0026-895X/97/030499-08$3.00/0

MOLECULAR PHARMACOLOGY, **51:**499–506 (1997).

Modulation of $\alpha 7$ Nicotinic Receptor-Mediated Calcium Influx by Nicotinic Agonists

M. QUIK, J. PHILIE, and J. CHOREMIS

Department of Pharmacology, McGill University, Montreal, Quebec H3G 1Y6, Canada

Received June 26, 1996; Accepted November 14, 1996

SUMMARY

Our previous work had demonstrated stable expression of rat $\alpha 7$ α-bungarotoxin (α-BGT) binding sites by GH_4C_1 rat pituitary cells, a clonal line that does not endogenously express nicotinic receptors. The stably expressed $\alpha 7$ sites had similar binding affinities, pharmacological profiles, kinetic properties, and molecular size as rat brain α-BGT receptors, suggesting that they represent a good model system for studying receptor function. The present data show that nicotinic receptor agonists increase intracellular calcium levels ($[Ca^{2+}]_i$), as assessed using Fura-2, in $\alpha 7/GH_4C_1$ cells in a dose-dependent manner with EC_{50} values that correlate well with the affinity of these ligands for $\alpha 7/GH_4C_1$ α-BGT receptors. Nicotinic receptor antagonists inhibited agonist-induced increases in $[Ca^{2+}]_i$, with IC_{50} values in the nanomolar to micromolar range. The nicotinic agonist-induced increase in $[Ca^{2+}]_i$ required extracellular calcium and did not occur in the presence of $CdCl_2$, suggesting that agonist-induced increases in $[Ca^{2+}]_i$ are due to an influx of extracellular calcium through voltage-gated calcium channels. Preexposure of the $\alpha 7/GH_4C_1$ cells to 8-bromo cAMP resulted in an enhanced $[Ca^{2+}]_i$ in response to agonist, suggesting that phosphorylation by adenylate cyclase may regulate receptor responsiveness. Interestingly, short-term preexposure (40–60 sec) of the cells to subthreshold concentrations of nicotinic agonist-enhanced receptor-stimulated calcium influx (up to 55%) while activating agonist concentrations completely blocked receptor-mediated responses. Long-term exposure of $\alpha 7/GH_4C_1$ cells to K^+ resulted in about a 2-fold increase in α-BGT receptors and in agonist-evoked calcium influx. The sensitivity of these up-regulated receptors were modulated by subthreshold and activating concentrations of agonist in a manner similar to control receptors. The present results, demonstrating a biphasic regulation of $\alpha 7$ receptor-mediated calcium influx by nicotinic agonists, suggest that these receptors may play an important role in neuronal function under control and depolarizing conditions.

Numerous studies have demonstrated that neuronal nicotinic receptors exhibit an extensive diversity. One nicotinic receptor subtype that is very widely distributed in both the central and peripheral nervous system is the α-BGT-sensitive nicotinic receptor (1, 2). This subtype contains the $\alpha 7$ α-BGT binding subunit in mammalian species (3–6) and an $\alpha 7$ and/or $\alpha 8$ subunit in chick (7–9). These subunits form functional homo-oligomeric channels after injection or transfection of $\alpha 7$ cDNA (cRNA) into oocytes or into an appropriate cell line, respectively (3, 5–7, 10). Although homo-oligomeric channels are formed under experimental conditions, the nature of the α-BGT receptor *in vivo* is still uncertain. Current evidence suggests that the receptor in chick nervous tissue also contains other as yet unidentified subunits in addition to $\alpha 7$ (11), whereas experiments with rat $\alpha 7/\alpha$-BGT receptors suggest that the properties of native rat brain receptors are similar to those for expressed $\alpha 7$ receptors (12).

α-BGT receptors are ligand-gated ion channels, which desensitize very rapidly, a property that had made their initial detection difficult (7, 13, 14). Studies indicate that $\alpha 7$ receptor activation results in an altered calcium flux across the membrane, which may be due to an increased calcium flux through the nicotinic receptor channel and/or to influx through voltage-dependent calcium channels. $\alpha 7$-Nicotinic receptor-mediated alterations in calcium flux have been demonstrated both for homo-oligomeric α receptors expressed in oocytes (3, 7, 15) and for endogenously expressed receptors in rat and chick ganglionic (16–18) and CNS neurons (19–21).

The functional role of the nicotinic $\alpha 7/\alpha$-BGT receptor in neuronal tissue is still under investigation. The very ancient lineage of the $\alpha 7$ receptor and its conservation throughout the course of evolution may suggest that it plays a significant role in neuronal function (22). Experimental evidence has

M.Q. was supported by the Canadian Medical Research Council and the Verum Foundation (Germany). J.C. received a Medical Research Council/Pharmaceutical Manufacturers Association of Canada scholarship.

ABBREVIATIONS: α-BGT, α-bungarotoxin; CNS, central nervous system; DMPP, 1,1-dimethyl-4-phenylpiperazinium; DMEM, Dulbecco's modified Eagles medium; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; AM, acetoxymethyl ester; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 5-HT, 5-hydroxytryptamine; MLA, methyllycaconitine; PKC, protein kinase C; TEA, tetraethylammonium; TTX, tetrodotoxin.

implicated this receptor in a number of different functions, including CNS development (23–26), the regulation of neurite outgrowth (27, 28), sensory gating (29), the modulation of synaptic transmission (21), as well as others (23). Recent evidence suggests that at least some of these activities may be mediated through nicotinic receptor-mediated alterations in intracellular calcium levels (3, 7, 15–21). The purpose of the present work is to further study the functional properties of the nicotinic receptor-mediated changes in calcium flux and the factors that regulate this response.

Because responses from endogenously expressed α7/α-BGT nicotinic receptors have proved difficult to study, possibly due to the rapidly desensitizing nature of the response and/or the relatively small magnitude of the response and the limited amount of tissue/cells available, we are using rat α7 receptors (α7/GH_4C_1) expressed in the GH_4C_1 pituitary cell line (12). The present results show that the functional characteristics of α7/GH_4C_1 receptors are similar to oocyte-expressed and endogenous α7 receptors, suggesting that these receptors represent an appropriate model to study receptor function. The data then show that α7/α-BGT receptors are regulated in a biphasic manner by nicotinic agonists, with an enhancement of receptor-mediated calcium influx after preexposure to subthreshold concentrations of agonists and desensitization after preexposure to high agonist concentrations.

Experimental Procedures

Materials. ^{125}I-α-BGT (10–20 μCi/μg) was purchased from DuPont-New England Nuclear (Boston, MA). Fura-2 AM was obtained from Molecular Probes, Inc. (Eugene, OR), MLA was obtained from Research Biochemicals, Inc. (Natick, MA), and nicotine, α-BGT, cytisine, DMPP, TEA and TTX, 8-bromo-cAMP, dibutyryl cAMP, and *d*-tubocurarine from Sigma Chemical (St. Louis, MO). Supplies and chemicals for culture were purchased from GIBCO (Grand Island, NY). All other chemicals were purchased from standard commercial sources.

