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Differential Regulation of Neuronal Nicotinic ACh Receptor Subunit Genes in Cultured Neonatal Rat Sympathetic Neurons: Specific Induction of α_7 by Membrane Depolarization Through a Ca^{2+}/Calmodulin-Dependent Kinase Pathway

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We have examined the regulation of neuronal nicotinic ACh receptor (nAChR) genes and ACh-evoked currents by neonatal rat sympathetic neurons developing in culture. These neurons contain 5 nAChR transcripts: α_3, α_5, α_7, β_2, and β_4. When developing in culture, the neurons express 4 of these transcripts, α_3, α_5, β_2, and β_4, at levels similar to those in neurons developing *in vivo*: α_3 mRNA levels increase two- to threefold over the first week, whereas the levels for α_5, β_2, and β_4 remain essentially constant. In contrast, α_7 mRNA levels drop by 60–75% within the first 48 hr and remain low. We show that during the first week, the ACh-evoked current densities on these cultured neurons increase twofold and correlate well with the increase in α_3 mRNA levels. Depolarizing the neurons with 40 mM KCl for 1–2 d upregulates the α_7 gene; this specific change in α_7 mRNA level correlates with an increase in α-bungarotoxin (α-BTX) binding on the surface of the neurons. Depolarization has little effect on the expression of the other four transcripts, or on the magnitude or kinetics of the ACh-evoked currents. Furthermore, activators or inhibitors of protein kinase A (PKA), protein kinase C (PKC), or tyrosine kinase do not affect nAChR transcript levels in these cultured neurons. The effect of membrane depolarization on α_7 expression is a result of Ca^{2+} influx through L-type Ca^{2+} channels, and we show that α_7 is upregulated through a Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase) pathway. The identification of CaM kinase as a link between activity and neurotransmitter receptor expression may indicate a novel mechanism that underlies some forms of synaptic plasticity.

[Key words: nicotinic ACh receptors, membrane depolarization, calcium influx, Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase), α-bungarotoxin, rat sympathetic neurons]

An important mechanism used by the nervous system to modify the strength of synaptic connections during development and in adult life involves altering the expression of postsynaptic receptors. However, many of the factors that control postsynaptic receptor numbers on neurons have not been identified. Much of what is known about the developmental regulation of postsynaptic receptor expression on neurons has come from studies on nAChRs expressed by autonomic neurons. Several genes coding for neuronal nAChRs have been identified and are part of a large family of ligand-gated receptor genes (reviewed in Sargent, 1993; McGehee and Role, 1995). Like muscle nAChRs, neuronal nAChRs have a pentameric structure containing two α subunits and three β subunits (Anand et al., 1991; Cooper et al., 1991). A number of studies have described the developmental appearance of nAChRs in autonomic neurons (Berg et al., 1989; Sargent, 1993; Devay et al., 1994; Mandelzys et al., 1994; Schwartz Levey et al., 1995; Zoli et al., 1995). Transcripts for nAChR subunits first appear early in development prior to pre- and postganglionic interactions (Devay et al., 1994; Zoli et al., 1995). During further development, nAChR transcript levels and ACh-evoked currents increase simultaneously with incoming innervation and target contact (Engisch and Fischbach, 1992; Corriveau and Berg, 1993; Moss and Role, 1993; Devay et al., 1994; Mandelzys et al., 1994; Schwartz Levey et al., 1995), suggesting that these interactions increase nAChR expression. Several studies have tested this hypothesis *in vivo* by removing these pre- and postganglionic interactions during development; the results differ, however, depending on whether one measures ACh-evoked currents, nAChR transcript levels or nAChR proteins. For example, removing preganglionic inputs causes little or no effect on the subsequent developmental increase in ACh-evoked current densities (McEachern et al., 1989; Engisch and Fischbach, 1992; Mandelzys et al., 1994; Schwartz Levey et al., 1995), a delayed increase in mRNA levels for nAChR subunit (Mandelzys et al., 1994; Schwartz Levey et al., 1995), and a large decrease in the intracellular pool of receptor proteins (Jacob and Berg, 1987; Arenella et al., 1993). This suggests that changes in both gene expression and posttranscriptional mechanisms regulate nAChR expression.

To learn more about factors that regulate nAChR expression, we have investigated neonatal rat superior cervical ganglion (SCG) neurons developing in culture. Rat SCG neurons contain mRNA for 5 nAChR subunits: α_3, α_5, α_7, β_2, and β_4 (Mandelzys et al., 1994). These neurons also express an α-BTX-nAChR (Fumagalli et al., 1976) which is likely to incorporate the α_7 subunit (Schoepfer et al., 1990; Couturier et al., 1990a; Séguéla et al., 1993). The function of this receptor is poorly understood (Clarke, 1992); however recent studies indicate that this receptor

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promotes Ca^{2+} influx (Vijayaraghavan et al., 1992; Séguéla et al., 1993; Pugh and Berg, 1994; Castro and Albuquerque, 1995) and may influence various Ca^{2+}-mediated processes possibly linked to synapse formation and synaptic transmission.

In this article, we have focused mainly on the effects of membrane depolarization on receptor expression. Studies with skeletal muscle have demonstrated that activity causes changes in nAChR expression as a consequence of Ca^{2+} influx and the activation of PKA (Walke et al., 1994) or PKC (Klarsfeld et al., 1989). Less is known about the link between Ca^{2+} influx and the regulation of neurotransmitter receptor genes in neurons. Here, we have measured the developmental expression of ACh-evoked currents and the relationship between changes in ACh-evoked current densities and nAChR transcript levels in these cultured neurons. We show that membrane depolarization, through Ca^{2+} influx, upregulates α_7 gene expression and α-BTX binding, and we show that this increase in α_7 expression occurs through a CaM kinase pathway. In contrast, membrane depolarization has little effect on the levels of the other four nAChR transcripts or on the ACh-evoked current densities.

Materials and Methods

Neuronal cultures

SCG ganglia were dissected from postnatal day 1 (P1) rats (Sprague–Dawley CD strain, Charles River, Canada) and dissociated mechanically and enzymatically as previously described (McFarlane and Cooper, 1992). Briefly, we dissected the ganglia under sterile conditions from animals that were killed by cervical dislocation. The ganglia were incubated for 15 min at 37°C in medium containing collagenase (1 mg/ml, Sigma) and a neutral protease (Dispase, grade II, 2.4 mg/ml; Boehringer-Mannheim), and then the ganglia were transferred to medium containing only the neutral protease and incubated at 37°C for 2–3 hr while triturating gently with a fire-polished Pasteur pipette every 15 min. After dissociation, the cells were centrifuged through a 35% percoll solution (Pharmacia) to partially separate neurons from non-neuronal cells. The neuronal suspension was washed and plated on aclar coverslips (Allied Chemicals) coated with 30 μg/ml of laminin (overnight at 4°C; gift from Dr. S. Carbonetto), at a density of 5000–8000 cells/cm^2. The neurons were grown in petri dishes (35 mm, Corning) that had been modified by boring a ~1 cm^2 hole in the center and covering it from beneath with the aclar coverslip glued with Sylgard (Dow Corning); this creates a ~2 mm deep well with a volume of ~100 μl into which the neurons are plated (Hawrot and Patterson, 1979).

The growth medium (1.5 ml/dish) consisted of: Leibovitz-15 medium (L15) supplemented with sodium bicarbonate, vitamins, cofactors, penicillin–streptomycin, 2.5S NGF (25 ng/ml), and 5% rat serum (Hawrot and Patterson, 1979). Cultures were maintained in a humid atmosphere of 95% air, 5% CO_2 at 37°C, and treated with cytosine arabinofuranoside (Ara-C; 5 μM, Sigma) for the first 2–3 d to eliminate the few remaining non-neuronal cells. For explant cultures, we cut the ganglia in half and plated one half-ganglion per petri dish; no Ara-C was added to explant cultures.

Treatments of cultures. To depolarize the neurons, we mixed a stock solution of 350 mM KCl in a 1:9 ratio with growth medium, which already contains 5 mM KCl. Many of the agents that were added to our cultures were first dissolved in dimethyl sulfoxide (DMSO) or ethanol, which typically amounted to 0.1% of the culture media. Treating neurons for 48 hr with any of the drugs listed below, with 0.1% DMSO, or with additional 35 mM KCl had no effect on neuronal survival, as measured by cell counts. Furthermore, these treatments had no noticeable effect on the morphological appearance of the neurons, as judged by phase contrast microscopy. Nifedipine, verapamil, and KN-62 had no effect on the nAChR mRNA levels when added to control cultures (data not shown). We used the following agents: 50 μg/ml α-amanitin (stock: 1 mg/ml in H_2O), 5 μM nifedipine (stock: 10 mM in DMSO), 5 μM verapamil (stock: 20 mM in ethanol), 10–50 μM nicotine (hydrogen tartatre salt, stock: 250 mM in H_2O), 10–100 μM carbachol (Cl salt, stock: 1 M in H_2O), 5 mM choline (Cl salt, stock: 1 M in H_2O), 100 μM hexamethonium (Cl salt, stock: 10 mM in H_2O), 100–200 nM α-BTX (stock: 125 μM in H_2O), 2 μM atropine (sulfate salt, stock: 1 mM in H_2O), (all from Sigma); 0.1–10 μM KN-62 (stock: 20 mM in DMSO), 1-20 μM chelerythrine (stock: 10 mM in DMSO), 1–10 μM H-89 (stock: 10 mM in DMSO), 1–100 μM genistein (stock: 100 mM in DMSO), 100–300 nM PMA (stock: 300 μM in DMSO), 1–10 μM forskolin (stock: 10 mM in DMSO) (all from Biomol).

RNA extraction

The RNA was extracted with a guanidinium isothyiocyanate (GTC)-phenol-chloroform extraction procedure as described by Chomczynski and Sacchi (1987), with some modifications. For RNA extraction from neurons at day 0, only the enriched neuronal fraction from the percoll step (see above) was used. For RNA extraction from cultured neurons, three to four petri dishes were typically pooled together and 300–400 μl of GTC solution (+ β-mercaptoethanol) was used to collect the RNA. The dishes were first rinsed twice with 2 ml of ice-cold Hank's balanced salt solution, made with diethyl pyrocarbonate-treated H_2O (DEPC-H_2O). To maximise the yield of extracted RNA, 100 μl of GTC solution was added to the center well of one petri dish, pipetted up and down five times and the solution was transferred to a second well. A second 100 μl of GTC solution was used to rinse the center well. Following standard phenol-chloroform extraction, the RNA was allowed to precipitate for 2 d at −20°C in equal volume of isopropanol. Precipitated RNA pellets were resuspended in 100 μl of DEPC-H_2O and reprecipitated immediately with ammonium acetate and ethanol for 1–2 d at −20°C. The optical density (OD) measurements on spectrophotometer were done using an RNase free microcuvette (100 μl, Beckman) that was precleaned with DEPC-H_2O containing 100 mM NaOH and 1 mM EDTA. The ODs were measured on the entire RNA samples which were resuspended in 100 μl of DEPC-H_2O. The RNA was reprecipitated and kept at −20°C until use for RNase protection assays. Our average yield of total cellular RNA from four petri dishes with each ~6000 neurons cultured for 7 d was approximately 2.0 ± 0.2 μg.

