FEBS Letters 411 (1997) 32–38 FEBS 18748

Activation of α_7 nicotinic acetylcholine receptor promotes survival of spinal cord motoneurons

M.L. Messi[a], M. Renganathan[b], E. Grigorenko[a], O. Delbono[a,b,*]

[a]*Department of Physiology and Pharmacology, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC 27157, USA*
[b]*Department of Internal Medicine (Gerontology), Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC 27157, USA*

Received 7 April 1997; revised version received 2 May 1997

Abstract Spinal cord motoneurons (MNs) undergo a process of cell death during embryonic development [1] and are the target of lethal acquired or inherited disorders, such as the amyotrophic lateral sclerosis. Therefore, the identification of mechanisms leading to MN survival is of crucial importance. Elevations in intracellular Ca^{2+} promote chicken MN survival during the embryonic period of naturally occurring cell death [2,3]. We have recently demonstrated that the α_7 nicotinic acetylcholine receptor (nAChR) mediates significant increases in free Ca^{2+} concentration at membrane potentials at which other pathways for Ca^{2+} influx are inactive [4,5]. Although it is possible that Ca^{2+} influx through α_7 nAChR promotes cell survival, the relation between α_7 nAChR activation, cytosolic free Ca^{2+} and mammalian spinal cord MN survival has not been established. In the present study we have now demonstrated that Ca^{2+} influx through the α_7-subunit is sufficient to rescue a significant number of cultured spinal cord MNs from programmed cell death induced by trophic factor deprivation. This is the first demonstration that neuronal nAChRs are involved in the regulation of MN survival.

1. Introduction

In the CNS, a major class of nicotinic receptors is defined by the high-affinity binding of [^{125}I]bungarotoxin and comprises predominantly, if not exclusively, the homomeric α_7 nAChR. The α_7 subtype exhibits a higher Ca^{2+} permeability relative to monovalent cations than other nAChR subtypes [6–8]. Influx of Ca^{2+} through the α_7 nAChR subtype has been suggested to be of particular importance in activating a number of Ca^{2+}-dependent processes including neurite growth, synaptic transmission and survival [9]. Recent studies have demonstrated that α-bungarotoxin (α-Bgt)-sensitive receptors mediate Ca^{2+} influx in ciliary ganglion neurons [10,11], hippocampal neurons [12,13] and at the synaptic junction of the medial habenula and interpeduncular nuclei [14]. Although the expression of the α_7 nAChR in whole spinal cord has been reported, the presence of this subunit in motoneurons is not known. The expression of α_7 nAChR in single embryonic (E_{15}) spinal cord motoneurons was studied using antisense RNA (aRNA) amplification technique combined with PCR. The role of Ca^{2+} influx through α_7 nAChR and raises in intracellular Ca^{2+} concentration in response to fast perfusion of (−)-nicotine was studied in a population of motoneurons undergoing programmed cell death.

*Corresponding author. Fax: (1) (910) 716 7359.
E-mail: odelbono@bgsm.edu

2. Materials and methods

2.1. Expression of α_7 nicotinic acetylcholine receptor in single rat spinal cord motoneurons

2.1.1. Antisense RNA amplification/PCR. The cytoplasmic content of single MNs was aspirated in whole-cell configuration of patch-clamp [15] and RNA pool was amplified by aRNA technique [16] with subsequent PCR [17] with some modifications in the RT reaction. A single MN content was exposed to 7 U/μl avian myeloblastosis virus reverse transcriptase, 500 mM dNTPs, an oligodeoxynucleotide containing an oligo-dT region together with the T7 RNA polymerase promoter [AAACGACGGCCAGTGAATTGTAATACGACTCACTATAGGCGC$(T)_{24}$] at 3 ng/ml, 10× RT buffer and 1.3 U/ml RNazin (Promega). The DNA obtained after the second round of aRNA amplification was used as a template for PCR with gene-specific primers for α_7 nAChR (sense: 5′-CCCTGATGGTGGCAAAATGCC-3′; antisense: 5′-CGTGCATGAGGTGCTCATCA-3′). The amplified α_7 nAChR fragment from single MN was labeled using a DECAprime II kit (Ambion) and then used as a probe. For Southern blot analysis we used a PCR fragment from the α_7-subunit amplified from adult rat spinal cord. RNA from adult rat spinal cord (n=5) was isolated and processed in RT-PCR as described [18,19]. Sequencing of the cDNA fragments amplified from single MNs and from adult spinal cords was performed on the Perkin-Elmer ABI Prism 377.