Cell culture. GH_4C_1 cells transfected with the expression vector pCEP4 containing the α7 nicotinic receptor cDNA insert have been described previously (12). A subclone of this cell line, expressing 30 fmol α7/α-BGT receptor per 10^6 cells, was used in the present study. Cells were grown in F10 medium supplemented with 8% fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin and maintained in a humidified, 95% air/5% carbon dioxide atmosphere at 37°.

$[Ca^{2+}]_i$ measurements. Cells were grown to confluency on a 100-mm dish (final cell density of approximately 10–15 × 10^6 cells per dish). The cells were removed from the dish following a 2- to 3-min exposure to 2 ml assay buffer (118 mM NaCl, 4.6 mM KCl, 20 mM HEPES, 10 mM D-glucose, and 1 mM $CaCl_2$, pH 7.2) supplemented with 5 mM EDTA. The cells were centrifuged for 3 min at 800 rpm and the resulting pellet resuspended in assay buffer at a concentration of 5 × 10^6 cells/ml. Intracellular calcium was measured using the calcium chelating dye Fura-2 AM; cytosolic esterases cleave off the ester moiety, leaving the membrane-impermeant species of the dye behind to chelate free intracellular calcium. Fura-2 AM (dissolved in dimethyl sulfoxide) was added to the cell suspension to achieve a final concentration of 4 μM and the cells were incubated for 40 min at 30° in a shaker to ensure proper mixing of the dye with the cells. To remove residual dye not taken up into the cells, the samples were centrifuged at 800 rpm for 3 min and resuspended in 10 ml of assay buffer. This procedure was repeated. Assays were performed in a cuvette equipped with a magnetic stirrer in a Perkin Elmer Luminescence Spectrofluorometer LS50 (Perkin-Elmer Cetus, Norwalk, CT) with excitation of 340/380 nm and emission of 540 nm. Drugs dissolved in assay buffer were added directly to the cuvette in aliquots of 20 μl. Intracellular free calcium was calculated according to the formula of Grynkiewicz *et al.* (30): $[Ca^{2+}]_i = K_d(R - R_{min}/R_{max} - R)B$, where K_d is the Fura-2 AM binding constant (224), R is the ratio of fluorescence of the cells at 340 and 380 nm, R_{max} and R_{min} are the ratios for Fura-2 free acid at 340 and 380 nm in the presence of saturating calcium (with 0.002% Triton X-100) and 10 mM EGTA, respectively, and B is the ratio of fluorescence at 380 nm in the presence of EGTA to the fluorescence at 380 nm in the presence of Triton X-100. Base-line $[Ca^{2+}]_i$ of α7/GH_4C_1 values ranged from 50 to 150 nM, agonist-stimulated values ranged from 40 to 100 nM, and potassium-evoked responses ranged from 250 to 600 nM (n = 50 experiments).

^{125}I-α-BGT receptor binding assays. Cells (0.4 × 10^6) were plated in a 24-multiwell dish and grown to confluency (10^6 cells/well). Before assay, the cells in culture were washed twice with 1 ml DMEM containing 3.7 mM $NaHCO_3$ and 0.1% bovine serum albumin (DMEM buffer). Cells were then preincubated for 60 min at 37° in the absence or presence of *d*-tubocurarine, followed by incubation in the presence of ^{125}I-α-BGT (1.0 nM) for 90 min at 37°. Binding was terminated by removal of the incubation medium followed by four 1-ml washes with DMEM buffer. The cells were resuspended in 500 μl of 0.5 N NaOH, with shaking, and the radioactivity was counted using a γ counter. Nonspecific binding was defined as binding in the presence of 10^{-4} M *d*-tubocurarine and represented approximately 5 to 10% of total binding at 1 nM of the radioligand.

Cell counts. α7/GH_4C_1 cells were washed twice with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution and subsequently incubated for 3 min in 0.05% trypsin to allow cell detachment. An aliquot of cells was removed from the well, trypan blue was added, and the cells were counted using a hemacytometer.

Data analysis. EC_{50} and IC_{50} values were determined by fitting the data to a sigmoidal logistic equation using the program Prism (GraphPAD Software, San Diego, CA). All values are expressed as the mean ± standard error. Results were analyzed using Student's *t* test or a one-way analysis of variance followed by the Newman-Keuls *post hoc* test, as indicated.

Results

Nicotinic receptor agonists increase $[Ca^{2+}]_i$ in α7/GH_4C_1 cells. Exposure of α7/GH_4C_1 cells to nicotinic agonists resulted in a dose-dependent increase in peak $[Ca^{2+}]_i$. No increase was observed in control GH_4C_1 cells or hygromycin-resistant cells transfected with α7/pCEP4 cDNA but not expressing α-BGT receptors. The rank order of potency (EC_{50} values) of the different agonists to evoke an increase in $[Ca^{2+}]_i$ correlated well (R^2 = 0.86) with the ability of these ligands to interact at the α-BGT receptor in binding assays (Table 1). Maximal responses for the different agonists were similar to those for DMPP (100 ± 9%; n = 11). On the other hand, the maximal response for cytisine was 69 ± 10% (n =

TABLE 1

Comparison of EC_{50} values of agonist-induced $[Ca^{2+}]_i$ and IC_{50} values of the drugs to inhibit ^{125}I-α-BGT binding to intact cells in culture

Each value represents the mean ± standard error of three to five experiments.

Agonist	$[Ca^{2+}]_i$	^{125}I-α-BGT binding
	EC_{50}	IC_{50}
	μM	
Epibatidine	0.17 ± 0.03	0.02 ± 0.01
Nicotine	2.0 ± 0.4	2.7 ± 0.3
DMPP	5.4 ± 1.2	2.0 ± 0.4
Cytisine	10 ± 2.3	5.3 ± 1.4
Acetylcholine	17 ± 3.3	19 ± 0.7

7), which was statistically significantly different from that for DMPP ($p < 0.05$ using Student's t test). As well, cytisine was the only agonist for which there was appreciable desensitization (data not shown) at higher concentrations of the agonist.

Inhibition of nicotinic receptor mediated increases in $[Ca^{2+}]_i$ by antagonists. Agonist-induced responses were blocked by the α7-selective nicotinic antagonists MLA and α-BGT as well as by d-tubocurarine. The data were fitted to one- and two-site competition curves. α-BGT and d-tubocurarine both fit best to a one-site model with R^2 values of 0.85 and 0.94, respectively. The IC_{50} value for α-BGT was $2.7 \pm 1.1 \times 10^{-10}$ M ($n = 3$) and d-tubocurarine $3.3 \pm 0.6 \times 10^{-4}$ M ($n = 4$). The MLA inhibition data were best fit by a two-site model (data not shown) with $R^2 = 0.86$, whereas that for a one-site model was 0.78. The EC_{50} values for MLA were $7.3 \pm 1.5 \times 10^{-13}$ M ($n = 3$) and $2.0 \pm 1.0 \times 10^{-10}$ M ($n = 4$). These data may indicate that MLA interacts with two distinct receptor sites; however, it is also possible that the biphasic curve occurred because of the extremely high affinity of MLA for the receptor such that the concentration of ligand did not saturate the receptor at the lower antagonist concentrations.