RNase protection assays

RNase protection assays were performed according to that described by Krieg and Melton (1987), with minor modifications (Mandelzys et al., 1994). Briefly, ^{32}P-labeled antisense RNA probes to α_2, α_3, α_4, α_5, α_7, β_2, β_3, and β_4 nAChR subunits were transcribed *in vitro* from linearized plasmids, pGEM, or pSP64, containing subcloned portions of the cDNA for each neuronal nAChR subunit (Mandelzys et al., 1994; kindly provided by E. Deneris). The thermal stabilities for all probes, as calculated from their melting temperatures, differed by less than 1°C. Each probe was gel purified before use. For each reaction, 0.5 μg of total cellular RNA was combined with two or three radiolabeled probes of 200,000 cpm each, and allowed to hybridize overnight at 60°C. The remaining single stranded RNAs were digested with RNase T1, and the protected RNA:RNA duplexes were denatured and run on a 5% polyacrylamide-8 M urea gel. The gels were first exposed to a phosphor imaging plate (Fujix BAS 2000, Bio Image Analyzer), to quantify the radioactive bands, and subsequently to film (Kodak XAR) with an intensifying screen for 48–72 hr at −70°C. The specific activity of each riboprobes was calculated from the number of adenine bases in the protected region. To quantify the levels of mRNA among different transcripts, the relative intensities of the hybridization signals were divided by the specific activity of the corresponding riboprobe. For each culture, every RNA sample was tested in duplicate or triplicate. mRNA levels for all nAChR subunits were similar between whole ganglia and dissociated neurons, as the contribution of the non-neuronal cells in total ganglionic RNA from P1 rat SCG is less than 10 % (Mandelzys et al., 1994). To estimate the number of nAChR transcripts expressed per neuron, we synthesized sense RNA for α_3 and β_2 and measured their OD. By comparing hybridization signals from known amounts of sense α_3 and β_2 RNA (e.g. 0.1, 0.5, 1, 2, 5, 10 pg) and hybridization signals from 0.5 μg of SCG RNA (≈6000 neurons), we estimated that an SCG neuron cultured for 7 d has on average 1000–1500 β_2 transcripts and 5000–7000 α_3 transcripts. In some experiments, we normalized nAChR subunit hybridization signals with signals for GAPDH to control for RNA loading. However, as both β subunit transcript levels showed little change in culture and with any treatment, they also served as internal controls in many experiments. As shown previously for SCG neurons *in vivo*, transcripts for α_2, α_4, and β_3 nAChR subunits were not detected in SCG neurons developing in culture (data not shown; see Mandelzys et al., 1994).

α-BTX binding

Neurons were plated at 15,000–20,000 neurons/3 cm^2 aclar coverslip. At day 5, sister cultures were treated with 40 mM KCl. At day 7, all cultures were washed twice with L15 plus 10% horse serum (HS), followed by a 90 min preincubation with or without 100 nM cold α-BTX at 37°C in L15+HS. ^{125}I-α-BTX (2.5 nM; 130 Ci/mmol; NEN) was then added to every dishes for a 90 min incubation at 37°C. The cells were subsequently washed six times with L15+HS over a 60 min period to remove all unbound ^{125}I-α-BTX. The neurons were finally extracted in 0.5 ml of 0.5 M NaOH, and the radioactivity was measured with a γ-counter. Four dishes per condition were used in each experiment (n = 3).

Electrophysiology

Whole-cell currents were measured with patch-clamp techniques, using a List EPC-7 amplifier. Pipette resistances were 2–6 MΩ and were balanced to zero with the pipette immersed in perfusion fluid. Membrane currents were filtered with an eight-pole Bessel filter (Frequency Devices, Inc.), and then sampled, displayed and stored on line with a 386-based PC computer (AT class with an EISA bus running at 33 MHz and a 64K cache and an A/D card; Omega); we used PATCHKIT (Alembic Software, Montreal) for data acquisition and analysis. All recordings were done at room temperature (22–24°C). We did not detect any synaptic interactions among the neurons, nor did we detect spontaneous action potential activity.

For recording ACh-evoked currents, we used a method of rapid agonist application (Mandelzys et al., 1995): we apply solutions from large double-barrel glass tubing attached to an electromechanical switching device. Each barrel has an opening of 200 μm and is positioned 30–50 μm from a neuron, with the central axis of one barrel in line with the neuron. Initially, the neuron is perfused by the control solution; by triggering the electromechanical device, the second barrel rapidly moves into position and perfuses the neuron with the agonist solution. The flow rate from each barrel is usually 8–12 μl/sec; higher rates are difficult because they detach the neurons from the substrate. The flow from each barrel is controlled by a valve so that the flow of agonist is not continuous, but limited to recording period (usually 2–5 sec for each trial); in addition, the recording chamber is perfused at 1–2 ml/min with perfusion media (see below) to minimize build up of agonist. To assess the speed of our agonist application technique, we filled each barrel with a different salt concentration and measured the time course for the change in electrode tip potential to occur upon switching from one barrel to the other. From this test, we demonstrated that our switching device changes the concentration in the vicinity of the electrode in less than 2 msec. For our experiments, we used 1 mM ACh to activate the currents rapidly (Mandelzys et al., 1995); this dose has been shown to activate rapidly desensitizing currents on chick ciliary neurons (Zhang et al., 1994). We also tested other nicotinic agonists (nicotine, DMPP, cytisine, and choline) at various doses (data not shown, Mandelzys et al., 1995). With this speed of activation, we found that adding 1.0 μM TTX to the perfusion media, as well as to the agonist solution, was essential to eliminate unclamped Na^+ currents. The ACh-evoked current densities were calculated by dividing the peak evoked currents by the whole-cell capacitance. The whole-cell capacitance was measured by integrating the current evoked by a 5 mV hyperpolarizing pulse from a holding potential of −60 mV.

For recording Ca^{2+} currents, we used two depolarizing protocols as shown in Figure 5. To quantify L-type Ca^{2+} tail currents, we added (+)-202-791 (1 μM; stock: 1 mM in ethanol, Sandoz Pharma) to the perfusion solution to prolong the L-type currents and allow better resolution of the tail currents, and we measured the amplitudes of the tail currents 3 msec after the repolarization (Plummer et al., 1989; Regan et al., 1991; Mathie et al., 1992). Currents were corrected for leakage and capacitive currents. The perfusion solution was the same as that shown below except that it contained 5 mM $CaCl_2$, 10 mM tetraethylammonium (TEA)-bromide (Sigma) and 2 mM 4-aminopyridine (4-AP)-Cl (grade II, Sigma). For some experiments, (+)-202-791 was not included to make sure that is was not interfering with a potential effect of KN-62 on the Ca^{2+} currents. To block Ca^{2+} currents, 5 μM nifedipine or 5 μM ω-CgTx (stock: 100 μM in H_2O, Sigma) were added to perfusion solution.

Solutions. For recording ACh-evoked currents, the perfusion solution consisted of 140 mM NaCl, 5.4 mM KCl, 0.33 mM NaH_2PO_4, 0.44 mM KH_2PO_4, 2.8 mM $CaCl_2$, 0.18 mM $MgCl_2$, 10 mM HEPES, 5.6 mM glucose, 2 mM glutamine, 5 μg/ml phenol red, and 1 μM tetrodotoxin (TTX; Sigma); pH was adjusted to 7.4 with 1 M NaOH (final ≈ 2 mM). The pipette solution contained 65 mM KF, 55 mM KAc, 5 mM NaCl, 1 mM $MgCl_2$, 10 mM EGTA, and 10 mM HEPES; pH was adjusted to 7.4 with 2 M KOH (final ≈ 20 mM). The pipette solution for recording Ca^{2+} currents, which was kept on ice during the experiments, consisted of 65 mM CsF, 50 mM CsAc, 4 mM $MgCl_2$, 4 mM Na_2ATP (Sigma), 0.2 mM NaGTP (Sigma), 14 mM phosphocreatine (Sigma), 10 mM HEPES, 10 mM EGTA; pH was adjusted to 7.4 with 2 M KOH (final ≈ 25 mM).

Statistics

Student's t tests were used to assess statistical differences.

Results

Expression of nAChR transcripts in cultured SCG neurons

Over the first 2 postnatal weeks *in vivo*, a period when much of the innervation from preganglionic neurons occurs (Smolen and Raismen, 1980; Rubin, 1985), SCG neurons undergo a significant change in the expression of nAChR transcripts: mRNA levels for α_3 increase fourfold, and levels for α_7 increase threefold, whereas those for α_5, β_2 and β_4 show little change (Mandelzys et al., 1994). The factors responsible for this differential regulation in nAChR subunit gene expression are unknown. To learn more, we have investigated nAChR expression by neonatal SCG neurons developing in dissociated cell cultures in the absence of non-neuronal cells (Fig. 1*a*). Figure 1*b* shows that SCG neurons developing in culture express four nAChR transcripts at levels similar to neurons developing *in vivo*: mRNA levels for α_3 increase two- to threefold over 7 d in culture, and levels of α_5, β_2, and β_4 remain essentially constant. However, the expression of α_7 is different: unlike the increase in mRNA levels during development *in vivo*, the α_7 mRNA levels in SCG neurons in culture decrease 60–75% within 2 d, and remain low for at least 2 weeks. This finding indicates that the regulation of α_7 mRNA by SCG neurons in culture differs from its regulation *in vivo*. Similar results were obtained with explant cultures indicating that the decrease in α_7 mRNA levels in these neurons is not a consequence of removing the non-neuronal cells (data not shown).

To investigate whether the drop in α_7 mRNA level was a result of decreased mRNA stability, we blocked mRNA synthesis by treating cultures at day 0 or at day 1 for 24 hr with a transcription inhibitor, α-amanitin (50 μM). We found no significant difference in the rate of decrease in α_7 mRNA levels between control- and α-amanitin-treated cultures 24 hr later, suggesting that the decreased α_7 mRNA levels reflect a reduction in mRNA synthesis and not an accelerated mRNA degradation (Fig. 1*c*). In addition, α_3 mRNA levels in α-amanitin-treated cultures decreased at a rate similar to that of α_7 mRNA (data not shown), suggesting that both transcripts have comparable stabilities.