2.2. Embryonic spinal cord motoneuron culture

For MN culture, the spinal cord was dissected from 15-day Sprague-Dawley rat embryos in phosphate-buffered saline and freed of meninges. Ventral spinal cords (10–14) were dissected, pooled and treated according to published procedures for dissociation and isolation of large MNs [20]. For counting, MNs were plated in poly-L-ornithine and laminin-coated 4-well Greiner dishes (Labortechnik) at 2000 cells/well in Leibovitz L15 media. The purity of the cultures was determined by immunostaining with a p75 Nerve Growth Factor Receptor monoclonal antibody (Accurate-Chemical), which selectively labels ventral spinal cord MNs [20]. The purity of the cultures in contrasting fluorescence and bright-field examination of 16 fields/dish in 4 dishes (from 2 different preparations) was 96 ± 2%. The correlation at late times after plating (day 5) was 97 ± 4%. These control studies argue against a change in cell morphology in response to nicotine treatment. For MN viability the following criteria was used: (a) multipolar neurite outgrowth (>3); (b) neurite >2× the soma diameter; (c) negative indication of vacuoles and/or degeneration of neurites (beading) suggesting cell death; and (d) uniform appearance of the nucleus (not condensed or lobed) [21]. These criteria were invariably associated with cell death in this preparation. Initial cell counting was performed 7 h after seeding when neurites were well developed. Drug treatment began after the first assessment of cell numbers. Control (no nicotine) and treated cells (3 pulses of nicotine/day) were plated on microgrid coverslips (Eppendorf) (3 experiments). These methods allowed us to follow changes in cell morphology. We did not detect changes in cell morphology in both experimental conditions. Neuronal survival was quantitated daily and expressed as percentage control ± S.E.M. MNs were counted in 80 fields/dish (4 wells) with a 20× objective. Each experimental group was seeded and counted twice in five different preparations.

2.3. Single-channel recording

Single channel activity was recorded for 5 min in cell-attached configuration of the patch-clamp technique [15] at −70 mV holding potential. Micropipettes were pulled from borosilicate glasses to obtain electrode resistance ranging from 2–4 MΩ. Single channel activity was recorded at 100 kHz using pClamp 6.0 with an Axopatch 200A am-

PII S0014-5793(97)00600-5

plifier (Axon Instruments). The data were digitally filtered (Gaussian filter, half-power frequency = 2–6 kHz). The threshold level for channel detection was set to half times the single channel amplitude estimate [22]. Baseline was accepted as the line which is crossed the maximum number of times by the records [23]. The same composition for the pipette and bathing solutions was used (mM): NaCl, 120; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 2; HEPES, 10; D-glucose, 25; pH adjusted to 7.4 with NaOH. Nicotine was diluted to 10 μM in the pipette solution. α-Bgt and DHβE were added to the bath 20 min before recording and were also included in the pipette.

2.4. Simultaneous measurement of intracellular Ca^{2+} and inward current in voltage-clamped motoneurons

Inward current and intracellular free Ca^{2+} concentration were recorded after loading the cells with 100 μM fluo-3 via the patch pipette in whole-cell configuration of the patch-clamp [15]. MNs were voltage-clamped using an Axopatch-200A amplifier. Currents were acquired at 5 kHz and filtered at 2 kHz with pClamp 6.0 (Axon). Coverslips were mounted in a small flow-through chamber on the stage of a inverted microscope. A fluar 100×, 1.3 NA microscope objective was used. For digital fluorescent imaging a frame-transfer cooled CCD camera was used. For fast drug delivery a glass theta tube was mounted in a piezoelectric device. Activation of the drug delivering system was synchronized with current and intracellular Ca^{2+} transients recordings [5]. The composition of the internal solution (pipette) was (mM): 120 KF; 20 KCl; 2.0 $MgCl_2$, 0.1 EGTA, 10 HEPES, pH was adjusted to 7.4 with KOH. The bathing solution contained (mM): NaCl, 120; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 2; HEPES, 10; D-glucose, 25; pH adjusted to 7.4 with NaOH.

3. Results and discussion

To investigate the expression of α_7 nAChR in embryonic (E_{15}) spinal cord motoneurons, we used antisense RNA (aRNA) amplification technique combined with PCR [16,17] to single cells. The cytoplasmic content of single motoneurons was extracted by applying negative pressure in the whole-cell configuration of the patch-clamp and after electrophysiological recordings (see below). A specific sequence corresponding to α_7 nAChR was found in 10 MNs tested from 5 different preparations (Fig. 1). The homology of the amplified fragment from single MN was 95% with the rat α_7-subunit [6] and was $<45\%$ with α_2–α_6 and α_9 nAChR subunits. These results demonstrate that the α_7-subunit is expressed in embryonic rat spinal cord motoneurons during the period of naturally occurring MN death.

To study the role of Ca^{2+} influx in response to α_7 nAChR activation on spinal cord motoneuron survival, in most of the experiments we used (−)-nicotine as an agonist. Motoneuron survival declines steeply when cells are plated at low density (2000 cells/well) in the absence of trophic factors (Fig. 2A, control). Nicotine was added to the culture medium for 5 min either 1× or 3× a day or continuously. A 50 μM nicotine concentration was used based on the EC_{50} for nicotine-induced inward current and intracellular Ca^{2+} increase in recombinant α_7 nAChR stably expressed in HEK 293 cells [5]. Experiments with ACh, the endogenous cholinergic neurotransmitter at the spinal cord [24], were performed at 100 μM concentration [5] given 3× per 24 h. These results indicate that treatment with nicotine and ACh promoted MN survival with the greatest survival occurring following treatment with 3 pulses/24 h (Fig. 2A). Although nicotine can reach significant concentrations in blood (0.5 μM) after smoking one cigarette [25], the actual concentrations in the neuronal microenvironment are not known. Therefore, we tested a range of nicotine concentrations to determine the dose exhibiting a maximum effect on survival (Fig. 2B). A dose of 50 μM nicotine was saturating and further increments in concentration (100, 500 and 1000 μM) did not significantly increase cell survival (data not shown). Based on these initial results, a treatment paradigm of 3 pulses/24 h of 50 μM nicotine was used in all subsequent experiments.