Nicotinic agonists increase $[Ca^{2+}]_i$ in $\alpha 7/GH_4C_1$ cells via voltage-gated calcium channels. The nicotinic agonist evoked increase in $[Ca^{2+}]_i$ was dependent on the presence of extracellular calcium. No significant increase in $[Ca^{2+}]_i$ was observed in the presence of $CdCl_2$, which blocks voltage-gated calcium channels but not nicotinic currents (data not shown). These results suggest that nicotinic agonists increase $[Ca^{2+}]_i$ through an enhanced flux through voltage-gated calcium channels. The voltage-gated calcium channel blockers nifedipine and methoxyverapamil, both at 10 μM, also blocked receptor-mediated responses (data not shown), although it is not clear that these agents are specific for only voltage-gated calcium channels.

To assess an involvement of voltage-gated sodium channels, the sodium channel blocker TTX was tested (data not shown). This agent had no effect on nicotinic receptor-mediated $[Ca^{2+}]_i$ at a concentration of 1 μM, which is considered a maximally effective concentration (17, 19). The use of TEA also was considered to determine an involvement of voltage-gated potassium channels. However, TEA, at concentrations much lower than those required to block potassium channels, blocks the nicotinic α-BGT receptor. Concentrations of TEA required to block voltage-gated potassium channels are on the order of 1–10 mM; the K_i value for inhibition of α-BGT binding is 0.1 mM (12). Receptor blockade would occur before block of the voltage-gated potassium channels.

Exposure to subthreshold and activating concentrations of agonist enhance and inhibit, respectively, subsequent agonist-induced calcium influx. Previous work has shown that nicotinic receptor responses mediated by varying α receptor subunits are desensitized after exposure to agonists at concentrations as low as 10^{-9} M. The present results (Fig. 1) show that preexposure to subthreshold concentrations of the agonists epibatidine, nicotine, and DMPP resulted in an enhanced response to a subsequent application of a maximal concentration of the same agonist. When the concentration of agonist is increased to one that induces a maximal effect, the response to a second activating concentration of agonist is reduced; this is most likely due to receptor desensitization. Preexposure of the cells to subthreshold

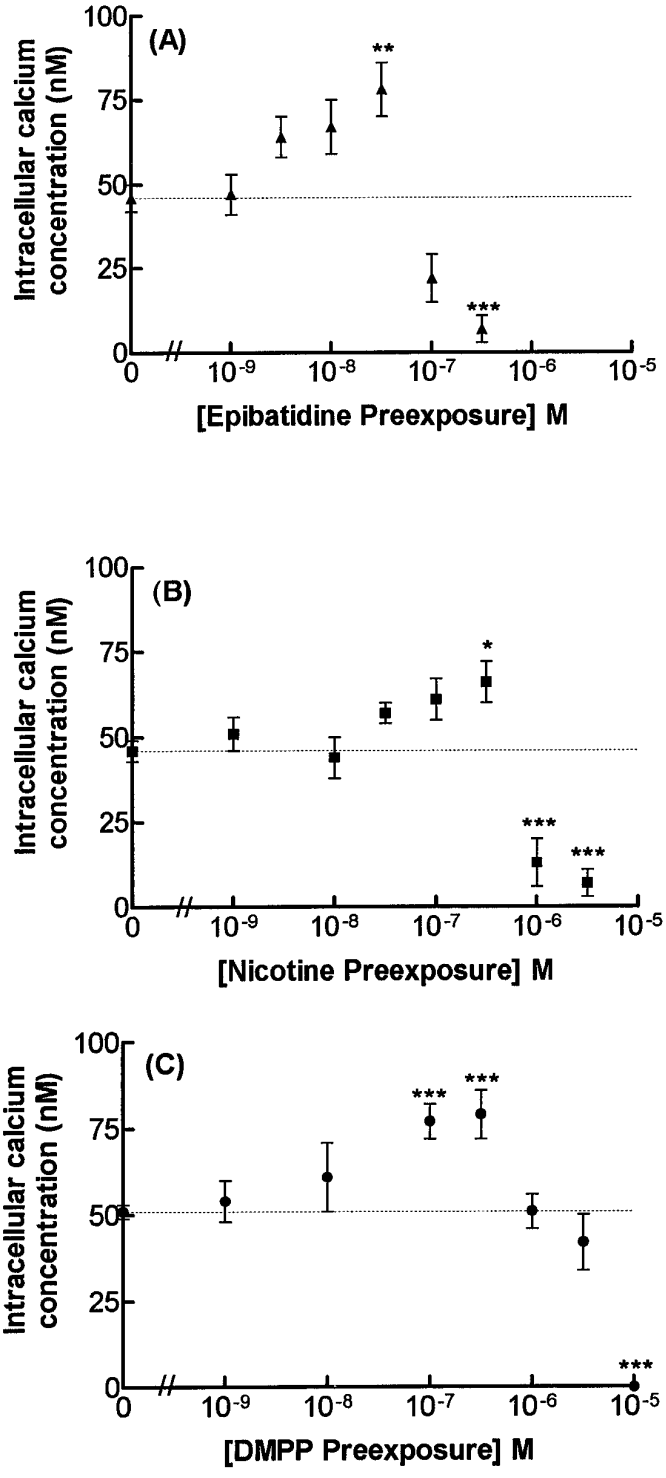

Fig. 1. Exposure to subthreshold concentrations of agonist enhance subsequent agonist-induced calcium influx, whereas activating agonist concentrations inhibit responsiveness. Dispersed $\alpha 7/GH_4C_1$ cells ($1–2 \times 10^6$ cells/ml) were preexposed to the indicated concentrations of agonists; a maximal concentration of the agonist (3×10^{-7} M epibatidine, 3×10^{-5} M nicotine or 3×10^{-5} M DMPP) was then added to the cuvette 40 sec later. The values represent the mean ± standard error of 4–25 determinations from two to eight experiments. *Dotted line*, control agonist-induced calcium responses. Significance of difference from control (no agonist preexposure) using one-way analysis of variance followed by Newman-Keuls *post hoc* test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

concentrations of agonist had no effect on potassium-evoked calcium influx.

Experiments were performed to determine whether a greater enhancement is observed in the agonist-induced response at a half-maximal rather than maximal concentration of agonist. The results in Table 2 show that the subthreshold concentrations of agonist result in a similarly enhanced increase in response whether a half-maximal or maximal concentration of agonist was used.