Membrane depolarization results in specific upregulation of α_7 mRNA

One consequence of placing neonatal neurons in culture is that initially they have a reduced electrical activity, in part, because the neurons are denervated. To test whether membrane depolarization plays a role in the expression of nAChR subunits, we added 40 mM KCl to sister cultures (high K^+). Exposing the neurons to high K^+ at the time of plating had little effect on mRNA levels for α_5, β_2, or β_4 48–72 hr later (Fig. 2*a*). After 3 d, mRNA levels for α_3 were only 1.3 (±0.1) fold greater in high K^+-treated neurons compared to control neurons. However, high K^+ produced a significant change in α_7 mRNA levels: unlike in

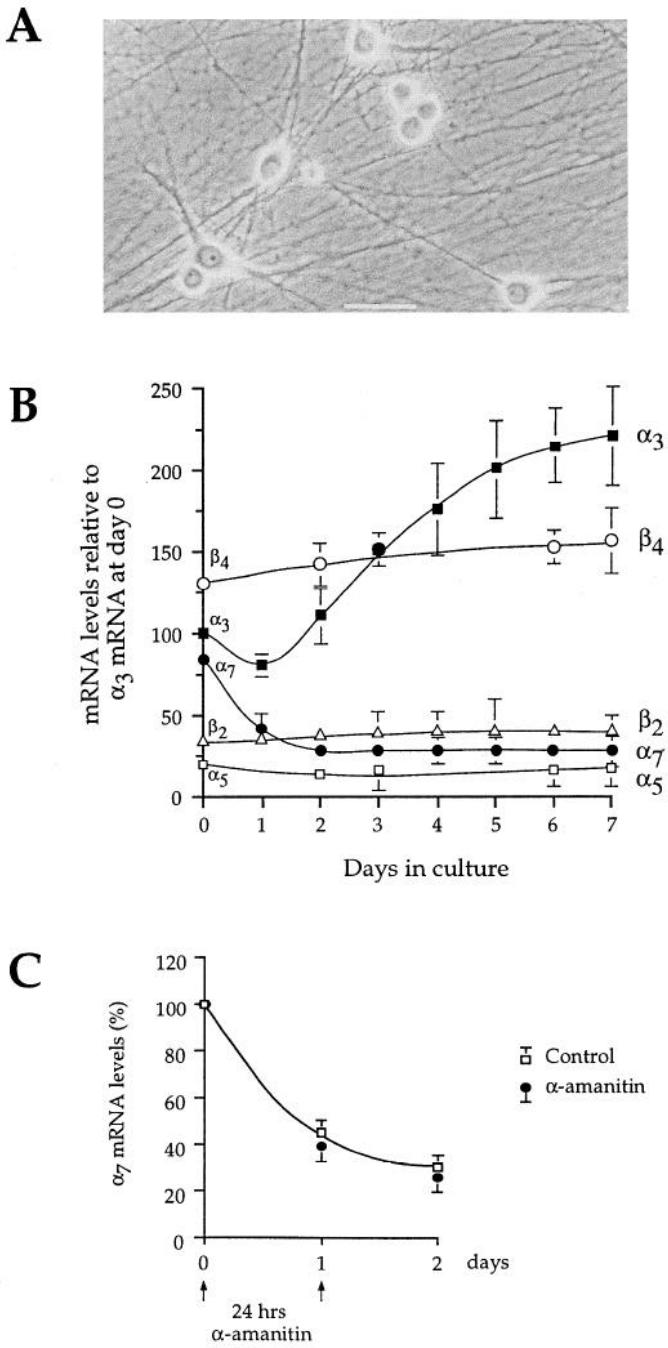

Figure 1. Developmental expression of nAChR transcripts by neonatal SCG neurons in culture. *A*, Phase contrast photomicrograph of SCG neurons that have developed in culture for 1 week. Scale bar, 60 μm. *B*, Relative mRNA levels for the five nAChR subunits expressed in freshly dissociated rat SCG neurons (day 0) and over the first 7 d in culture. The mRNA levels for each subunit were measured by RNase protection assays, quantified with a phosphor imager and expressed relative to the levels of α_3 subunit mRNA at day 0 (=100%). The measured α_3 transcript levels correspond approximately to 2000–3000 copies per neuron (see Materials and Methods). Examples of the hybridization signals for the five nAChR subunits are shown in Figure 2, *a* and *c*, at day 0, 3 and 7. Each value represent an average of four to six cultures (±SEM). *C*, Decay of mRNA levels for α_7 subunit in control and α-amanitin-treated cultures. α-amanitin (50 μM) was added for 24 hr either at day 0 or at day 1. Values represent means (±SEM) of four cultures.

control neurons, α_7 mRNA levels remained at their initial value for several days in high K^+-treated neurons (Fig. 2*a,b*). High K^+ appears to increase α_7 mRNA synthesis because addition of α-amanitin to both control and high K^+ cultures resulted in similar decay rates for α_7 mRNA levels, suggesting that high K^+ does not increase α_7 mRNA stability (data not shown). Furthermore, by delaying the addition of high K^+ to day 5, we observed a two- to threefold increase in α_7 transcript levels which reached 70% of their plateau in 24 hr and 100% by 48 hr (Fig. 2*c*). High K^+ added at day 5 caused little change in the other nAChR transcript levels, including α_3, indicating that the effects of membrane depolarization are specific for α_7 (Fig. 2*c*, Table 1).

The role of Ca^{2+} influx

Several studies have shown that sustained membrane depolarization, produced by high K^+, induces the expression of many genes by increasing Ca^{2+} influx through 1,4-dihydropyridine (DHP)-sensitive L-type Ca^{2+} channels (Murphy et al., 1991; Bading et al., 1993; Bessho et al., 1994; Rosen et al., 1994). To test whether this is the case for α_7 gene expression, we added L-type Ca^{2+} channel blockers, verapamil (5 μM) or DHP-antagonist nifedipine (5 μM), to cultures treated with high K^+ from day 5 to day 7. Figure 2, *c* and *d*, shows that both nifedipine and verapamil specifically block the increase in α_7 mRNA levels, while having no effect on other nAChR transcript levels (see also Table 1). These data indicate that Ca^{2+} influx through L-type Ca^{2+} channels is necessary for the induction of α_7 gene expression by membrane depolarization.

The effects of protein kinase activity on nAChR subunit expression

Next, we investigated whether the induction of α_7 gene expression by Ca^{2+} influx is mediated by protein kinase activity. To test if PKA is involved, we either treated control and high K^+ cultures at day 5 with forskolin (10–50 μM) for 48 hr to activate PKA, or treated high K^+ cultures with H-89 (1–10 μM) to inhibit PKA. Neither treatments had any effect on α_7 transcript levels (Fig. 3). To determine if PKC is involved, we either treated control and high K^+ cultures at day 5 with phorbol-12-myristate-13-acetate (PMA; 100–300 nM) for 48 hr to activate PKC, or treated high K^+ cultures with chelerythrine (1–20 μM) to inhibit PKC. Similarly, neither the activation nor the inhibition of PKC had any effect on α_7 transcript levels (Fig. 3). High K^+ has been shown to support the short term survival of NGF-deprived SCG neurons (Franklin and Johnson, 1992). To test whether the effects of high K^+ on α_7 gene expression is linked to *trk*A activity, we increased the concentration of NGF fourfold in both control and high K^+ cultures (from 25 ng/ml to 100 ng/ml), in attempt to increase signaling through the *trk*A receptors (Ma et al., 1992). This treatment did not affect any of the nAChR transcript levels (data not shown). In addition, we treated high K^+ cultures with a tyrosine kinase inhibitor, genistein (10–100 μM); this had no effect on the increase in α_7 transcripts (Fig. 3). Furthermore, treatment of high K^+ cultures with 100 nM K-252a, which inhibits *trk* receptors preferentially at this concentration (Koizumi et al., 1988; Berg et al., 1992), had no effect on α_7 or the other nAChR transcript levels (data not shown).

These results suggest that neither PKA, PKC nor tyrosine kinase is involved in the Ca^{2+} signaling cascade that affects α_7 gene expression. Table 1 shows that activators or inhibitors of PKA, PKC or *trk*A-related tyrosine kinases appear not to be involved in the regulation of any nAChR transcripts expressed

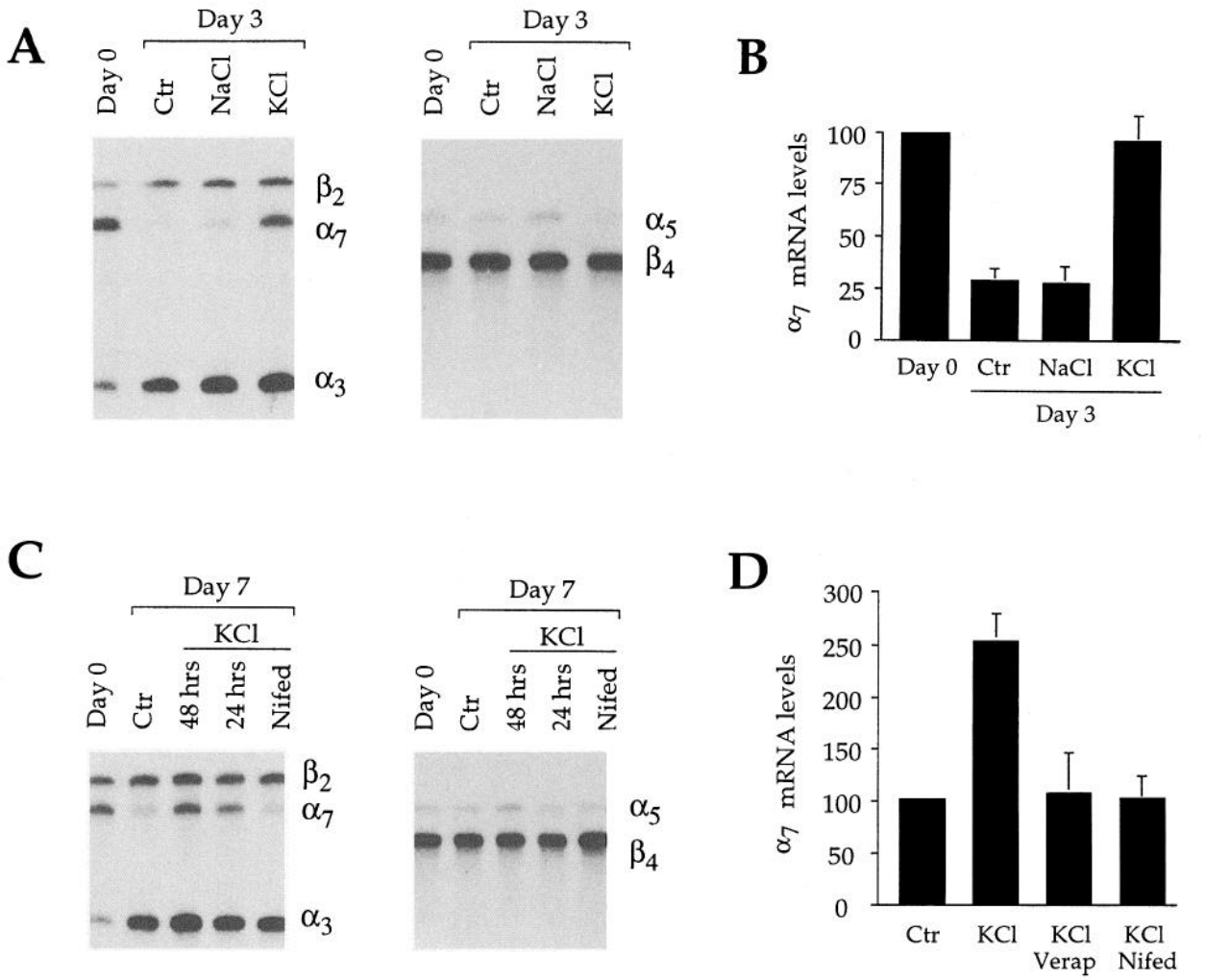

Figure 2. Membrane depolarization and Ca^{2+} influx induce α_7 gene expression. *A*, RNase protection assays for α_3, α_7, β_2 (*left*), α_5 and β_4 (*right*) nAChR subunit mRNAs. The protected riboprobe base pair sizes are: β_2= 571, α_7 = 467, α_3 = 258, α_5 = 479, and β_4 = 411. In both gels, the first lane (*Day 0*) is total cellular RNA from freshly dissociated SCG. RNA from neurons grown for 3 d in control medium (*2nd lane; Ctr*), with additional 35 mM NaCl (*3rd lane; NaCl*), as a control for osmolarity change, or with additional 35 mM KCl (*3rd lane; KCl*) to depolarize the neurons. α_7 mRNA levels drop in culture, but are sustained in high K^+. α_5, β_2, and β_4 mRNA levels change little in culture and are not affected by high K^+. α_3 mRNA levels increase in culture and are 1.3 ± 0.1 greater in high K^+. *B*, Means (±SEM) of mRNA levels for α_7 subunit relative to Day 0 (=100%); n = 3 cultures. *C*, RNase protection assays, as in *A*, for RNA extracted from freshly dissociated neurons (*Day 0*) or neurons that have been cultured for 7 d in control medium (*Ctr*), with additional 35 mM KCl for 24 hr (added at day 6) or for 48 hr (added at day 5), or for 48 hr with both KCl and 5 μM nifedipine, an L-type Ca^{2+} channel blocker. High K^+ induces α_7 expression after its drop in culture; nifedipine blocks this high K^+ induction, but has no effect on other nAChR transcripts (see also Table 1). *D*, Means (±SEM) of mRNA levels for α_7 subunit relative to Day 7 control (=100%); n = 6 cultures. In some experiments, L-type Ca^{2+} channel blocker verapamil (5 μM) was added with high K^+ (n = 3). Each reaction, in this figure and in Figures 3, 4, and 6, contained 0.5 μg of total cellular RNA and 200,000 cpm for each riboprobe.