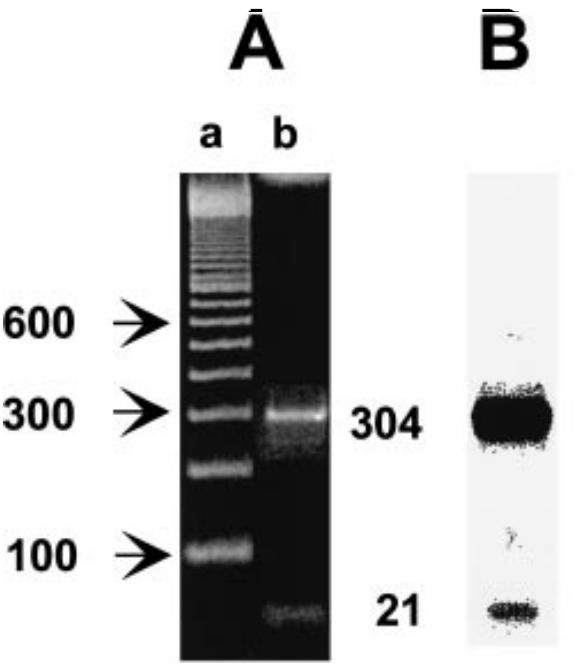


Fig. 1. Expression of α_7 nicotinic acetylcholine receptor in single rat spinal cord motoneuron. (A) Agarose gel (3%) showing an α_7 nAChR 304-bp fragment (from bases 963 to 1267, in the 3′–5′ direction) amplified by RT-PCR from total RNA of adult rat spinal cord (a, 100-bp ladder; b, rat α_7-subunit cDNA fragment). (B) Labeled PCR product from α_7-subunit amplified from single embryonic MN used as a probe in Southern blot analysis with the α_7 nAChR cDNA from adult spinal cord.

The role of extracellular Ca^{2+} on nicotine-induced MN survival was examined in MNs pre-incubated in a medium with zero Ca^{2+} and 100 μM BAPTA. This treatment completely blocked the effects of nicotine on cell survival (Fig. 2C). To determine whether the nicotine effect on cell survival was mediated by membrane depolarization, Na^+ channels and voltage-gated Ca^{2+} channel (VGCC) activation, 1 μM tetrodotoxin (TTX) and 500 μM Cd^{2+} were used to block these events (Fig. 2C). Because nicotine still promoted survival in this situation, we conclude that MN survival is mediated by Ca^{2+} influx through α_7 nAChR rather than by Ca^{2+} influx through VGCC. The specific involvement of the α_7-subunit in MN survival was assessed using the antagonist methyllycaconitine (MLA) at a concentration (2 nM) that is specific for the α_7-subunit [26]. Pre-incubation in MLA prevented the nicotine-dependent effect on cell survival. Sustained incubation in MLA or α-Bgt alone did not modify the time course of MN death (Fig. 2D). These results do not support previous suggestions that *d*-tubocurare and α-Bgt save avian MNs from death in vivo by blocking neuronal nAChRs in the CNS [27,28]. The interpretation of these in vivo experiments is not simple because in addition to a potential effect of these drugs on neuronal nAChRs, they also have a potent effect in blocking peripheral neuromuscular transmission [29]. It has been argued that muscle inactivity in this situation may either

