP. Red color indicates staining with an α subunit antibody, green the expression of GFP, and yellow the colocalization of both proteins. Note that only one of the cells expresses GFP alone. Approximately 90% of the cells expressing GFP also expressed nAChR.

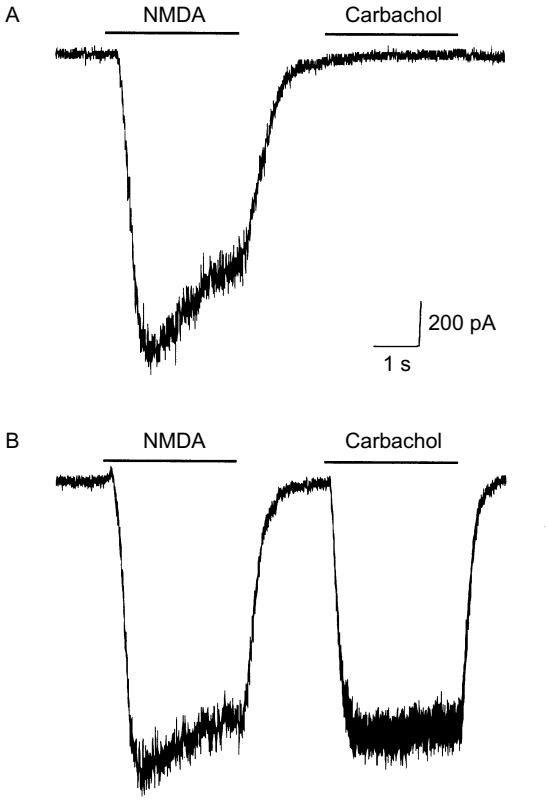

FIG. 2. Functional expression of muscle nAChR in cultured neurons. A) Whole-cell recording from a GFP-negative neuron during application of 30 μM NMDA and 10 μM carbachol for the durations indicated by the lines above the traces. The cell was voltage-clamped at –60 mV in this and subsequent traces. Note the lack of carbachol-induced current in this cell. Similar findings were observed in a total of 16 cells. B) NMDA and carbachol-elicited responses in a GFP positive cell demonstrating the functional expression of nAChR in this cell. Similar results were obtained in a total of 22 out of 25 GFP-positive neurons.

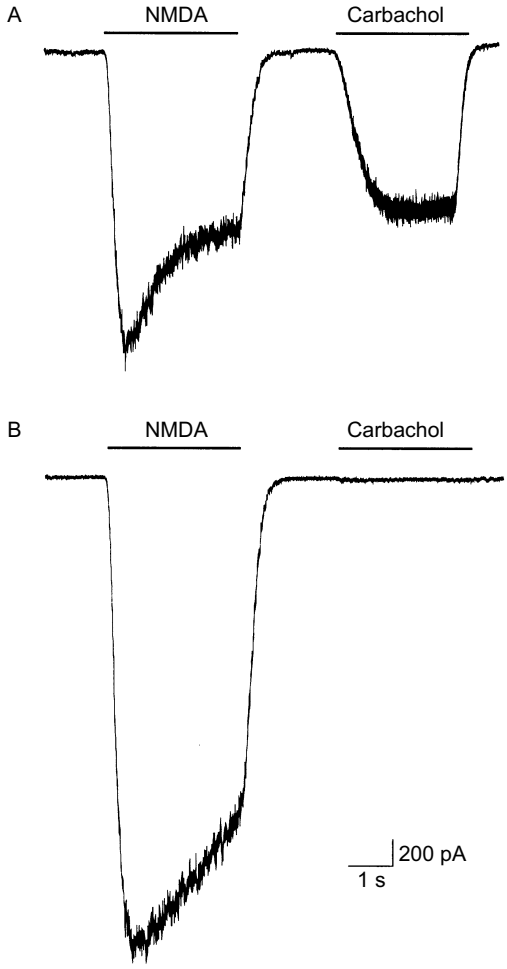

FIG. 3. Effects of α-cobratoxin treatment on NMDA and nAChR. Whole-cell responses obtained from a GFP-positive neuron in culture to 30 μM NMDA and 10 μM carbachol before(A) and after (B) a 2-min treatment with 1 μM α-cobratoxin. Responses following toxin treatment were obtained following a 2-min wash with control solution. Note the complete block of the carbachol-induced current by the toxin treatment, as well as the unanticipated enhancement of the NMDA response. Similar findings were observed in a total of 4 transfected cells.

such, requires low agonist concentrations and rapid drug applications to be properly resolved electrophysiologically (16). In contrast to our findings with GFP-negative cells, 22 out of the 25 GFP-positive cells tested exhibited robust responses to carbachol (Fig. 2B). Similar to the NMDA-elicited responses, the amplitude of the carbachol-induced currents varied from neuron to neuron, with peak amplitudes ranging from –47 to –2200 pA at a holding voltage of –60 mV. These currents were mostly non-desensitizing at the concentration of carbachol utilized, strongly suggesting that they were not mediated by endogenous neuronal α7 receptors.

To validate the pharmacological identity of the transfected nAChR, we decided to investigate if α-cobratoxin

was able to inhibit carbachol-induced responses in transfected neurons. Among the *Naja naja kaouthia* toxins, α-cobratoxin is an α peptide antagonist that binds with high affinity to regions overlapping acetylcholine binding sites in the a1 subunit (17). As demonstrated in Figure 3, a 2-min treatment with 1 μM α-cobratoxin was sufficient to completely eliminate the response to carbachol in the 4 GFP-positive neurons tested. Interestingly, treatment with α-cobratoxin had an unexpected effect on the NMDA receptor-mediated response. The toxin produced a nearly 2-fold potentiation of the NMDA response, as well as induced a measurable change in the overall desensitization of the currents (Fig. 3B). We are unaware of any prior reports in the literature describing this effect of α-cobratoxin on the NMDA receptor. However, it is possible that this phenomenon could be explained by the actions of a potential contaminant in the toxin preparation utilized. Indeed, another fraction of the cobra venom, α-cardiotoxin, has been shown to activate phospholipase C (18), and a recent study has suggested that NMDA receptor activity can be enhanced via activation of this enzyme (19). Future studies will be performed to clarify this issue.

The results presented in this study demonstrate that as many as 5 plasmids can be effectively delivered into a single neuron utilizing a simple transfection technique, and that proteins encoded by 4 of these can be coassembled into a pentamer, packaged and delivered to the cell membrane where they can form a functional neurotransmitter receptor channel. Although the overall efficiency of the transfection procedure was relatively low, the functional efficacy for a positively transfected cell was actually very high. This is reflected by the fact that approximately 90% of the GFP-positive cells had functional nAChRs, suggesting that the gene transfer event, in and of itself, occurs in a nearly all or none fashion. This apparent nonrandomness of total plasmid delivery for a given cell may be important in future experiments as it would allow for the experimental manipulation of gene dosage ratios without affecting the overall transfection efficiency. Nonetheless, we have yet to fully optimize the transfection procedure and it may be possible to achieve higher percentages of transfected cells by varying parameters such as cell density or total DNA added. These studies open the door for new approaches in the study of ligand-gated ion channel pharmacology, assembly and trafficking in cultured neurons.

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