Table 1. Effects of membrane depolarization, Ca^{2+} influx, and protein kinase activity on nAChR expression

	40 mM KCl									
NAChR transcript	(6)	Nifedipine (5 μM; 6)	K-252a (1 μM; 3)	KT-5926 (1 μM; 3)	KN-62 (4 μM; 6)	Chelerythrine (2 μM; 3)	H-89 (1 μM; 3)	Genistein (10 μM; 3)	PMA (100 nM; 3)	Forskolin (10 μM; 3)
α_3	104 ± 5	105 ± 2	95 ± 8	107 ± 10	117 ± 7	111 ± 10	117 ± 10	103 ± 7	107 ± 7	108 ± 10
α_5	120 ± 15	120 ± 4	ND	ND	99 ± 17	116 ± 15	110 ± 23	101 ± 20	120 ± 28	100 ± 20
α_7	256 ± 15*	98 ± 9	166 ± 15§	143 ± 17§	103 ± 4	272 ± 12*	257 ± 5*	250 ± 20*	112 ± 20	116 ± 15
β_2	109 ± 7	94 ± 5	100 ± 10	123 ± 16	106 ± 6	108 ± 7	106 ± 14	114 ± 12	94 ± 12	111 ± 8
β_4	101 ± 3	109 ± 5	ND	ND	97 ± 15	94 ± 12	104 ± 6	105 ± 9	98 ± 7	110 ± 15

Mean (±SEM) mRNA levels, measured with RNase protection assays, expressed relative to control neurons at day 7 (=100%). All treatments were done from day 5 to day 7. n is in parentheses. ND, Not determined.

*Significantly different from control neurons ($p < 0.001$).

§Significantly different from control neurons and high K^+-treated neurons ($p < 0.001$).

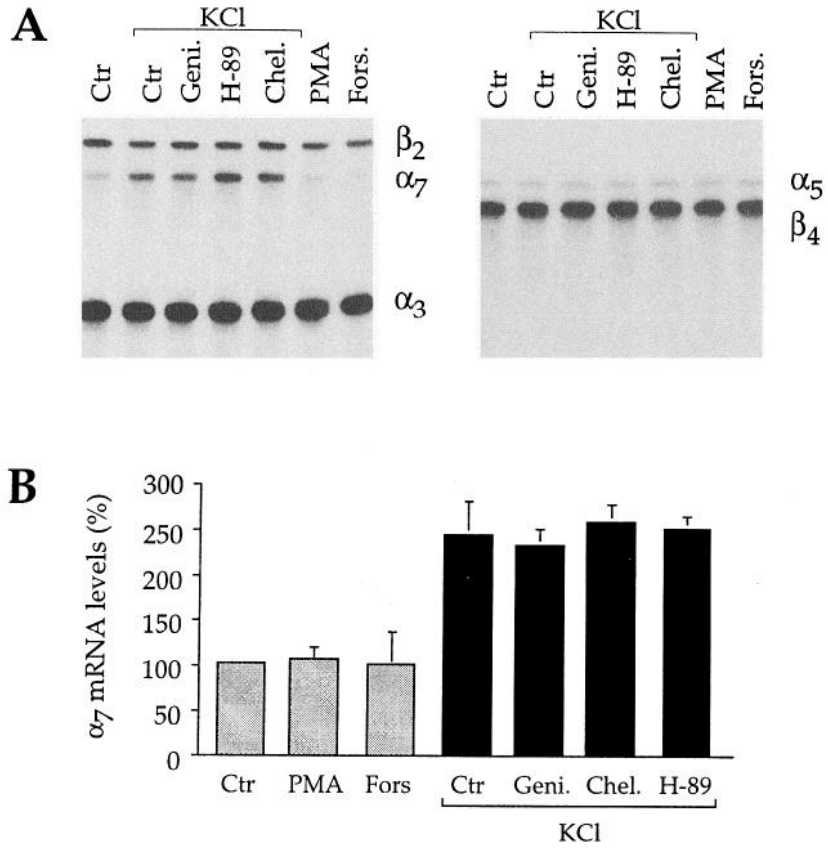

Figure 3. PKA, PKC, and tyrosine kinase do not regulate nAChR subunit expression. *A*, RNase protection assays performed as in Figure 2 on RNA extracted from SCG neurons cultured for 7 d in control medium (*Ctr*), or treated at day 5 with PKC activator PMA (100 nM), PKA activator forskolin (10 μM), or with 40 mM KCl with either 0.1% DMSO (*Ctr*), tyrosine kinase inhibitor genistein (10 μM), PKA inhibitor H-89 (2 μM) or PKC inhibitor chelerythrine (2 μM). In addition, PMA, forskolin, and NGF were added to some high K^+ cultures (not shown). None of these agents affected nAChR subunit expression (see also Table 1). *B*, Mean (±SEM) mRNA levels for α_7 subunit relative to control (=100%); $n = 3$ cultures. Higher doses of these agents were also tested: genistein, 100 μM; H-89, 10 μM; chelerythrin, 20 μM; PMA, 300 nM; forskolin 30 μM; no significant differences on the nAChR subunit mRNA levels were observed at these doses ($n = 2$).

by SCG neurons developing in culture. However, we did observe that higher concentrations of K-252a and KT-5926 (1 μM) partially blocked the α_7 induction by high K^+ (Table 1); these agents at higher concentrations inhibit a number of protein kinases, including CaM kinase II (Hashimoto et al., 1991).

CaM kinase activity upregulates α_7 gene expression

To test whether CaM kinase activity is involved in the induction of α_7 gene expression by membrane depolarization, we treated control and high K^+ cultures with KN-62. KN-62 is a highly specific CaM kinase inhibitor that competes with calmodulin for its binding site on CaM kinase (Tokumistu et al., 1990). In control cultures, KN-62 (10 μM) for 48 hr had no effect on nAChR transcript levels (data not shown); however, when added to high K^+ cultures, KN-62 completely blocked the increase in α_7 mRNA levels (Fig. 4). The block by KN-62 is dose dependent with an IC_{50} of ~1.2 μM and full inhibition at 3–4 μM (Fig. 4*b*); this is consistent with the dose-dependent effects of KN-62 on purified CaM kinase activity *in vitro* (Tokumistu et al., 1990; Enslen et al., 1994), suggesting that the effects of KN-62 are specific for CaM kinase activity in SCG neurons. These above results indicate that CaM kinase activity is involved in the Ca^{2+} signaling cascade that regulates α_7 gene expression.

Lack of effects of KN-62 on L-type Ca^{2+} channels

Although it is unlikely that KN-62 produces its effects by inhibiting protein kinases other than CaM kinase (Tokumistu et al., 1990), a potential difficulty in the interpretation the above results could arise if, in addition to its inhibition of CaM kinase activity, KN-62 also blocked Ca^{2+} influx through the DHP-sensitive L-type Ca^{2+} channels. Therefore, we investigated this possibility by measuring Ca^{2+} currents on SCG neurons with whole-cell voltage clamp techniques. To distinguish Ca^{2+} currents flowing through L-type Ca^{2+} channels from those flowing through other Ca^{2+} channels we focused on the tail currents; several studies have shown that the Ca^{2+} tail current recorded at the end of a positive voltage step from a depolarized holding potential is due largely to DHP-sensitive L-type Ca^{2+} channels (Plummer et al., 1989; Regan et al., 1991; Mathie et al., 1992), and can be prolonged by DHP analogues, such as Bay K 8644 or (+)-202-791 (Hess et al., 1984; Plummer et al., 1989). Figure 5*a* shows an example of Ca^{2+} currents recorded from a neuron in the presence of TTX (1 μM), TEA (10 mM), and 4-AP (2 mM) to block voltage-dependent Na^+ and K^+ currents. Tail currents were evoked by a two step voltage protocol: first we held the neurons at −30 mV to partially inactivate non L-type Ca^{2+} channels, and then we applied a 150 msec depolarizing voltage step to +30 mV, followed by a repolarizating step to −40 mV. This resulted in a large Ca^{2+} tail current (Fig. 5*a*, left) which was prolonged two- to threefold in the presence of 1 μM (+)-202-791 (Fig. 5*a*, right), indicating that much of the tail current was due to the deactivation of L-type Ca^{2+} channels. In addition, as shown in Figure 5*b*, these tail currents were blocked by the DHP-antagonist, nifedipine (5 μM), but were unaffected by ω-conotoxin GV1A (ω-CgTx; 5 μM), a specific blocker of N-type Ca^{2+} channels (Plummer et al., 1989; Regan et al., 1991).

To test whether KN-62 affects L-type Ca^{2+} channels, we recorded Ca^{2+} tail currents from neurons treated with 4 μM KN-62 both acutely and for 24 hr prior to recording. Figure 5*b* shows that KN-62 had no significant effect on L-type Ca^{2+} channels on these neurons. Therefore, we conclude that the inhibition by

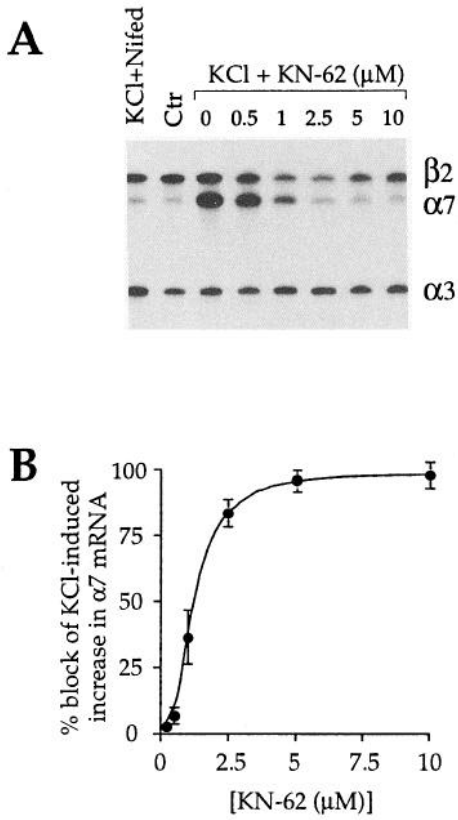

Figure 4. CaM kinase activity mediates the induction of α_7 nAChR expression by membrane depolarization. *A*, RNase protection assay performed as in Figure 2 on RNA extracted from neurons cultured for 7 d. High K^+ was added at day 5, either with 5 μM nifedipine, 0.1% DMSO (0), or with 0.5 to 10 μM KN-62, a CaM kinase inhibitor. In this experiment, α_3 riboprobe was added at 100 000 cpm/reaction. *B*, KN-62 inhibits high K^+ induction of α_7 expression in a dose dependent manner with an IC_{50} of ~1.2 μM and full inhibition at 3–4 μM. The means (±SEM) were obtained from three cultures.

KN-62 on α_7 gene expression after high K^+ is due most likely to its inhibitory action on CaM kinase activity.