To determine whether the time of exposure to subthreshold concentrations of agonist altered the extent of the enhanced response to a maximal concentration of agonist, the experiments depicted in Table 3 were performed. As can be seen in the table, optimal enhancement of the agonist-induced response was observed 40–60 sec after exposure to a submaximal agonist concentration.

Potassium pretreatment enhances α7 receptor binding and function. The results in Fig. 2A show that potassium treatment of $\alpha7/GH_4C_1$ cells results in a dose-dependent increase in α-BGT receptor binding, which is due to an increase in B_{max} (12). Fig. 2B shows that these up-regulated receptors are functional and that the increase in the agonist-induced responses for all three agonists correspond well with the increase in receptor number. Although there were significant increases in nicotinic receptor-mediated responses after potassium pretreatment, both base-line $[Ca^{2+}]_i$ and potassium-evoked responses were similar to those observed in control cells. Values of the base-line $[Ca^{2+}]_i$ for control and potassium-treated cells were 63 ± 4 nM (n = 20) and 67 ± 6 nM (n = 19), respectively, whereas peak potassium-evoked $[Ca^{2+}]_i$ was 255 ± 22 nM (n = 20) and 234 ± 11 (n = 19) for control and potassium-treated cells, respectively. Potassium treatment did not increase cell number compared with control cells.

To determine whether the functional characteristics of up-regulated α7 receptors were similar to control cells, agonist dose-response curves were performed. Table 4 showed that the EC_{50} values for DMPP, nicotine, and epibatidine were not statistically different from nontreated cells. Therefore, sensitivity of up-regulated receptors to agonists is similar to control.

Preexposure to subthreshold concentrations of agonist enhances nicotinic agonist sensitivity after potassium pretreatment. The results of Fig. 1 show that exposure to subthreshold concentrations of agonists resulted in an enhanced responsiveness to a subsequent exposure of a maximal concentration of agonist. To determine whether receptors up-regulated in the presence of potassium exhibited similar characteristics, experiments were performed to determine the effect of subthreshold concentrations of agonist on a subsequent agonist-evoked response in cells pretreated

TABLE 2

Modulation of nicotinic receptor sensitivity by varying agonist concentrations

Dispersed cells (1–2 × 10^6) were exposed to subthreshold concentrations (S1) of DMPP (10^{-7} M or 3 × 10^{-7}M) followed 40 sec later by exposure (S2) to a half-maximal (3 × 10^{-6} M) or maximal concentration of agonist (10^{-5} M). The values represent the mean ± standard error of 8 to 20 determinations from two to five experiments. Significance of difference from control: *p < 0.01; **p < 0.001.

S1	S2	$[Ca^{2+}]_i$	Control
	M	nM	%
No drug	3 × 10^{-6} DMPP	37 ± 7	100
Subthreshold DMPP	3 × 10^{-6} DMPP	65 ± 6**	175
No drug	1 × 10^{-5} DMPP	51 ± 7	100
Subthreshold DMPP	1 × 10^{-5} DMPP	78 ± 6*	153

TABLE 3

Effect of length of exposure time with subthreshold agonist on the agonist-induced response

Dispersed cells (1–2 × 10^6) were exposed to subthreshold concentrations of DMPP or buffer followed 20–160 sec later by exposure to a maximal concentration of DMPP (3 × 10^{-5} M). The values represent the mean ± standard error of four to six determinations from two experiments. Significance of difference from control: *p < 0.01.

Time of application of 3 × 10^{-5} M DMPP	Pretreatment (t = 0 sec)	Agonist-induced $[Ca^{2+}]_i$	Control
			%
20 sec	Buffer	65 ± 8	100
	Subthreshold DMPP	80 ± 10	123
40 sec	Buffer	57 ± 4	100
	Subthreshold DMPP	93 ± 8*	163
60 sec	Buffer	60 ± 2	100
	Subthreshold DMPP	92 ± 7*	153
80 sec	Buffer	55 ± 6	100
	Subthreshold DMPP	73 ± 5	130
120 sec	Buffer	49 ± 9	100
	Subthreshold DMPP	51 ± 8	104
160 sec	Buffer	58 ± 4	100
	Subthreshold DMPP	64 ± 12	109

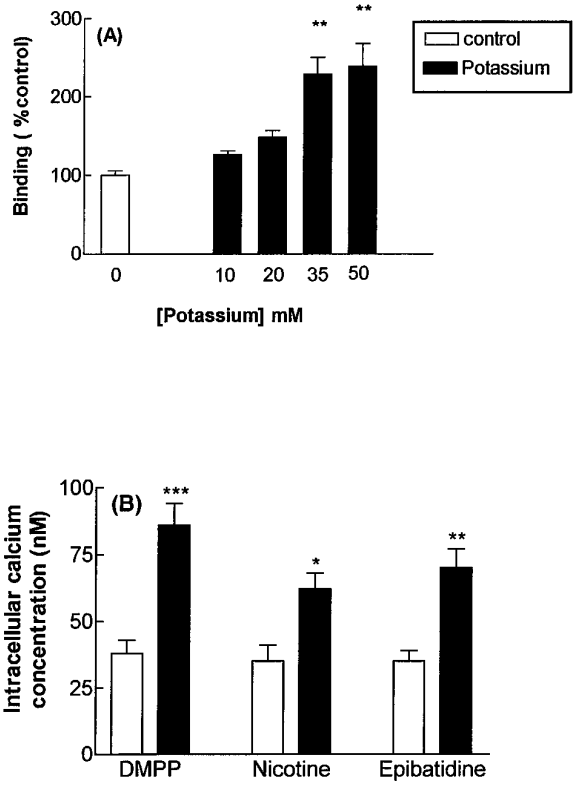

Fig. 2. Potassium pretreatment enhances α7 receptor binding and function. $\alpha7/GH_4C_1$ cells were exposed to increasing potassium concentrations for 2–3 days in culture. The potassium-containing medium was removed 3–5 hr before assay. ^{125}I-α-BGT binding (A) was determined as described in Experimental Procedures. Control binding was 27.6 ± 0.8 fmol/10^6 cells; *bars*, mean ± standard error of three to seven experiments. (B) The effects of 3 × 10^{-5} M DMPP, 3 × 10^{-5} M nicotine, or 3 × 10^{-8} M epibatidine on $[Ca^{2+}]_i$ in control and potassium-pretreated cells. *Bars*, mean ± standard error of six to seven determinations from two experiments. Significance of difference from its own control using one-way analysis of variance followed by Newman-Keuls *post hoc* test: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

TABLE 4

Comparison of EC_{50} values of agonist-induced $[Ca^{2+}]_i$ in control cells and cells treated for 3 days with potassium

Agonist-induced responsiveness is similar in control cells and cells treated with potassium for 2 to 3 days. Each value represents the mean ± standard error of three experiments.