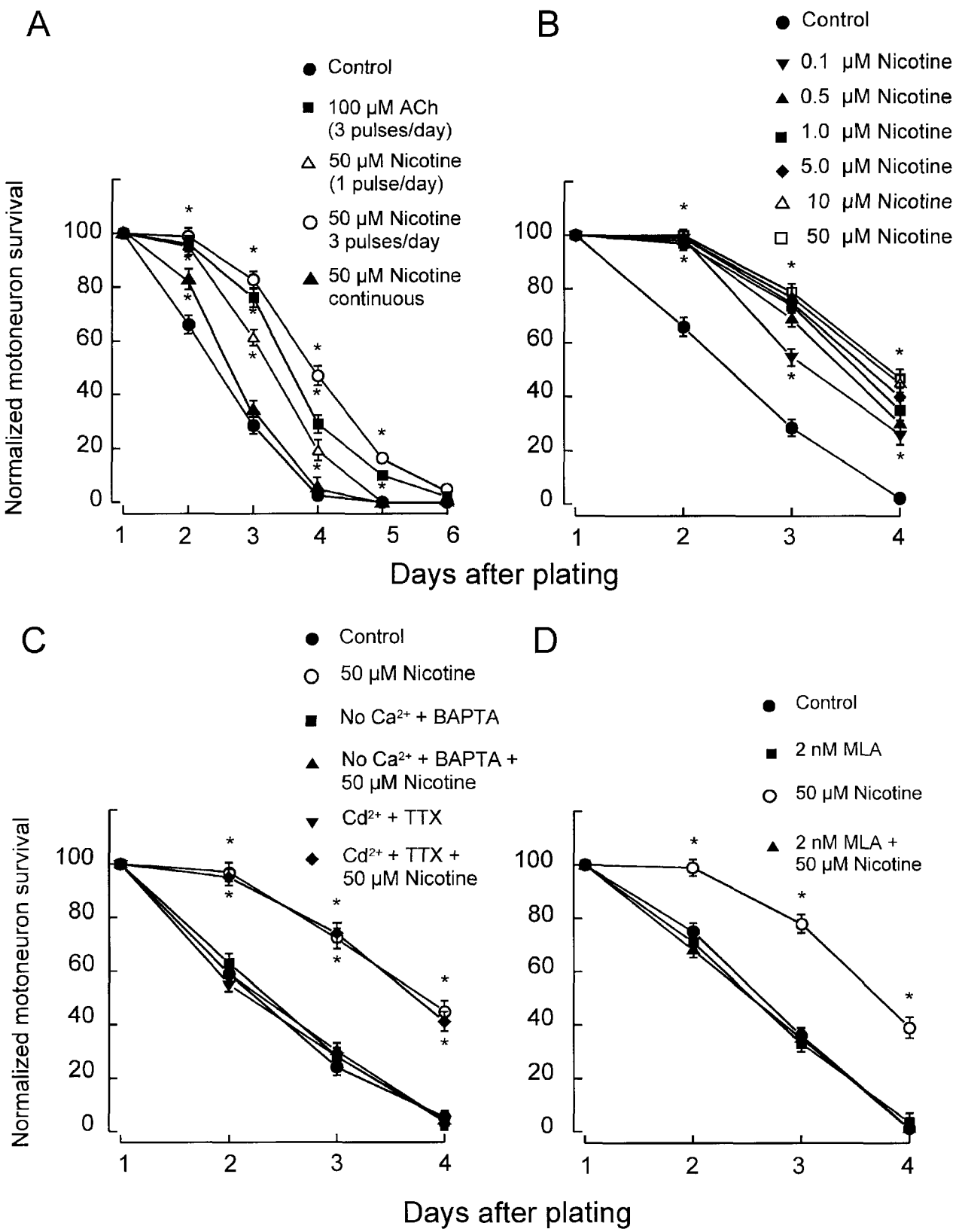


Fig. 2. (−)-Nicotine promotes Ca^{2+}-dependent motoneuron survival. (A) Effect of 50 μM nicotine or 100 μM acetylcholine in a continuous- or pulsed-application scheme (5 min every 8 h or once a day) on MN survival. (B) Nicotine concentration dependence of MN survival in response to a pulsed drug application. Effect of zero Ca^{2+} (Ca^{2+} omission plus 100 μM BAPTA) and Na^{+}-, Ca^{2+}-voltage-gated channels blockade (1 μM tetrodotoxin and 500 μM Cd^{2+}, respectively), and chronic incubation in methyllycaconitine (MLA) on nicotine-dependent increase in MN survival (D). Asterisks denote statistically significant differences with respect to control ($P < 0.001$). In plot B, all the points for nicotine were significantly different from control.