It had been reported, however, that KN-62 may reduce Ca^{2+} influx in other preparations (Li et al., 1992). To investigate whether this could occur through other classes of Ca^{2+} channels, we recorded from neurons at more negative holding potentials (−60 mV) to remove inactivation of non-L-type Ca^{2+} currents. Figure 5*c* shows that ω-CgTx (5 μM) blocks a large portion of these Ca^{2+} currents, without any significant effect on the tail currents. We observed similar results in neurons treated with 4 μM KN-62; furthermore, even concentration as low as 0.2 μM, which had little effect on α_7 expression (Fig. 4), also blocked these currents. Nifedipine (5 μM), on the other hand, affected only a small fraction of the current elicited by the depolarizing step, and blocked most of the tail current (Fig. 5*c*), consistent with L-type Ca^{2+} currents representing only a small proportion of the total Ca^{2+} currents on rat SCG neurons (Plummer et al., 1989; Regan et al., 1991). Therefore, it appears that KN-62 can inhibit non-L-type Ca^{2+} channels.

Choline induces α_7 expression

Several studies have shown that activation of nAChRs increases intracellular Ca^{2+} concentrations, either by depolarizing neurons and activating voltage-depende Ca^{2+} channels, or by Ca^{2+} influx through nAChRs directly (Mulle et al., 1992; Vernino et al., 1992, 1994; Vijayaraghavan et al., 1992; Trouslard et al., 1993; Rathouz and Berg, 1994). Therefore, we investigated whether application of nicotinic agonists to cultured SCG neurons, either at day 0 or day 5, would increase α_7 transcript levels. We found that neither the addition of nicotine (10–50 μM) nor carbachol (10–100 μM) for 48 hr had any effect on the mRNA levels of the five nAChR subunits present in these neurons (data not shown), possibly because these long agonist applications desensitize the receptors. It has been shown that choline, a byproduct of ACh degradation and a weak nicotinic agonist, increases intracellular Ca^{2+} in SCG neurons by releasing Ca^{2+} from internal stores (Koike et al., 1989). To test whether elevated extracellular choline leads to an increase in α_7 expression, we applied 5 mM choline to cultured neurons for 48 hr at day 5. Choline caused a moderate increase in α_7 mRNA levels (Fig. 6); the other four nAChR transcript levels were not affected. This choline-mediated increase in α_7 mRNA was prevented by KN-62 (4 μM; Fig. 6), but was unaffected by nifedipine (5 μM; Fig. 6), hexamethonium (100 μM), atropine (2 μM) or α-BTX (100 nM) (data not shown), consistent with choline causing Ca^{2+} release from internal stores.

Correlation between changes in α_7 mRNA levels and changes in α-BTX-nAChR numbers

As the α_7 gene codes for an α-BTX-nAChR (Schoepfer et al., 1990; Couturier et al., 1990a; Séguéla et al., 1993), we investigated whether the induction of α_7 gene expression by membrane depolarization in cultured SCG neurons results in an increase in the number of α-BTX binding sites on the surface of these neurons. Figure 7 shows that neurons exposed to high K^+ for 48 hr had three- to fivefold more surface ^{125}I-α-BTX binding than control neurons. These results demonstrate a good correlation between increases in α_7 mRNA levels and α-BTX-nAChRs surface density.

Relationship between nAChR mRNA levels and ACh-evoked current densities

To investigate whether changes in nAChR transcript levels (Fig. 1) affect the magnitude and/or kinetics of the macroscopic ACh-evoked currents, we measured ACh-evoked current densities on these neurons with whole-cell voltage-clamp techniques. For these measurements, we applied the agonists with a fast perfusion system (see Materials and Methods) to avoid underestimating any rapidly desensitizing components.

α_7. Figure 8*a* shows examples of ACh-evoked currents from a control neuron in culture for 7 d, and from a neuron in culture for the same time but exposed to 40 mM KCl for 48 hr starting at day 5. We observed no significant differences in the magnitudes of the ACh-evoked current densities between the two groups (Fig. 8*b*). In addition, we did not observe any rapidly desensitizing current components from either control neurons or neurons treated with high K^+, nor did we observe any significant difference in the time course of the ACh-evoked currents. Furthermore, these ACh-evoked currents were not reduced by pretreating the neurons with α-BTX (500 nM) for 2–3 hr prior to the recordings (data not shown; Mandelzys et al., 1995). Thus, in spite of the two- to threefold difference in α_7 transcripts between these two groups and the three- to fivefold difference in surface α-BTX-nAChRs, there are no differences in the density, time course or α-BTX sensitivity of the ACh-evoked currents. These results suggest that the α_7 gene product has little or no

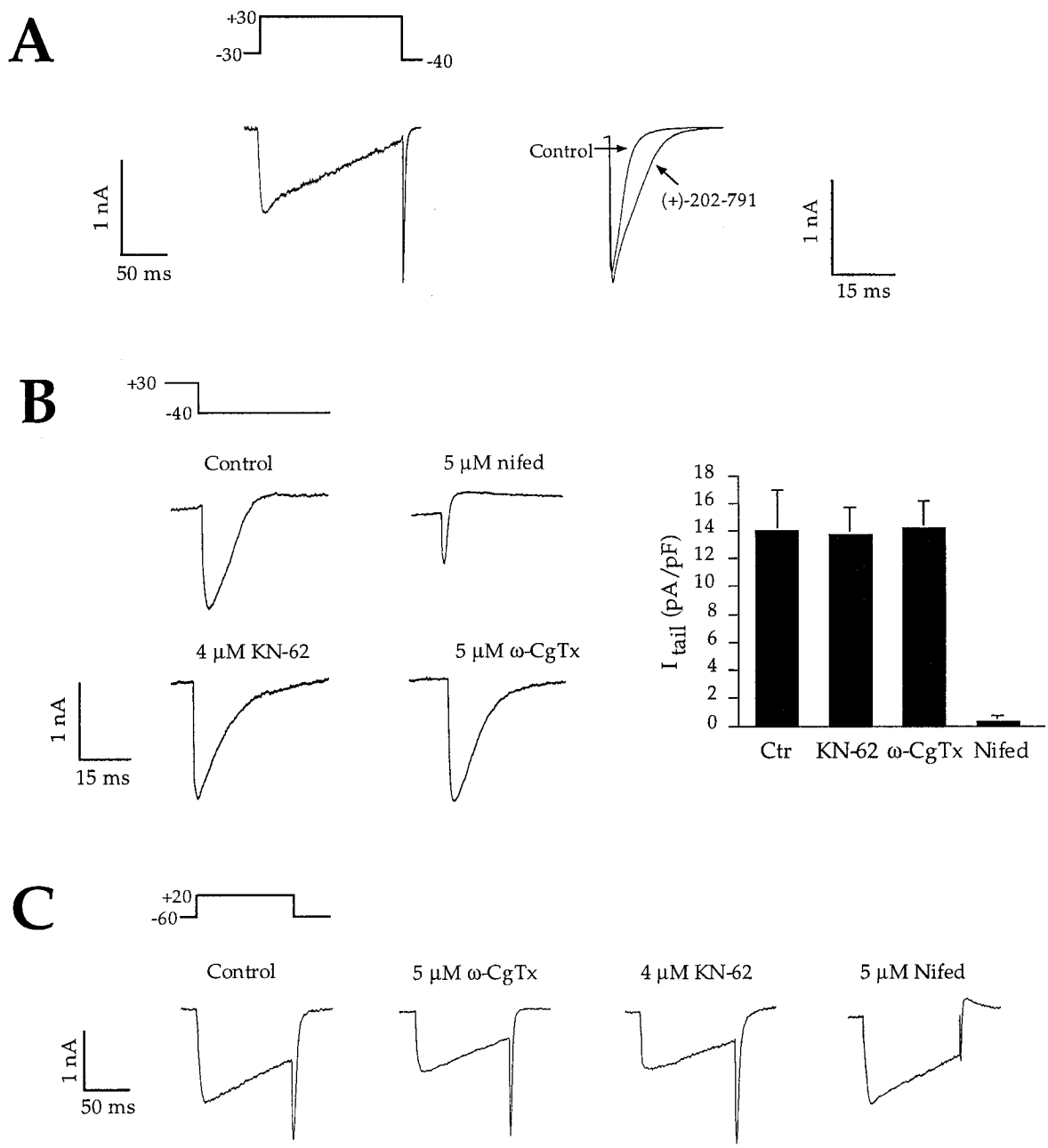

Figure 5. Effects of KN-62 on Ca^{2+} currents recorded with whole-cell voltage clamp. Leak-subtracted Ca^{2+} currents recorded in presence of 1 μM TTX, 10 mM TEA, and 2 mM 4-AP. *A*, The neuron was held at −30 mV to partially inactivate non L-type Ca^{2+} currents and a depolarizing step to +30 mV was applied for 150 msec. Repolarization to −40 mV at the end of the step evoked a Ca^{2+} tail current. This tail current is prolonged by DHP-agonist (+)-202-791 (1 μM; *right trace*). *B*, Tail currents elicited as in *A* from a neuron in control medium or in presence of either 5 μM nifedipine, 5 μM ω-CgTx, or 4 μM KN-62. Nifedipine blocks the tail current, whereas ω-CgTx and KN-62 do not. The histogram shows the mean tail current densities (±SEM) from six neurons in each condition, obtained by measuring the amplitude of the tail currents at 3 msec after repolarization and dividing by whole-cell capacitance. *C*, The neurons were held at −60 mV to remove inactivation of transient Ca^{2+} currents and depolarization step to +20 mV were applied for 125 msec. At least 50% of the current was blocked by ω-CgTx (5 μM); a similar blockade was observed with KN-62 (4 μM). This effect of KN-62 was observed with concentrations as low as 0.2 μM (data not shown). As in *B*, the tail currents were unaffected by both drugs. Nifedipine (5 μM) had a small effect on the Ca^{2+} currents during the step, while eliminating most of the tail current. All drugs were applied as in *B*).

detectable effect on the macroscopic ACh-gated currents on these neurons.

α_3. In contrast, we measured a two- to threefold increase in the ACh-evoked current densities on control neurons from day 0 to day 7 (Fig. 8*b*). This increase parallels the increase in α_3 mRNA levels over the same period (see Fig. 1). In addition, in neurons treated with high K^+ for 3 d starting from day 0, both the α_3 mRNA levels and the ACh-evoked current densities were significantly greater than in control neurons (see Figs. 2, 8). These results indicate that increases in α_3 mRNA levels correlate with increases in ACh-evoked current densities in SCG neurons developing in culture. A similar correlation also exists for these neurons during postnatal development *in vivo* (Mandelzys et al., 1994).