Agonist	Control	Potassium-treated
	EC_{50}	EC_{50}
	μM	
Epibatidine	0.17 ± 0.03	0.13 ± 0.04
Nicotine	2.0 ± 0.4	3.3 ± 1.2
DMPP	5.4 ± 1.2	5.0 ± 1.7

with 50 mM potassium for 2–3 days in culture. The data in Fig. 3 show that a similarly enhanced response to agonist was observed after potassium treatment (Fig. 3; Table 5). These results indicate that a greatly enhanced receptor response can occur under conditions associated with chronic depolarization, if receptors are exposed initially to low agonist. Preexposure of up-regulated receptors to high agonist resulted in receptor desensitization similar to control cells (Table 5).

Experiments were performed to address the question of the molecular site of action of the enhanced response to agonist, that is, whether the nicotinic receptor itself, the voltage-gated calcium channel, or some alternate site may be involved. As indicated earlier, no agonist-induced response was observed in the presence of 10 μM nifedipine or 10 μM methoxyverapamil. Similarly, no enhanced responsiveness to agonist was observed under these same conditions. These results would suggest that calcium is entering via voltage-gated calcium channels after the sensitization because the voltage-gated calcium channel blockers would be expected to inhibit calcium entry regardless of the site of sensitization.

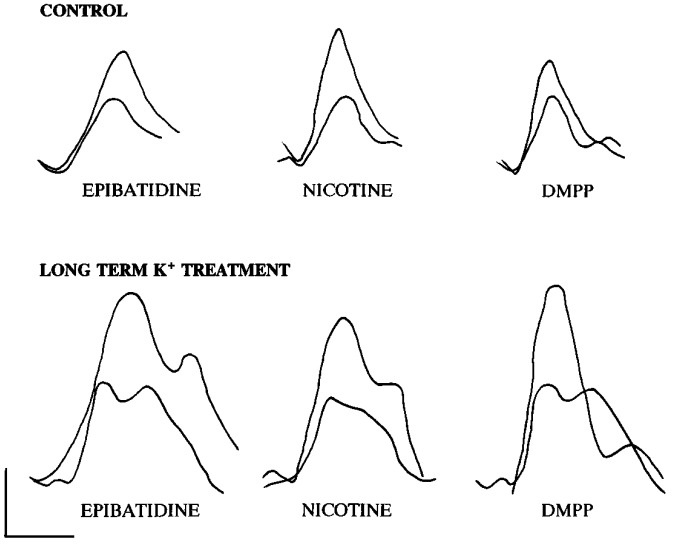

Fig. 3. Preexposure to subthreshold concentrations of agonist enhances nicotinic agonist sensitivity after potassium pretreatment. *Lower traces*, control agonist-induced increases in $[Ca^{2+}]_i$; *upper traces*, response that occurs after preexposure to subthreshold doses of agonist (10^{-8} M epibatidine, 3×10^{-7} M nicotine, or 3×10^{-7} M DMPP) followed 40 sec later by exposure to a concentration of the same agonist, which elicits a maximal response (3×10^{-7} M epibatidine, 3×10^{-5} M nicotine or 3×10^{-5} M DMPP). *Horizontal bar*, 10 sec; *vertical bar*, 50 nM $[Ca^{2+}]_i$. The response to nicotinic agonists is enhanced after potassium treatment (compare lower traces in each set). Preexposure to agonist results in a larger response compared with control in both untreated and potassium-treated cells.

Effect of the neurotransmitters substance P and 5-HT on nicotinic receptor mediated $[Ca^{2+}]_i$. Experiments were performed to determine whether neurotransmitter compounds that had been shown previously to alter nicotinic receptor-mediated activity affected α7 responses. One such compound is substance P (31), which modulates nicotinic receptor responsiveness in both neuronal and muscle cells in a noncompetitive manner. Table 6 shows that substance P decreased nicotinic receptor-mediated calcium influx. The decrease was maximal with 1×10^{-6} M; higher concentrations did not further decrease calcium influx. On the other hand, 5-HT, which also has been shown to result in noncompetitive blocking effects or to modulate desensitization at some nicotinic receptors (32, 33), had no appreciable effect (Table 6).

Modulation of nicotinic receptor-mediated $[Ca^{2+}]_i$ by second messenger systems. An extensive body of evidence has shown that alterations in the activity of protein kinase A and/or protein kinase C activity may modulate both muscle and neuronal nicotinic receptor-mediated responses under specific experimental conditions (2). Therefore, experiments were performed to determine whether these second messenger systems might be involved in α7-mediated calcium influx (Table 6). Pretreatment of the cells with 10^{-3} M 8-bromo-cAMP for 5–40 min resulted in a significant increase in agonist-induced $[Ca^{2+}]_i$, with a trend for an increase at the lower concentrations tested. A tendency for an enhanced responsiveness also was observed with 10^{-3} M dibutyryl cAMP; however, this was not statistically significantly different from control. Neither 8-bromo-cAMP nor dibutyryl cAMP altered basal or potassium-stimulated $[Ca^{2+}]_i$ at any concentrations tested. As an alternate approach to elevate cyclic nucleotide levels, the effect of forskolin, which stimulates adenylate cyclase, was also tested. It resulted in a complete block of α7 receptor-mediated responses at concentrations (1 μM) much lower than those required to inhibit enzymic activity (data not shown). This is most likely due to forskolin's ability to block nicotinic receptor-mediated activity (34), particularly because addition of 8-bromo-cAMP and dibutyryl cAMP enhanced or did not affect α7-induced responses.

Agents that alter PKC also have been shown to modulate both muscle and neuronal nicotinic receptor-mediated activity (2). Phorbol 12-myristate 13-acetate (at 10^{-8} M and 10^{-7} M) and phorbol-12, 13-dibutyrate (at 10^{-8} M, 3×10^{-8} M, 10^{-7} M and 3×10^{-7} M), at preexposure times varying from 5 to 35 min, did not change agonist-induced $[Ca^{2+}]_i$. Drugs were subsequently tested that inhibit PKC. Calphostin C (10^{-9} M, 10^{-8} M and 10^{-7} M) and H7 (10^{-7} M, 10^{-6} M and 10^{-5} M) did not alter α7 receptor-mediated calcium influx; preincubation with calphostin C or H7 was for 10–30 min. There were no effects of the PKC activators or inhibitors on basal or potassium-stimulated $[Ca^{2+}]_i$. Results using a maximal concentration of these agents is depicted in Table 6.