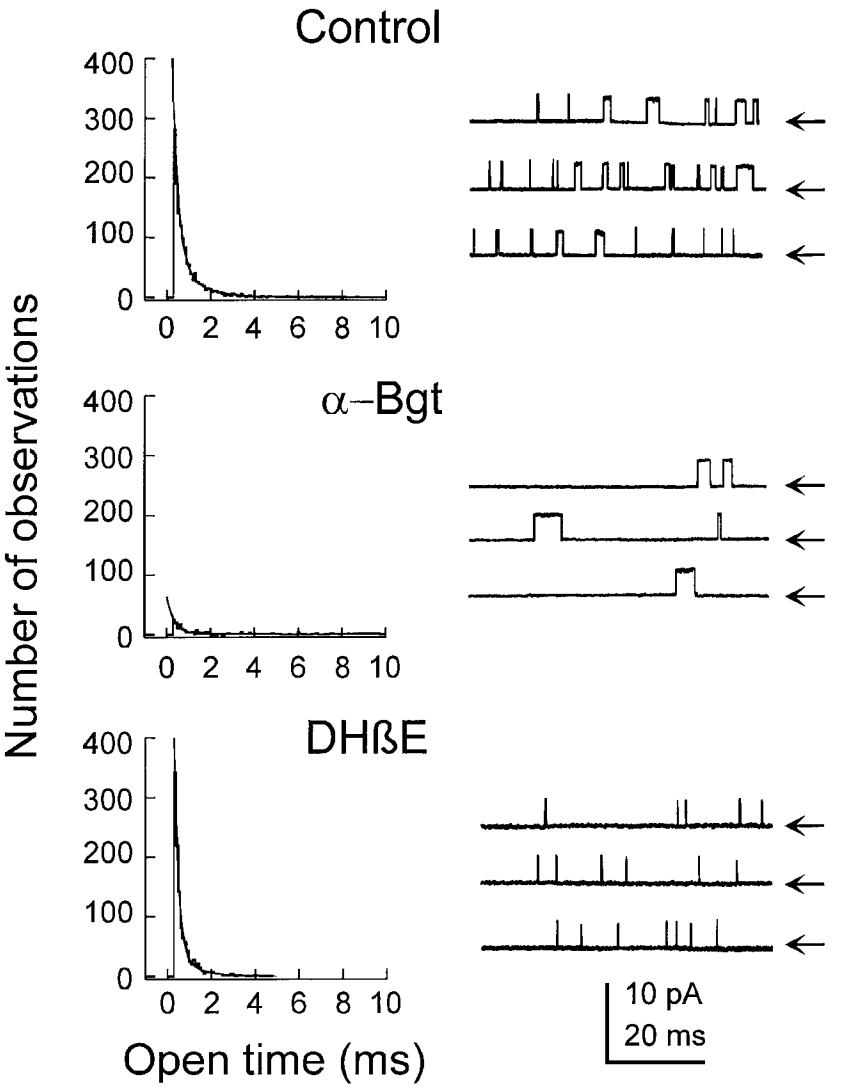


Fig. 3. Expression of α_7 nAChR and DHβE-sensitive receptors in spinal cord motoneurons. Open times and fitted probability density functions of single channel activity induced by 10 μM nicotine in control, 2 nM α-Bgt and 10 nM DHβE. The fitted values were (mean ± S.E.M.): $\tau_1 = 0.19 \pm 0.02$ ms and $\tau_2 = 2.45 \pm 0.26$ ms in control ($n = 15$), $\tau_1 = 2.23 \pm 0.31$ ms in α-Bgt ($n = 10$) and $\tau_1 = 0.26 \pm 0.04$ ms in DHβE ($n = 7$). Traces on the right illustrate single channel activity for each group. Arrows indicate the closed state.

increase the expression of muscle-derived trophic agents that promote MN survival or may increase MN access to muscle-derived trophic agents by inducing axonal branching and synapse formation [30,31]. The specific mechanism by which neuromuscular blocking agents rescue MNs in vivo remains to be determined.

The α_7 nAChR subtype is expressed in MNs together with other nicotine-sensitive AChR subunits. The analysis of the single channel activity elicited by 10 μM nicotine revealed two populations of channels distinguished by distinct, clearly defined mean open time distributions (Fig. 3). Brief openings correspond to α_7 nAChR activity (conductance 65 pS) and are completely blocked by pre-incubation in 2 nM α-Bgt [12], whereas longer openings (conductance 46 pS) were completely blocked with 10 nM DHβE [4,26]. This longer channel activity corresponded to the $\alpha_4\beta_2$-subunits based on single channel kinetics and the pharmacological response profile [26]. The role of these two receptor subtypes on MN survival was tested using specific agonists and antagonists for the α_7- and $\alpha_4\beta_2$-nAChR subtypes. As summarized in Fig. 4, α-Bgt but not DHβE prevented the nicotine-dependent maintenance of MN survival. These results support the notion that the promotion of embryonic MN survival in this situation is mediated by the α_7 nAChR subtype.

The activation of α_7 nAChR by fast solution exchange shows macroscopic inward currents with fast activation and desensitization kinetics [5] (Fig. 5A, upper panel). The activation of α_7 nAChR increases intracellular Ca^{2+} concentration with a maximum at the end of the desensitizing phase (Fig. 5A, lower panel). The time course of the intracellular Ca^{2+} transient was constructed by averaging intracellular digital fluorescent images (Fig. 5B). The increase in intracellular fluorescence, expressed as $\Delta F/F$ (Fig. 5A, lower panel) was 2.1 ± 0.19. A separate group of experiments was performed in motoneurons loaded with 500 μM of the Ca^{2+} indicator fura-2 pentapotassium salt via the patch pipette to determine the increase in free Ca^{2+} concentration [5]. The elevation in cytoplasmic Ca^{2+} concentration in response to 100 μM nico-