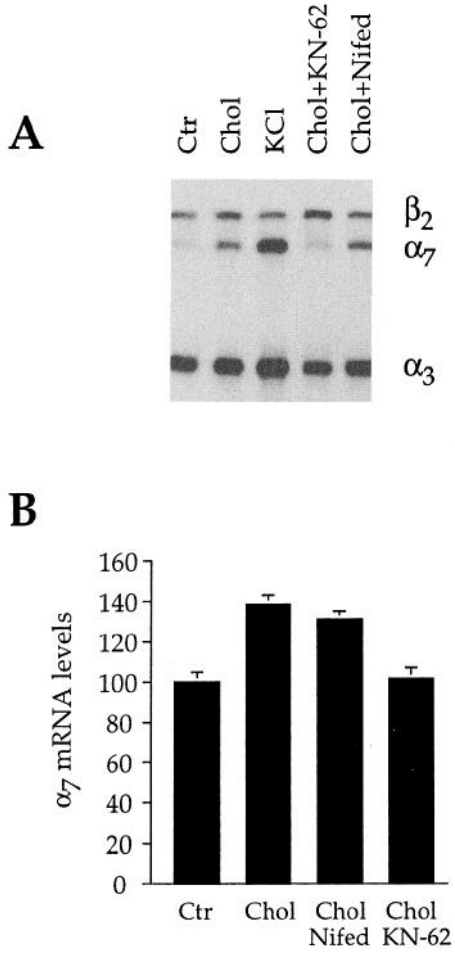

Figure 6. Choline induces α_7 expression. RNase protection assay on RNA extracted from SCG neurons cultured for 7 d. At day 5, additional 35 mM KCl, 5 mM choline (*Chol*), or 5 mM choline with either KN-62 (4 μM) or nifedipine (5 μM) were added to sister cultures. Choline caused a small increase in α_7 mRNA levels without affecting the levels of α_3 and β_2; β_4 and α_5 were also unaffected (data not shown). The choline effect was not blocked by nifedipine, but was blocked by KN-62. Mean (±SEM) mRNA levels for α_7 subunit expressed relative to control (=100%); $n = 4$ cultures.

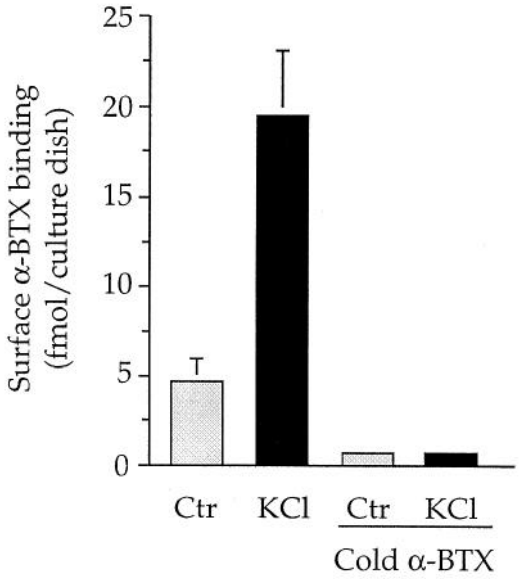

Figure 7. Membrane depolarization increases surface α-BTX binding on SCG neurons. ^{125}I-α-BTX surface binding was performed on neurons cultured for 7 d in control condition or after 48 hr with high K^+ cultures from day 5. Surface binding was increased three- to fivefold with high K^+. Only low levels of binding was detected when cells were pretreated with cold α-BTX (100 nM) for 90 min. Mean (±SEM) binding (fmol/dish) from three cultures.

Discussion

In this article, we have investigated factors that influence nAChR expression by neonatal rat SCG neurons developing in culture. A novel aspect of this study is that it links neuronal activity with neurotransmitter receptor gene expression through a CaM kinase pathway.

Role of CaM kinase activity on α_7 subunit expression

To demonstrate that CaM kinase activity is essential for the induction of the α_7 gene, we used a potent and selective CaM kinase inhibitor, KN-62 (Tokumitsu et al., 1990). Several studies have addressed the specificity of KN-62. Tokumitsu et al. (1990) showed that KN-62 competes with calmodulin for its binding site on CaM kinase, but does not interact with calmodulin directly. *In vitro*, KN-62 inhibits CaM kinase autophosphorylation with an IC_{50} of ~1.0 μM (Tokumitsu et al., 1990) and inhibits phosphorylation of a purified substrate by CaM kinase with an IC_{50} of 1–2 μM (Enslen et al., 1994). In contrast, 100-fold higher concentrations have little effect on calmodulin-dependent kinases, such as myosin light chain kinase, phosphodiesterase, or on PKA or PKC (Tokumitsu et al., 1990). We found that KN-62 inhibits the increase in α_7 expression induced by membrane depolarization in a dose-dependent manner with an IC_{50} of ~1.2 μM and full inhibition at 3–4 μM; this tight correspondence between these values and those for the action of KN-62 on CaM kinase *in vitro* (Tokumitsu et al., 1990; Enslen et al., 1994) strongly suggests that KN-62 acts to inhibit CaM kinase in SCG neurons.

The induction of α_7 expression by membrane depolarization is due to an influx of Ca^{2+} through L-type Ca^{2+} channels. One concern was whether the effects of KN-62 were due, in part, to inhibition of Ca^{2+} influx, possibly by interfering with L-type Ca^{2+} channels directly; this possibility has also been addressed by others (Li et al., 1992; Hack et al., 1993; Wyllie and Nicoll, 1994). However, by measuring Ca^{2+} tail currents, we showed that 4 μM KN-62 has no effect on L-type Ca^{2+} currents in SCG neurons. Our results are consistent with those of Hack et al. (1993) who showed that KN-62 (2.5–10 μM) had no effect on intracellular $^{45}Ca^{2+}$ levels in rat cerebellar granule cells grown in culture with high K^+ for 1 week.

Our finding that Ca^{2+} influx through L-type Ca^{2+} channels is essential for high K^+ induction of α_7 expression is consistent with the sustained mode of depolarization used in our experiments. In fact, the sustained elevation in intracellular Ca^{2+} in rat SCG neurons with high K^+ is generated only by the activation of DHP sensitive L-type Ca^{2+} channels, and not by ω-CgTx-sensitive N-type Ca^{2+} channels (Franklin and Johnson, 1992; Franklin et al., 1995). Moreover, several studies have implicated L-type Ca^{2+} channels in regulating gene expression in neurons (Murphy et al., 1991; Bading et al., 1993; Bessho et al., 1994; Rosen et al., 1994).

We have not determined the signaling events downstream of CaM kinase that are responsible for the induction of α_7 expres-

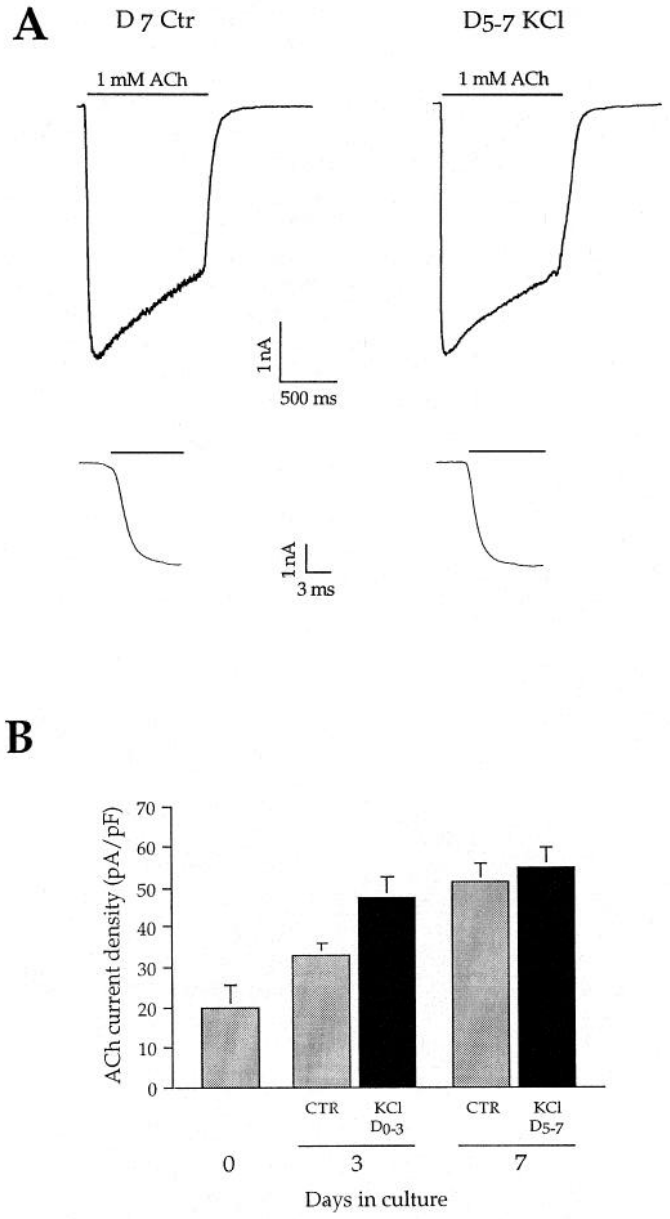

Figure 8 ACh-evoked current densities correlate with changes in α_3 but not α_7 transcript levels. Whole-cell voltage-clamp recording from freshly plated neurons (3–16 hr; $n = 10$), or neurons that have developed for 3 ($n = 12$) and 7 ($n = 12$) d in control culture. Some cultures were treated with high K^+ either at the time of plating ($n = 12$) or at day 5 ($n = 10$). *A* Representative currents evoked by fast application of 1 mM ACh. *Lower traces* show the first 15 msec of the response on a faster time scale to illustrate the speed of activation. ACh-evoked currents usually reached 90% of maximal value within 5–10 msec. No rapidly desensitizating components were observed in the currents either in control- or high K^+-treated neurons at any time in culture or from freshly dissociated neurons. *B*, Mean (±SEM) ACh-evoked current densities increase over time in culture at a rate similar to the increase in α_3 mRNA (see Fig. 1). No significant differences between 7 d old control neurons and neurons treated with high K^+ for 48 hr at day 5. Neurons treated with high K^+ at the time of plating have significantly larger ACh-evoked current densities than control neurons ($p < 0.005$), similar to the differences seen in α_3 mRNA levels.

sion. Our data using α-amanitin suggest that the changes in α_7 mRNA levels result from altered mRNA synthesis rather than from a change in mRNA stability: the approximate rate of decay in α_7 mRNA was unaltered by α-amanitin, and was comparable to the decay of other nAChR transcripts in neurons treated with α-amanitin, although we cannot exclude the possibility that α-amanitin blocked transcription of a gene(s) coding for a protein(s) that preferentially destabilizes α_7 mRNA. From the relatively slow time course for α_7 mRNA to plateau after treatment with high K^+ (greater than 24 hr), it seems likely that transcription of other genes, such as immediate early genes, precedes α_7 expression (Bading et al., 1993; Enslen and Soderling, 1994; Ghosh and Greenberg, 1995). If so, the α_7 gene may represent a good model in which to investigate the link between immediate early gene expression and the expression of delayed response genes.

Several types of CaM kinases have been characterized and recently it has been shown that, in addition to CaM kinase II (Tokumitsu et al., 1990), similar concentrations of KN-62 inhibit CaM kinase IV and V (Enslen et al., 1994; Mochizuki et al., 1993). While little is known about the function and distribution of CaM kinase V, CaM kinase IV has been shown to target to the nucleus (Jensen et al., 1991) suggesting that it has a role in regulating gene expression (Enslen et al., 1994). However, PKA activity has been shown to inhibit CaM kinase IV activity (Kameshita et al., 1991), whereas neither activation nor inhibition of PKA has any effect on α_7 expression in SCG neurons making it less likely that CaM kinase IV is involved. On the other hand, the biochemical properties and cellular distribution of CaM kinase II make it a more attractive candidate for mediating the effects of activity on α_7 expression (Hanson et al., 1994; reviewed by Hanson and Schulman, 1992; Schulman, 1993). CaM kinase II is involved in long-term modifications of synaptic transmission and has been implicated in mechanisms that underlie certain forms of memory, and several experiments have demonstrated that CaM kinase II can modulate synaptic transmission through posttranslation modification of neurotransmitter receptors, ion channels, and by affecting neurotransmitter release (reviewed by Hanson and Schulman, 1992; Schulman, 1993; Lisman, 1994). However, CaM kinase II can also phosphorylate transcription factors (Wegner et al., 1992), regulate the expression of immediate early genes (Bading et al., 1993; Enslen and Soderling, 1994; Ghosh and Greenberg, 1995), and target to the nucleus (Srinivasan et al., 1994). Our finding that activity regulates the expression of a neurotransmitter receptor subunit through a CaM kinase pathway suggest another mechanism that could underlie some forms of synaptic plasticity.