Discussion

The present results show that nicotinic receptor-mediated increases in $[Ca^{2+}]_i$ are readily detectable in α7/GH_4C_1 cells, with no responses occurring in untransfected cells or α7 transfected cells not expressing α-BGT receptors. The rank

TABLE 5

Agonist preexposure modulates nicotinic receptor sensitivity following long-term potassium treatment

$\alpha 7/GH_4C_1$ cells were exposed to 50 mM potassium for 2 to 3 days in culture. The potassium-containing medium was removed 3 to 5 hr before measurement of $[Ca^{2+}]_i$. For assay, dispersed cells (1–2 × 10^6) were exposed to a low or high concentrations of agonist (S1) followed 40 sec later by exposure (S2) to a maximal concentration of agonist, as indicted. The values represent the mean ± standard error of 4 to 10 determinations from two to four experiments. Significance of difference from control: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

S1	S2	$[Ca^{2+}]_i$	Control
M	*M*	*nM*	%
No drug	3×10^{-5} DMPP	84 ± 8	100
3×10^{-7} DMPP	3×10^{-5} DMPP	126 ± 18**	150
3×10^{-5} DMPP	3×10^{-5} DMPP	0 ± 0***	0
No drug	3×10^{-7} epibatidine	72 ± 5	100
1×10^{-8} epibatidine	3×10^{-7} epibatidine	115 ± 14*	159
3×10^{-7} epibatidine	3×10^{-7} epibatidine	0 ± 0**	0
No drug	3×10^{-5} nicotine	63 ± 6	100
3×10^{-7} nicotine	3×10^{-5} nicotine	100 ± 11*	158
3×10^{-5} nicotine	3×10^{-5} nicotine	0 ± 0***	0

order of potency of the different nicotinic receptor agonists correlated well ($R^2 = 0.86$) with the interaction of these ligands at the α-BGT receptor in binding assays and is similar to that observed for the homomeric rat, chick, human, and bovine α7 receptors expressed in oocytes and/or human embryonic kidney 293 cells (3–6). The α7 mediated responses were blocked by the selective α-BGT sensitive nicotinic receptor antagonists α-BGT and MLA (35) and also by *d*-tubocurarine, which inhibits most nicotinic receptor-mediated responses; these results would suggest that the agonist-induced increase in intracellular calcium concentrations is mediated by an α-BGT-sensitive nicotinic receptor.

The responses to nicotinic receptor agonists were dependent on the presence of calcium in the external medium, as were potassium-evoked responses. These results suggest that the increase in $[Ca^{2+}]_i$ requires the influx of extracellular calcium and is not the result of a mobilization of intracellular stores. The observation that the nicotinic receptor-mediated responses were blocked by $CdCl_2$, which blocks voltage-gated calcium channels but does not inhibit nicotinic α-BGT receptor-mediated currents (17), suggests that the increase in $[Ca^{2+}]_i$ elicited by nicotinic agonists occurs through voltage-gated calcium channels. The voltage-gated sodium channel blocker TTX had no effect on the agonist-induced response at maximally effective concentrations (17, 19). The present data are thus in agreement with previous work which showed that, in cultured ciliary ganglion (16, 17, 28) and in sympathetic (18) and hippocampal neurons (19), the α7/α-BGT-mediated rise in $[Ca^{2+}]_i$ in response to nicotinic agonist also occurs through voltage-gated calcium channels.

TABLE 6

Effect of various drugs on nicotinic receptor-mediated calcium mobilization in $\alpha 7/GH_4C_1$ cells

Dispersed $\alpha 7/GH_4C_1$ cells (1–2 × 10^6) were exposed to the indicated concentration of drug at least 20 min before exposure to 3×10^{-5} M DMPP. Each value represents the mean ± standard error determinations from two to four experiments. Control $[Ca^{2+}]_i$ was 68 ± 3 ($n = 43$). Significance of difference from control: *$p < 0.05$; **$p < 0.001$.

Drug	Concentration	Agonist-induced $[Ca^{2+}]_i$ (control)
	M	%
Control		100 ± 4
5-HT	1×10^{-3}	97 ± 3
Substance P	1×10^{-5}	69 ± 11*
	1×10^{-6}	62 ± 6**
	1×10^{-7}	101 ± 5
8-bromo-cAMP	1×10^{-3}	165 ± 34**
	3×10^{-4}	113 ± 10
	1×10^{-4}	93 ± 21
Dibutyryl cAMP	1×10^{-3}	121 ± 7
	3×10^{-4}	113 ± 14
	1×10^{-4}	111 ± 15
Calphostin C	1×10^{-7}	121 ± 22
H7	1×10^{-5}	117 ± 17
Phorbol-12-myristate 13-acetate	1×10^{-7}	93 ± 20
Phorbol-12, 13-dibutyrate	3×10^{-7}	88 ± 21

Desensitization of nicotinic α-BGT-sensitive nicotinic responses is a well-documented phenomenon (3, 5–7, 10, 13, 14). The present results show that agonist-induced $[Ca^{2+}]_i$ similarly desensitizes. The novel aspect of the present work is the observation that exposure of the receptors to subthreshold concentrations of agonist (i.e., concentrations that do not themselves elicit a response) results in an enhanced response to a subsequent application of a higher concentration of agonist. Similar types of studies in which the effect of varying agonist concentrations were determined on brain and peripheral nervous tissue nicotinic responses, possibly mediated by α3- or α4-containing receptors (36, 37), indicate that desensitization is the general response. Therefore, α7 receptors seem to respond to subthreshold in a manner distinct from other nicotinic receptor subtypes.

Time-course studies to determine the effect of varying the time period between the addition of subthreshold agonist and a subsequent maximal concentration of agonist showed that the enhanced response was maximal 40–60 sec after the exposure to subthreshold agonist; the response declined back to control after longer exposure periods. These results suggest that there is a desensitization to subthreshold agonist with continued exposure time. Therefore, desensitization may be dependent on both length of time of exposure to agonist, as suggested from the present results, and on agonist concentration, as shown previously by others. Although the molecular mechanisms responsible for this effect are not clear, they may involve receptor phosphorylation or dephosphorylation by various protein kinases or phosphatases, especially as the present data and that of others has shown that protein kinase A modulates nicotinic receptor responsiveness. These phenomena may represent receptor regulatory mechanisms that allow for optimal neuronal responsiveness *in vivo* to varying agonist exposure.

Electrical activity plays an important role in the regulation of the functional properties of neurons. Depolarization-induced increases have been demonstrated both for neuronal and muscle α-BGT receptors. Exposure of cells in culture to elevated potassium for several days results in an increase in receptor number and receptor-mediated activity (38). In the present study, treatment of cultured cells to potassium for 2–3 days resulted in a significant increase in α-BGT receptor binding, which is due to an increase in B_{max} (12). The increase in receptor number is associated with a corresponding increase in α7 receptor-mediated function. The characteristics of these up-regulated receptors were similar to control receptors in terms of agonist potencies. Interestingly, these

up-regulated receptors also exhibited an enhanced response after preexposure to subthreshold concentrations of agonist. Previous studies have shown that neuronal nicotinic acetylcholine responses on PC12 cells and sympathetic neurons in culture are not regulated by depolarization, indicating that this regulatory mechanism exhibits specificity (18, 39).