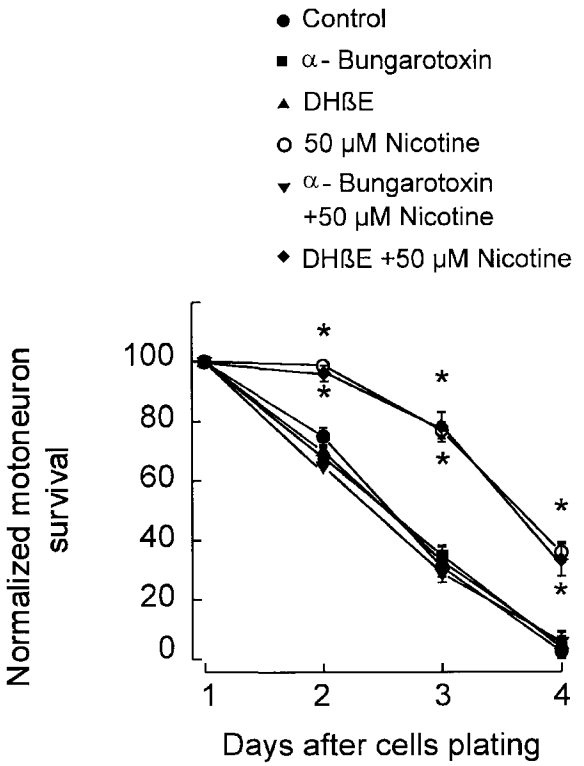


Fig. 4. Nicotine effect on MN survival is blocked by α-bungarotoxin but not by DHβE. Incubation in the nAChRs blockers α-Bgt and DHβE on MN survival and the effect of pre-incubation in both antagonists on nicotine-dependent promotion of MN survival. Methods. Each experimental group was seeded and counted twice in five different preparations. Asterisks denote statistically significant differences with respect to control ($P < 0.001$).

tine was 587 ± 43 nM ($n = 25$) (resting $[Ca^{2+}] = 96 \pm 12$ nM; $n = 25$) [5]. The potential contribution of intracellular Ca^{2+} stores to the measured $[Ca^{2+}]$ response to nicotine was also examined. Pre-incubation in heparin (20 mg/ml) and ryanodine (5 μM), blockers of the endoplasmic reticulum inositol 1,4,5-triphosphate (IP_3) receptor and Ca^{2+} release channel/ryanodine receptor, respectively, for 10 min, did not significantly change the amplitude of the Ca^{2+} response ($[Ca^{2+}] = 559 \pm 63$, $n = 7$).

We conclude that Ca^{2+} influx through α_7 nAChR may account for the observed effects of nicotine on MN survival. However, it is also known that α_7 and $\alpha_4\beta_2$ nAChR subtypes are up-regulated by chronic exposure to both agonists and antagonists compounds [31–33]. If receptor up-regulation mediates the reported effects of nicotine on MN survival, it would be expected that MNs continuously exposed to nicotine would survive better than those treated with 3 pulses/24 h. However, MNs exposed to 3 short pulses of the agonist exhibited a more significant increase in survival than following continuous exposure. These results support our argument that Ca^{2+} mobilization in response to α_7 nAChR activation by nicotine is involved in MN survival. Consistent with this, pre-treatment with α-Bgt blocks the effect of nicotine on MN survival. Because, both α_7 AChR agonists and antagonists promote receptor up-regulation, whereas only agonists induce MN survival, it can be concluded that this effect is current-mediated.

In summary, these studies demonstrate for the first time that α_7 nicotinic AChR expression in cultured embryonic spinal cord MNs plays a role in cell survival through a Ca^{2+}-dependent mechanism. Further studies are needed to examine the role of neuronal nAChRs in the normal programmed cell death of developing MNs in vivo and in pathological conditions involving the loss of motoneurons.

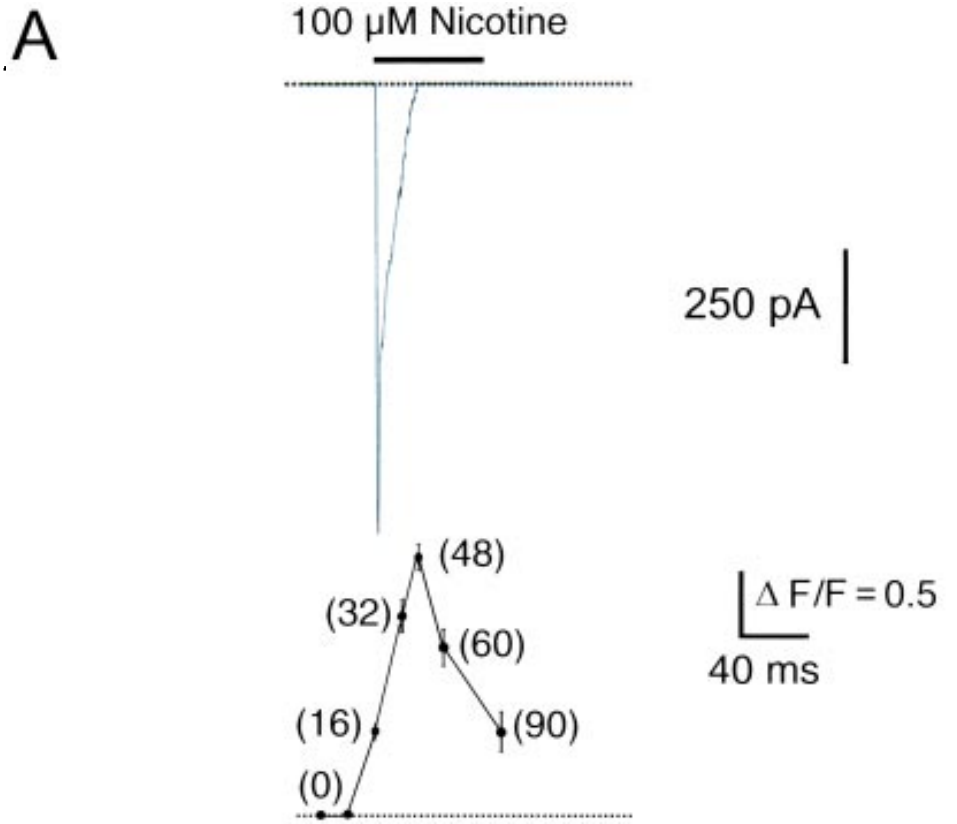
Acknowledgements: We thank Drs. R.W. Oppenheim and John Dani for helpful comments on the manuscript. This work was supported by the National Institutes of Health and Muscular Dystrophy Association (O.D.).