Effects of choline on α_7 expression

Nicotinic agonists, nicotine and carbachol, did not mimic the effects of high K^+ presumably because nAChRs desensitize. However, we observed that choline produced a small but significant increase in α_7 expression. Choline has been shown to cause an increase in intracellular Ca^{2+} in SCG neurons by releasing Ca^{2+} from internal stores (Koike et al., 1989). Consistent with this, we found that the choline effect on α_7 expression was not blocked by nifedipine, hexamethonium, α-BTX, or atropine; however, it was blocked by KN-62, suggesting that CaM kinase-induced expression of α_7 can be activated by Ca^{2+} released from internal stores. However, further studies are needed to confirm this point.

Expression of α_3, α_5, β_2, and β_4 nAChR transcripts

An interesting aspect of the effect of membrane depolarization on nAChR expression is that it is specific for the α_7 gene. The regulation of α_3, α_5, β_2, and β_4 in cultured SCG neurons was similar to that of SCG neurons *in vivo*, suggesting that factors other than innervation are important for nAChR expression (Mandelzys et al., 1994; Schwartz Levey et al., 1995). One possibility is that intrinsic mechanisms related to their neuronal phenotype regulate these transcripts. This latter possibility is consistent with the results of Zoli et al. (1995) showing that nAChR transcripts appear early in neurogenesis. Moreover, treating neurons with activators or inhibitors of PKA, PKC, or tyrosine kinases produced little change in the mRNA levels of these four subunits, although it was difficult to determine to what extent the activities of these kinases were altered by these treaments. Furthermore, while the tyrosine kinase inhibitors used in this study would have blocked *trk*A-related tyrosine kinases, it would not have blocked all subtypes of these enzymes.

Transcript levels for both β_2 and β_4 changed little in these neurons, even if denervated *in vivo* (Mandelzys et al., 1994), or depolarized in culture. β_2 is widely expressed in the nervous system and appears to be under little developmental regulation (Hill et al., 1993; Mandelzys et al., 1994; Zoli et al., 1995), except in the chick optic tectum (Matter et al., 1990). The β_4 gene is located upstream of the α_3 gene in the same cluster (Boulter et al., 1990; Couturier et al., 1990b), and their initial expression appears to be coordinately regulated during neurogenesis (Zoli et al., 1995). The promoter for α_3, which lies in the intervening 2 kb segment between β_4 and α_3 (Boyd 1994; Yang et al., 1994), could allow for specific upregulation of the α_3 gene as these neurons develop further. The α_5 gene is part of this same cluster but oriented in the opposite direction (Boulter et al., 1990; Couturier et al., 1990b), suggesting that its regulation is distinct from β_4 and α_3. Of the five transcripts present in SCG neurons, α_5 is expressed at the lowest levels.

Relationship between nAChR transcript levels, ACh-evoked currents, and α-BTX-nAChRs

The increase in α_3 mRNA levels in cultured SCG neurons correlates well with the increase in ACh-evoked currents. A similar correlation exists *in vivo* (Mandelzys et al., 1994). As none of the other nAChR transcripts increase significantly over this period, these findings emphasize the importance of α_3 gene expression for the appearance of functional nAChRs on these neurons. As it is likely that these receptors are pentameters composed of 2 α and 3 β subunits (Anand et al., 1991; Cooper et al., 1991), our results suggest that α_3 is rate-limiting for the appearance of functional receptors. Presumably, SCG neurons have an excess pool of β subunit in the cytoplasm (see Hill et al., 1993) and that newly expressed α_3 subunits recruit β subunits from this pool to coassemble into functional receptors. Consistent with this possibility, a large pool of intracellular nAChR-immunoreactivity has been described in chick ciliary neurons (Jacob et al. 1986).

In contrast to α_3, the levels of α_7 mRNA decrease threefold in culture, suggesting that the α_7 subunit does not contribute significantly to the macroscopic ACh-evoked currents in rat SCG neurons. In addition, increasing α_7 mRNA levels by treating neurons with high K^+ for 48 hr at day 5 had no effect on the magnitude, kinetics, or α-BTX sensitivity of the ACh-evoked currents. In addition, in none of these neurons did we detect any rapidly desensitizing currents (see also Mandelzys et al., 1995). Our results contrast those of chick ciliary neurons which also contain significant levels of α_7 mRNA (Corriveau and Berg, 1993) and α-BTX binding sites (Jacob and Berg, 1983; Loring et al., 1985), but do have rapidly desensitizing ACh-evoked currents that are blocked by α-BTX (Zhang et al., 1994). The reasons for the difference in α-BTX-sensitive currents between chick and rat autonomic neurons are unclear (Zhang et al., 1994; Mandelzys et al., 1995). In chick ciliary neurons, α_7 does not associate with any of the known nAChR subunits (Vernallis et al., 1993), but possibly associates with other unidentified proteins. In fact, there is evidence to indicate that α_7 can coassemble with other subunits to form nAChRs (Schoepfer et al., 1990; Listerud et al., 1991; Helekar et al., 1994). Conceivably, by coassembling with different proteins, or by undergoing differential posttranslational modifications (Helekar et al., 1994), α_7 may assemble into receptors with different functions.

Nonetheless, we observed that treating neurons with high K^+ increases surface binding of α-BTX; this correlates well with the changes seen in α_7 mRNA levels in these neurons and is consistent with the α_7 gene coding for an α-BTX-nAChR (Couturier et al., 1990a; Schoepfer et al., 1990; Séguéla et al., 1993). The role of the α-BTX-nAChR on SCG neurons, however, remains unclear. α_7's regulation by activity in SCG neurons shown here, its regulation during synapse formation on embryonic muscle (Corriveau et al., 1995), and its high permeability to Ca^{2+} (Séguéla et al., 1993; Vijayaraghavan et al., 1992; Pugh and Berg, 1994; Castro and Albuquerque, 1995) suggest a role for this receptor in events related to synaptogenesis. Further work is needed to determine whether α-BTX-nAChRs have a role in synapse formation on rat SCG neurons.

References

Anand R, Conroy WG, Schoepfer R, Lindstrom J (1991) Neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes have a pentameric quaternary structure. J Biol Chem 266:11192–11198.

Arenella LS, Oliva JM, Jacob MH (1993) Reduced levels of acetylcholine receptor expression in chick ciliary ganglion neurons developing in the absence of innervation. J Neurosci 13:4525–4537.

Bading H, Ginty DD, Greenberg ME (1993) Regulation of gene expression in hippocampal neurons by distinct calcium signaling pathways. Science 260:181–186.

Berg DK, Boyd RT, Halvorsen SW, Higgins LS, Jacob MH, Margiotta JF (1989) Regulating the number and function of neuronal acetylcholine receptors. Trends Neurosci 12:16–21.

Berg MM, Sternberg DW, Parada LF, Chao MV (1992) K-252a inhibits nerve growth factor-induced trk proto-oncogene tyrosine phosphorylation and kinase activity. J Biol Chem 267:13–16.

Bessho Y, Nawa H, Nakanishi S (1994) Selective up-regulation of an NMDA receptor subunit mRNA in cultured cerebellar granule cells by K^+-induced depolarization and NMDA treatment. Neuron 12:87–95.

Boulter J, O'Shea-Greenfield M, Duvoisin RM, Connolly JG, Wada E, Jensen A, Gardner PD, Ballivet M, Deneris ES, McKinnon D, Heinemann S, Patrick J (1990) α3, α5, and β4: three members of the rat neuronal nicotinic acetylycholine receptor-related gene family form a gene cluster. J Biol Chem 265:4472–4482.

Boyd TR (1994) Sequencing and promoter analysis of the genomic region between the rat neuronal nicotinic acetylcholine receptor β4 and α3 genes. J Neurobiol 25:960–973.

Castro NG, Albuquerque EX (1995) α-Bungarotoxin-sensitive hippocampal nicotinic receptor channel has a high calcium permeability. Biophys J 68:516–524.

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Clarke PBS (1992) The fall and rise of neuronal α-bungarotoxin binding protein Trends Pharmacol 131:407–413.

Cooper E, Couturier S, Ballivet M (1991) Pentameric structure and subunit stoichiometry of a neuronal acetylcholine receptor. Nature 350:235–238.

Corriveau RA, Berg DK (1993) Coexpression of multiple acetylcholine receptor genes in neurons: quantification of transcripts during development. J Neurosci 13:2662–2671.

Corriveau RA, Romano SJ, Conroy WG, Oliva L, Berg DK (1995) Expression of neuronal acetylcholine receptor genes in vertebrate skeletal muscle during development J Neurosci 15:1372–1383.

Couturier S, Bertrand D, Matter J-M, Hernandez M-C, Bertrand S, Millar N, Valera S, Barkas T, Ballivet M (1990a) A neuronal nicotinic acetylcholine receptor subunit (α_7) is developmentally regulated and forms a homo-oligomeric channel blocked by α-Btx. Neuron 5:847–856.

Couturier S, Erkman L, Valera S, Rungger D, Bertrand, S, Boulter, J, Ballivet M, Bertrand D (1990b) α5, α3 and non-α 3: three clustered avian genes encoding neuronal nicotinic acetylcholine receptors-related subunits. J Biol Chem 265:17560–17567.

Devay P, Qu X, Role LW (1994) Regulation of nAChR subunit gene expression relative to the development of pre- and postsynaptic projections of embryonic chick sympathetic neurons. Dev Biol 162:56–70.

Engisch KL, Fischbach GD (1990) The development of ACh- and GABA-activated currents in normal and target-deprived embryonic chick ciliary ganglia. Dev Biol 139:417–426.

Engisch KL, Fischbach GD (1992) The development of ACh- and GABA-activated currents in embryonic chick ciliary ganglion neurons in the absence of innervation *in vivo*. J Neurosci 12:1115–1125.

Enslen H, Soderling TR (1994) Roles of calmodulin-dependent protein kinase and phosphatase in calcium-dependent transcription of immediate early genes. J Biol Chem 269:20872–20877.

Enslen H, Sun P, Brickey D, Soderling S, Klamo E, Soderling TR (1994) Characterization of Ca^{2+}/calmodulin-dependent protein kinase IV. J Biol Chem 269:15520–15527.

Franklin JL, Johnson EM (1992) Suppression of programmed neuronal death by sustained elevation of cytoplasmic calcium. Trends Neurosci 15:501–508.

Franklin JL, Sanz-Rodriguez C, Juhasz A, Deckwerth L, Johnson EM Jr (1995) Chronic depolarization prevents programmed death of sympathetic neurons in vitro but does not support growth: Requirement for Ca^{2+} influx but not trk activation. J Neurosci 15:643–664.

Fumagalli L, De Renzis G, Miani N (1976) Acetylcholine receptors: Number and distribution in intact and deafferented superior cervical ganglion of the rat. J Neurochem 27:47–52.

Ghosh A, Greenberg ME (1995) Calcium signaling in neurons: molecular mechanisms and cellular consequences. Science 268:239–247.

Hack N, Hidaka H, Wakefield MJ, Balazs R (1993) Promotion of granule cell survival by high K^+ or excitatory amino acid treatment and Ca^{2+}/calmodulin-dependent protein kinase activity. Neuroscience 57: 9–20.