The above results indicate that low agonist exposure and depolarizing inputs may act alone or in combination to result in an amplified neuronal responsiveness. It is possible that *in vivo*, α7 nicotinic receptor-bearing cells may be exposed to a diffuse release of agonist, which may result in a concentration of agonist insufficient to elicit a receptor-mediated response. On the other hand, this subthreshold agonist exposure may serve as a priming influence for a more enhanced response to subsequent pulses of the transmitter. The results with α7/GH_4C_1 cells exposed to long-term potassium suggest that even when the cells are chronically stimulated to result in receptor up-regulation, they are still more susceptible to nicotinic receptor-mediated depolarization after low agonist preexposure when compared with the normal resting condition. Numerous studies have shown that α-BGT receptor are located extrasynaptically (40), a finding that suggests that these receptors may be involved in nonsynaptic signaling. If one of these nonsynaptically mediated functions for the α-BGT receptors is neuritic retraction/extension and remodeling, as has been suggested (27, 28), the above described regulatory mechanisms may provide the means for optimal growth and development.

The neuropeptide substance P, at μM concentrations, acts as a noncompetitive inhibitor of nicotinic receptor function in both neuronal and muscle. Furthermore, α-BGT has been shown to interact with rat brain tachykinin receptors and substance P to inhibit binding of ^{125}I-α-BGT to muscle and brain membranes with a maximal inhibition of 60% (31). The present results show that substance P also decreases α7 receptor-mediated $[Ca^{2+}]_i$, with a maximal inhibition of approximately 40% at 10^{-6} M. Concentrations up to 10^{-5} M did not result in a further decrease in function. These results correlate well with the receptor-binding studies of Weiland *et al.* (31), which suggest that substance P may have a modulatory action on α-BGT-sensitive nicotinic receptors.

The α7 nicotinic receptor and 5-HT_3 receptor are both ligand-gated ion channels with a similar molecular organization and some similar activation and desensitization properties. Furthermore, serotonergic ligands have been shown to affect nicotinic receptors. Garcia-Colunga and Miledi (32) had shown that serotonergic agents block neuronal nicotinic acetylcholine receptors in a noncompetitive manner, whereas Cross *et al.* (33) found that 5-HT enhanced the rate of desensitization of the acetylcholine current response in muscle and neuronal receptor subtypes. Despite these effects on muscle and other neuronal nicotinic receptors, the present experiments suggest that 5-HT exposure does not affect α7-mediated responses.

Second messenger have been implicated in the regulation of nicotinic receptor-mediated function, particularly cAMP (1, 2). The present data show that exposure of the α7/GH_4C_1 cells to a nonhydrolyzable analog of cAMP resulted in a significant enhancement of the response at the higher concentrations of the nucleotide, with a trend for an increase at the lower concentrations. These data suggest that phosphorylation by protein kinase A may be a mechanism involved in mediating α7 nicotinic receptor-mediated changes in $[Ca^{2+}]_i$.

In summary, nicotinic agonist-mediated increases in calcium influx in α7/GH_4C_1 cells have the same characteristics as those observed in cultured peripheral and CNS neurons with respect to agonist/antagonist potencies and up-regulation by potassium, suggesting that they represent a good model system to study α7 receptor-mediated function. The present data further show that α7 receptors exhibit an enhanced response to agonist after preexposure of the cells to subthreshold concentrations and that this increased responsiveness also occurs after up-regulation of receptor activity in response to membrane depolarization. Therefore, multiple mechanisms may be present to increase α7 receptor-mediated neuronal responsiveness.

References

1. Sargent, P. The diversity of neuronal nicotinic acetylcholine receptors. *Annu. Rev. Neurosci.* **16:**403–443 (1993).
2. McGehee, D. S., and L. Role. Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. *Annu. Rev. Physiol.* **56:** 521–546 (1995).
3. Séguéla, P., J. Wadiche, K. Dineley-Miller, J. A. Dani, and J. W. Patrick. Molecular cloning, functional properties, and distribution of rat brain α7: a nicotinic cation channel highly permeable to calcium. *J. Neurosci.* **13:** 596–604 (1993).
4. Peng, X., M. Katz, V. Gerzanich, R. Anand, and J. Lindstrom. Human α7 acetylcholine receptor: cloning of the α7 subunit from the SH-SY5Y cell line and determination of pharmacological properties of native receptors and functional α7 homomers expressed in *Xenopus oocytes. Mol. Pharmacol.* **45:**546–554 (1994).
5. Garcia-Guzman, M., F. Sala, S. Sala, A. Campos-Caro, W. Stuhmer, L. M. Gutierrez, and M. Criado. α-Bungarotoxin sensitive nicotinic receptors on bovine chromaffin cells: molecular cloning, functional expression and alternative splicing of the α7 subunit. *Eur. J. Neurosci.* **7:**647–655 (1995).
6. Gopalakrishnan, M., B. Buisson, E. Touma, T. Giordano, J. E. Campbell, I. C. Hu, D. Donnelly-Roberts, S. P. Arneric, D. Bertrand, and J. P. Sullivan. Stable expression and pharmacological properties of the human α7 nicotinic acetylcholine receptor. *Eur. J. Pharmacol.* **290:**237–246 (1995).
7. Couturier, S., D. Bertrand, J. M. Matter, M. C. Hernandez, S. Bertrand, N. Millar, S. Valera, T. Barkas, and M. Ballivet. A neuronal nicotinic acetylcholine receptor subunit (α7) is developmentally regulated and forms a homo-oligomeric channel blocked by α-BTX. *Neuron* **5:**847–856 (1990).
8. Schoepfer, R., W. G. Conroy, P. Whiting, M. Gore, and J. Lindstrom. Brain α-bungarotoxin binding protein cDNAs and mAbs reveal subtype of this branch of the ligand-gated ion channel gene superfamily. *Neuron* **5:**35–48 (1990).
9. Keyser, K. T., L. R. G. Britto, R. Schoepfer, P. Whiting, J. Cooper, W. Conroy, A. Brozozowska-Precht, J. H. Karten, and J. M. Lindstrom. Three subtypes of α-bungarotoxin sensitive nicotinic acetylcholine receptors are expressed in chick retina. *J. Neurosci.* **13:**442–454 (1993).
10. Gerzanich, V., R. Anand, and J. Lindstrom. Homomers of α8 and α7 subunits of nicotinic receptors exhibit similar channel but contrasting binding site properties. *Mol. Pharmacol.* **45:**212–220 (1994).
11. Anand, R., X. Peng, and J. Lindstrom. Homomeric and native α7 acetylcholine receptors exhibit remarkably similar but nonidentical pharmacological properties, suggesting that the native receptor is a heteromeric protein complex. *FEBS Lett.* **327:**241–246 (1993).
12. Quik, M., J. Choremis, J. Komourian, R. J. Lukas, and E. Puchacz. Similarity between rat brain nicotinic α-bungarotoxin receptors and stably expressed α-bungarotoxin binding sites. *J. Neurochem.* **67:**145–154 (1996).
13. Revah, F., D. Bertrand, J. L. Galzi, A. Devillers-Thiery, C. Mulle, N. Hussy, S. Bertrand, M. Ballivet, and J. P. Changeux. Mutations in the channel domain alter desensitization of a neuronal nicotinic receptor. *Nature (Lond.)* **353:**846–849 (1991).
14. Alkondon, M., E. F. R. Pereira, S. Wonnacott, and E. X. Albuquerque. Blockade of nicotinic currents in hippocampal neurons defines methyllycaconitine as a potent and specific receptor antagonist. *Mol. Pharmacol.* **41:**802–808 (1992).
15. Bertrand, D., J. L. Galzi, A. Devillers-Thiery, S. Bertand, and J. P. Changeux. Mutations at two distinct sites within the channel domain M2 alter calcium permeability of neuronal α7 nicotinic receptor. *Proc. Natl. Acad. Sci. USA* **90:**6971–6975 (1993).
16. Vijayaraghavan, S., P. C. Pugh, Z. Zhang, M. M. Rathouz, and D. K. Berg. Nicotinic receptors that bind α-bungarotoxin on neurons raise intracellular free calcium. *Neuron* **8:**353–362 (1992).
17. Zhang, Z., S. Vijayaraghavan, and D. K. Berg. Neuronal acetylcholine