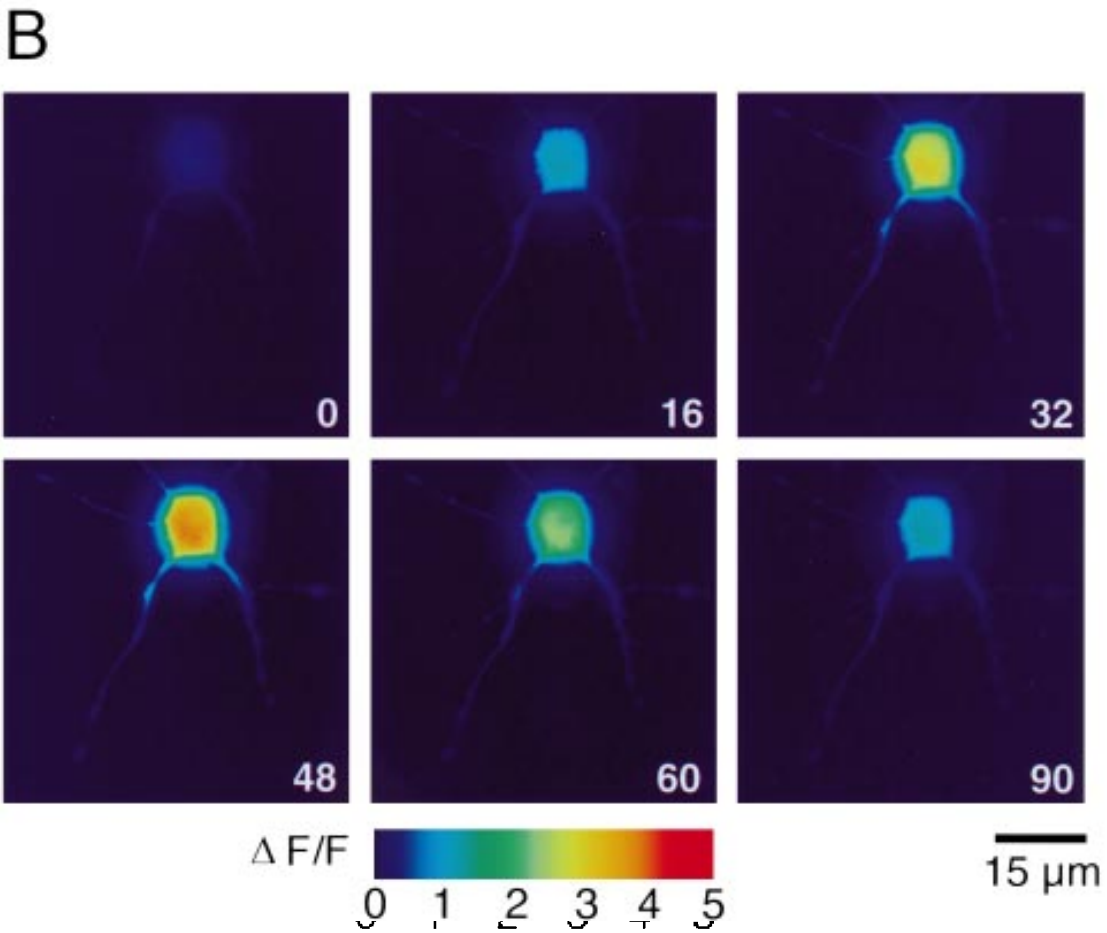
References

[1] Oppenheim, R.W. (1986) J. Comp. Neurol. 246, 281–286.
[2] Lloyd, E.D., Lo, A.C., Oppenheim, R.W. and Houenou, L.J. (1994) Soc. Neurosci. Abstr. 20, 683.
[3] Comella, J.X., Soler, R.M., Egea, J., Gine, E. and Sanz-Rodriguez, C. (1996) Soc. Neurosci. Abstr. 22, 1477.
[4] Messi, M.L., Sullivan, J., Renganathan, M., Gopalakrishnan, M. and Delbono, O. (1996) Biophys. J. 70, A52.
[5] Delbono, O., Gopalakrishnan, M., Renganathan, M., Monteggia, L.M., Messi, M.L. and Sullivan, J.P. (1997) J. Pharmacol. Exp. Ther. 280, (1) 428–438.
[6] Séguéla, P., Wadiche, J., Dineley-Miller, K., Dani, J. and Patrick, J. (1993) J. Neurosci. 13, 596–604.
[7] Bertrand, D., Galzi, J.-L., Devillers-Thiery, A., Bertrand, S. and Changeux, J.-P. (1993) Proc. Natl. Acad. Sci. USA 90, 6971–6975.
[8] Sands, S.B., Costa, A.C.S. and Patrick, J.W. (1993) Biophys. J. 65, 2614–2621.
[9] Rathouz, M.M., Vijayaraghavan, S. and Berg, D.K. (1996) Mol. Neurobiol. 12, 117–131.
[10] Vijayaraghavan, S., Pugh, P.C., Zhang, Z., Rathouz, M.M. and Berg, D.K. (1992) Neuron 8, 353–362.
[11] Zhang, Z., Vijayaraghavan, S. and Berg, D.K. (1994) Neuron 12, 167–177.
[12] Alkondon, M. and Albuquerque, E.X. (1993) J. Pharmacol. Exp. Ther. 265, 1455–1473.
[13] Castro, N.C. and Albuquerque, E.X. (1993) Neurosci. Lett. 164, 137–140.
[14] McGehee, D.S., Heath, M.J.S., Gelber, S., Devay, P. and Role, L.W. (1995) Science 269, 1692–1696.
[15] Hamill, O.P., Marty, A., Neher, E., Sackmann, B. and Sigworth, F.J. (1981) Pflüg. Arch. 391, 85–100.
[16] Eberwine, J., Yeh, H., Miyashiro, K., Cao, Y., Nair, S., Finnell, R., Zettel, M. and Coleman, P. (1992) Proc. Natl. Acad. Sci. USA 89, 3010–3014.
[17] Mackler, S.A., Brooks, B.P. and Eberwine, J.H. (1992) Neuron 9, 539–548.
[18] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[19] Grigorenko, E.V. and Yeh, H.H. (1994) Vis. Neurosci. 11, 379–387.
[20] Camu, W. and Henderson, C.E. (1992) J. Neurosci. Methods 44, 59–70.