Hanson PI, Schulman H (1992) Neuronal Ca^{2+}/calmodulin-dependent protein kinases. Annu Rev Biochem 61:559–601.

Hanson PI, Meyer T, Stryer L, Schulman H (1994) Dual role of calmodulin in autophosphorylation of multifunctional CaM kinase may underlie decoding of calcium signals. Neuron 12:943–956.

Hashimoto Y, Nakayama T, Teramoto T, Kato H, Watanabe T, Kinoshita M, Tsukamoto K, Tokunaga K, Kurokawa K, Nakanishi S, Matsuda Y, Nonomura Y (1991) Potent and preferential inhibition of Ca^{2+}/calmodulin-dependent protein kinase II by K252a and its derivative, KT 5926. Biochem Biophys Res Commun 181:423–429.

Hawrot E, Patterson PH (1979) Long-term cultures of dissociated sympathetic neurons. Methods Enzymol 58:574–584.

Helekar SA, Char D, Neff S, Patrick J (1994) Prolyl isomerase requirement for the expression of functional homo-oligomeric ligand-gated ion channels. Neuron 12:179–189.

Hess P, Lansman JB, Tsien RW (1984) Different modes of Ca channel gating behavior favoured by dihydropyridine Ca agonists and antagonists. Nature 311:538–544.

Hill JA, Zoli M, Bourgeois JP, Changeuz JP (1993) Immunocytochemical localization of a neuronal nicotinic receptor: the β2 subunit. J Neurosci 13:1551–1568.

Jacob MH, Berg DK (1983) The ultrastructural localization of α-bungarotoxin binding sites in relation to synapses on chick ciliary ganglion neurons. J Neurosci 3:260–271.

Jacob MH, Berg DK (1987) Effects of preganglionic denervation and postganglionic axotomy on acetylcholine receptors in the chick ciliary ganglion. J Cell Biol 105:1847–1854.

Jacob MH, Lindstrom JM, Berg DK (1986) Surface and intracellular distribution of a putative neuronal nicotinic acetylcholine. J Cell Biol 103:205–214.

Jensen KF, Ohmstede C-A, Fisher RS, Sahyoun N (1991) Nuclear and axonal localization of Ca^{2+}/calmodulin-dependent protein kinase type Gr in rat cerebellar cortex. Proc Natl Acad Sci USA 88:2850–2853.

Kameshita I, Fujisawa H (1991) Phosphorylation and functional modification of calmodulin-dependent protein kinase IV by cAMP-dependent protein kinase. Biochem Biophys Res Commun 180:191–196.

Klarsfeld A, Laufer R, Fontaine B, Devillers-Thiéry A, Dubreuil C, Changeux J-P (1989) Regulation of muscle AChR α subunit gene expression by electrical activity: involvement of protein kinase C and Ca^{2+}. Neuron 2:1229–1236.

Koike T, Martin DP, Johnson EM Jr (1989) Role of Ca^{2+} channels in the ability of membrane depolarization to prevent neuronal death induced by trophic-factor deprivation: evidence that levels of internal Ca^{2+} determine nerve growth factor dependence of sympathetic ganglion cells. Proc Natl Acad Sci USA 86:6421–6425.

Koizumi S, Contreras ML, Matsuda Y, Hama T, Lazarovici L, Guroff G (1988) K-252a: a specific inhibitor of the action of nerve growth factor on PC12 cells. J Neurosci 8:715–721.

Krieg PA, Melton DA (1987) *In vitro* RNA synthesis with SP6 polymerase. Methods Enzymol 155:397–415.

Li G, Hidaka H, Wollheim CB (1992) Inhibition of voltage-gated Ca^{2+} channels and insulin secretion in hit cells by the Ca^{2+}/calmodulin-dependent protein kinase II inhibitor KN-62: comparison with antagonists of calmodulin and L-type Ca^{2+} channels. Mol Pharmacol 42: 489–.

Lisman J (1994) The CaM kinase II hypothesis for the storage of synaptic memory. Trends Neurosci 17:406–412.

Listerud M, Brusssaard AB, Devay P, Colman DR, Role LW (1991) Functional contribution of neuronal AChR subunits revealed by antisense oligonucleotides. Science 254:1518–1521.

Loring RH, Dahm LM, Zigmond RE (1985) Localization of α-bungarotoxin binding sites in the ciliary ganglion of the embryonic chick: an autoradiographic study at the light and electron microscopic level. Neurosci 14:645–660.

Ma Y, Campenot RB, Miller FD (1992) Concentration-dependent regulation of neuronal gene expression by nerve growth factor. J Cell Biol 117:135–141.

Mandelzys A, Pié B, Deneris ES, Cooper E (1994) The developmental increase in ACh current densities on rat sympathetic neurons correlates with changes in nicotinic ACh receptor α-subunit gene expression and occurs independent of innervation. J Neurosci 14:2357–2364.

Mandelzys A, De Koninck P, Cooper E (1995) Agonist and toxin sensitivities of ACh-evoked currents on neurons expressing multiple nicotinic ACh receptor subunits. J Neurophysiol 74:1212–1221.

Mathie A, Bernheim L, Hille B (1992) Inhibition of N-type calcium channels by muscarinic receptor activation in rat sympathetic neurons. Neuron 8:907–914.

Matter J-M, Matter-Sadzinski L, Ballivet M (1990) Expression of neuronal nicotinic acetylcholine receptor genes in the developing chick visual system. EMBO J 9:1021–1026.

McEachern AE, Jacob MH, Berg DK (1989) Differential effects of nerve transection on the ACh and GABA receptors of chick ciliary ganglion neurons. J Neurosci 9:3899–3907.

McFarlane S, Cooper E (1992) Postnatal development of voltage-gated K currents on rat sympathetic neurons. J Neurophysiol 67:1291–1300.

McGehee DS, Role LW (1995) Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. Annu Rev Physiol 57:521–546.

Mochizuki H, Ito T, Hidaka H (1993) Purification and characterization of Ca^{2+}/calmodulin-dependent protein kinase V from rat cerebrum. J Biol Chem 268:9143–9147.

Moss BL, Role LW (1993) Enhanced ACh sensitivity is accompanied by changes in ACh receptor channel properties and segregation of ACh receptor subtypes on sympathetic neurons during innervation *in vivo*. J Neurosci 13:13–28.

Mulle C, Choquet D, Korn H, Changeux J-P (1992) Calcium influx

through nicotinic receptor in rat central neurons: its relevance to cellular regulation. Neuron 8:135–143.

Murphy TH, Worley PF, Baraban JM (1991) L-Type voltage-sensitive calcium channels mediate synaptic activation of immediate early genes. Neuron 7:625–635.

Plummer MR, Logothesis DE, Hess P (1989) Elementary properties and pharmacological sensitivities of calcium channels in mammalian peripheral neurons. Neuron 2:1453–1463.

Pugh PC, Berg DK (1994) Neuronal acetylcholine receptors that bind α-bungarotoxin mediate neurite retraction in a calcium-dependent manner. J Neurosci 14:889–896.

Rathouz MM, Berg D (1994) Synaptic-type acetylcholine receptors raise intracellular calcium levels in neurons by two mechanisms. J Neurosci 14:6935–6945.

Regan LJ, Sah DWY, Bean BP (1991) Ca^{2+} channels in rat central and peripheral neurons: high-threshold current resistant to dihydropyridine blockers and ω-conotoxin. Neuron 6:269–280.

Rosen LB, Ginty DD, Weber MJ, Greenberg ME (1994) Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of Ras. Neuron 12:1207–1221.

Rubin E (1985) Development of the rat superior cervical ganglion: initial stages of synapse formation. J Neurosci 5:697–704.

Sargent PB (1993) The diversity of neuronal nicotinic acetylcholine receptors. Annu Rev Neurosci 16:403–443.

Schoepfer R, Conroy WG, Whiting P, Gore M, Lindstrom J (1990) Brain α-bungarotoxin binding protein cDNAs and MAbs reveal subtypes of this branch of the ligand-gated ion channel gene superfamily. Neuron 5:35–48.

Schulman H (1993) The multifunctional Ca^{2+}/calmodulin-dependent protein kinases. Curr Opin Cell Biol 5:247–253.

Schwartz Levey M, Brumwell CL, Dryer SE, Jacob MH (1995) Innervation and target tissue interactions differentially regulate acetylcholine receptor subunit mRNA levels in developing neurons *in situ*. Neuron 14:153–162.

Séguéla P, Wadiche J, Dineley-Miller K, Dani J, Patrick JW (1993) Molecular cloning, functional properties, and distribution of rat brain α_7 nicotinic cation channel highly permeable to calcium. J Neurosci 13:596–604.

Smolen AJ, Raisman G (1980) Synapse formation in the rat superior cervical ganglion during normal development and after neonatal deafferentation. Brain Res 181:315–323.

Srinivasan M, Edman CF, Schulman H (1994) Alternative splicing introduces a nuclear localization signal that targets multifunctional CaM kinase to the nucleus. J Cell Biol 126:839–852.

Tokumitsu H, Chijiwa T, Hagiwara M, Mizuntani A, Terasawa M, Hidaka H (1990) KN-62, 1-[*N,O*-Bis(5-isoquinolinesulfonyl)-*N*-methyl-l-tyrosyl]-4-phenylpiperizine, a specific inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II. J Biol Chem 265:4315–4320.

Trouslard J, Marsh SJ, Brown DA (1993) Calcium entry through nicotinic receptor channels and calcium channels in cultured rat superior cervical ganglion cells. J Physiol (Lond) 468:53–71

Vernallis AB, Conroy WG, Berg DK (1993) Neurons assemble acetylcholine receptors with as many as three kinds of subunits while maintaining subunit segregation among receptor subtypes. Neuron 10:451–464.

Vernino S, Amador M, Luetje CW, Patrick J, Dani JA (1992) Calcium modulation and high calcium permeability of neuronal nicotinic acetylcholine receptors. Neuron 8:127–134.

Vernino S, Rogers M, Radcliffe KA, Dani JA (1994) Quantative measurement of calcium flux through muscle and neuronal nicotinic acetylcholine receptors. J Neurosci 14:5514–5524.

Vijayaraghavan S, Pugh PC, Zhang Z, Rathouz M.M, Berg DK (1992) Nicotinic receptors that bind α-bungarotoxin on neurons raise intracellular free calcium. Neuron 8:353–362.

Walke W, Staple J, Adams L, Gnedy M, Chahine K, Goldman D (1994) Calcium-dependent regulation of rat and chick muscle nicotinic acetylcholine receptor (nAChR) gene expression. J Biol Chem 269: 19447–19456.

Wegner M, Zhaodan C, Rosenfeld MG (1992) Calcium-regulated phosphorylation within the leucine zipper of C/EBPβ. Science 256:370–373.

Wyllie DJA, Nicoll RA (1994) A role for protein kinases and phosphatases in the Ca^{2+}-induced enhancement of hippocampal AMPA receptor-mediated synaptic responses. Neuron 13:635–643.

Yang X, McDonough J, Fyodorov D, Morris M, Wang F, Deneris ES (1994) Characterization of an acetylcholine receptor α3 gene promoter and its activation by the POU domain factor SCIP/Tst-1. J Biol Chem 269:10252–10264.

Zhang Z-w, Vijayaraghavan, S, Berg DK (1994) Neuronal acetylcholine receptors that bind α-bungarotoxin with high affinity function as ligand-gated ion channels. Neuron 12:167–177.

Zoli M, Le Novere M, Hill JA, Changeux JP (1995) Developmental regulation of nicotinic Ach receptor subunit mRNA in the rat central and peripheral nervous system. J Neurosci 15:1912–1939.