receptors that bind α-bungarotoxin with high affinity function as ligand gated ion channels. *Neuron* **12:**167–177 (1994).
18. De Koninck, P., and E. Cooper. Differential regulation of neuronal nicotinic ACh receptor subunit genes in cultured neonatal rat sympathetic neurons: specific induction of α7 by membrane depolarization through a Ca^{2+}-calmodulin dependent kinase pathway. *J. Neurosci.* **15:**7966–7978 (1995).
19. Barrantes, G. E., C. T. Murphy, J. Westwick, and S. Wonnacott. Nicotine increases intracellular calcium in rat hippocampal neurons via voltage gated calcium channels. *Neurosci. Lett.* **198:**101–104 (1995).
20. Castro, N. G., and E. X. Albuquerque. α-Bungarotoxin sensitive hippocampal nicotinic receptor channel has a high calcium permeability. *Biophys. J.* **68:**516–524 (1995).
21. McGehee, D. S., M. J. S. Heath, S. Gelber, P. Devay, and L. W. Role. Nicotine enhancement of fast excitatory synaptic transmission in CNS by presynaptic receptors. *Science (Washington D. C.)* **269:**1692–1696 (1995).
22. Ballivet, M., C. Alliod, S. Bertrand, and D. Bertrand. Nicotinic acetylcholine receptors in the nematode *Caenorhabditis elegans. J. Mol. Biol.* **258:** 261–269 (1996).
23. Quik, M., and S. Geertsen. Neuronal nicotinic α-bungarotoxin sites. *Can. J. Physiol. Pharmacol.* **66:**971–979 (1988).
24. Fuchs, J. L. [^{125}I]α-Bungarotoxin binding marks primary sensory areas of developing rat neocortex. *Brain Res.* **501:**223–234 (1989).
25. Hamassaki-Britto, D. E., P. F. Gardino, J. N. Hokoc, K. T. Keyser, H. J. Karten, J. M. Lindstrom, and L. R. G. Britto. Differential development of α-bungarotoxin sensitive and α-bungarotoxin insensitive nicotinic acetylcholine receptors in the chick retina. *J. Comp. Neurol.* **347:**161–170 (1994).
26. Broide, R. S., R. T. Robertson, and F. M. Leslie. Regulation of α7 nicotinic acetylcholine receptors in the developing rat somatosensory cortex by thalamocortical afferents. *J. Neurosci.* **16:**2956–2971 (1996).
27. Chan, J., and M. Quik. A role for the neuronal nicotinic α-bungarotoxin receptor in neurite outgrowth in PC12 cells. *Neuroscience* **56:**441–451 (1993).
28. Pugh, P. C., and D. K. Berg. Neuronal acetylcholine receptors that bind α-bungarotoxin mediate neurite retraction in a calcium dependent manner. *J. Neurosci.* **14:**889–895 (1994).
29. Freedman, R., M. Hall, L. E. Adler, and S. Leonard. Evidence in postmortem brain tissue for decreased numbers of hippocampal nicotinic receptors in schizophrenia. *Biol. Psychol.* **38:**22–33 (1995).
30. Grynkiewicz, G., M. Poenie, and T. Y. Tsien. A new generation of calcium indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260:**3440–3450 (1985).
31. Weiland, G. A., J. A. Durkin, J. M. Henley, and S. M. Simasko. Effects of substance P on the binding of ligands to nicotinic acetylcholine receptors. *Mol. Pharmacol.* **32:**625–632 (1987).
32. Garcia-Colunga, J., and R. Miledi. Effects of serotonergic agents on neuronal nicotinic acetylcholine receptors. *Proc. Natl. Acad. Sci. USA* **92:** 2919–2923 (1995).
33. Cross, K. M. L., R. C. Foreman, and J. E. Chad. Enhancement of 5-hydroxytryptamine and analogues of desensitization of neuronal and muscle nicotinic receptors expressed in *Xenopus* oocytes. *Br. J. Pharmacol.* **114:** 1636–1640 (1995).
34. Aylwin, M. L., and M. M. White. Forskolin acts as a noncompetitive inhibitor of nicotinic acetylcholine receptors. *Mol. Pharmacol.* **41:**908–913 (1992).
35. Palma, E., S. Bertrand, T. Binzoni, and D. Bertrand. Neuronal nicotinic α7 receptor expressed in *Xenopus* oocytes presents five putative binding sites for methyllycaconitine. *J. Physiol.* **491:**151–161 (1996).
36. Wonnacott, S. The paradox of nicotinic acetylcholine receptor upregulation by nicotine. *Trends Pharmacol. Sci.* **11:**216–219 (1990).
37. Grady, S., M. J. Marks, and A. C. Collins. Desensitization of nicotine stimulated [^{3}H]dopamine release from mouse striatal synaptosomes. *J. Neurochem.* **62:**1390–1398 (1994).
38. Geertsen, S., R. Afar, J. M. Trifaro, and M. Quik. Regulation of α-bungarotoxin sites in chromaffin cells in culture by nicotinic ligands, K^+ and cAMP. *Mol. Pharmacol.* **34:**549–556 (1988).
39. DeLorme, E. M., and R. McGee. Effects of prolonged depolarization on the nicotinic acetylcholine receptors of PC12 cells. *J. Neurochem.* **50:**1248–1252 (1988).
40. Wilson Horch, H. L., and P. B. Sargent. Perisynaptic surface distribution of multiple classes of nicotinic acetylcholine receptors on neurons in the chicken ciliary ganglion. *J. Neurosci.* **15:**7778–7795 (1995).

Send reprint requests to: Dr. Maryka Quik, The Parkinson's Institute, 1170 Morse Avenue, Sunnyvale, CA 94089. E-mail: mquik@ix.netcom.com