[21] Milligan, C.E., Oppenheim, R.W. and Schwartz, L.M. (1994) J. Neurobiol. 25, 1005–1016.

→

Fig. 5. Fast exposure to nicotine induces inward current and raises intracellular Ca^{2+} in spinal cord motoneurons. (A) Inward current (upper trace) in a MN voltage-clamped at −70 mV, and intracellular Ca^{2+} transient elevation (lower trace) in response to 100 μM nicotine for 75 ms. Numbers near the experimental points indicate the times at which images were grabbed and correspond to the intervals shown in B. The time course of the Ca^{2+} transient was reconstructed from digital fluorescent images from 25 MNs taken 16 ms apart and using fluo-3 pentapotassium salt as a Ca^{2+} probe (B).


A
100 μM Nicotine
250 pA
(48)
(32)
(60)
(16)
(90)
(0)
Δ F/F = 0.5
40 ms


B
0
16
32
48
60
90
Δ F/F
0 1 2 3 4 5
15 μm

[22] Benndorf, K. (1995) in: Single Channel Recording (Sakmann, B., Neher, E., Eds.), pp. 129–145, Plenum, New York, NY.
[23] Colquhoun, D., Sigworth, F.J. (1995) in: Single Channel Recording (Sakmann, B., Neher, E., Eds.), pp. 483–587, Plenum, New York, NY.
[24] Phelps, P.E., Barber, R.P., Brennan, L.A., Maines, V.M., Salvaterra, P.M. and Vaughn, J.E. (1984) J. Comp. Neurol. 229, 347–361.
[25] Henningfield, J.E., Stapleton, J.M., Benowitz, N.L., Grayson, R.F. and London, E.D. (1993) Drug Alcohol Depend. 33, 23–29.
[26] Buisson, B., Gopalakrishnan, M., Arneric, S.P., Sullivan, J.P. and Bertrand, D. (1996) J. Neurosci. 16, 7880–7891.
[27] Renshaw, G., Rigby, P., Self, G., Lamb, A. and Goldie, R. (1993) Neuroscience 53, 1163–1172.
[28] Hory-Lee, F. and Frank, E. (1995) J. Neurosci. 15, (10) 6453–6460.
[29] Pittman, R. and Oppenheim, R.W. (1978) Nature (London) 271, 364–366.
[30] Oppenheim, R.W. (1989) Trends Neurosci. 12, 252–254.
[31] Landmesser, L. (1994) Prog. Brain Res. 103, 67–73.
[32] Peng, X., Gerzanich, V., Anand, R., Whiting, P.J. and Lindstrom, J. (1994) Mol. Pharmacol. 45, 546–554.
[33] Gopalakrishnan, M., Delbono, O., Molinari, E.J., Renganathan, M., Messi, M.L., Arneric, S.P. and Sullivan, J. (1996) Neurosci. Abstr. 22, 603.18.