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Agonist-Induced Regulation of the Neuronal Nicotinic Acetylcholine Receptor of PC12 Cells

DENISE ROBINSON AND RICHARD MCGEE, JR.

Department of Pharmacology, Georgetown University, Washington, D. C. 20007

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SUMMARY

Pheochromocytoma cells, clone PC12, possess neuronal nicotinic acetylcholine (ACh) receptors which can be activated by an agonist to produce a transient transmembrane cation flux similar to that seen with neuromuscular nicotinic ACh receptors. We have observed that these neuronal nicotinic ACh receptors are subject to long term regulation by the cholinergic agonist carbamoylcholine (carbachol). Receptor function was measured by agonist-induced uptake of $^{86}Rb^{+}$ into the cells. Chronic exposure to concentrations of carbachol that caused receptor activation induced an adaptation (down-regulation) of the receptors, seen as a decrease in the responsiveness of the cells to a subsequent exposure to carbachol. The extent of the decreased responsiveness was proportional to the concentration of carbachol between 50 μM and 10 mM and was distinct from acute receptor desensitization. The concentrations of carbachol that caused down-regulation were greater than those that caused maximum receptor activation, but were similar to those that greatly enhanced desensitization. This suggested that promotion of the desensitized state of the receptor could be the true stimulus for down-regulation. Adaptation was observed initially after several hours of exposure and maximally within about 7 days. Recovery from the down-regulated state required several days of growth in the absence of carbachol. Treatment with the antagonist mecamylamine did not cause a similar change in the responsiveness of the cells. However, concurrent exposure to carbachol and mecamylamine prevented down-regulation. Therefore, it appeared that receptor activation was necessary for regulation to occur. Comparison of the cellular responses of chronically treated and control cells to acute stimulation with carbachol supported the hypothesis that the mechanism for this down-regulation in PC12 cells was a decrease in the number of functional ACh receptors.

INTRODUCTION

Most work on regulation of nicotinic receptors has focused on the neuromuscular junction (1). An early observation related to receptor regulation was the appearance of supersensitivity of muscle to ACh[1] after denervation of the motor end plate. Following denervation, the rate of *de novo* synthesis and insertion of extrajunctional ACh receptors greatly increased during a period of days to weeks (2). The appearance of supersensitivity could be prevented by direct electrical stimulation of the denervated muscle (3). Exposure of the normally innervated muscle to receptor antagonists also led to increased receptor numbers (4). Finally, localized treatment of the nerve with tetrodotoxin to block conduction resulted in receptor increases, although not as dramatic as those induced by complete nerve section (5).

To characterize further the molecular mechanism of supersensitivity, tissue culture muscle systems have been employed. Upon introduction of the muscle to culture, innervation is terminated, leading to "denervation supersensitivity." The increase in receptor number again was shown to be due to increased rates of receptor synthesis and could be blocked by direct electrical stimulation (6). Treatment of the cultures with nicotinic agonists resulted in a decrease in receptor numbers, while treatment with the antagonist *d*-tubocurarine had no effect (2). From these studies *in vivo* and *in vitro*, it is clear that receptor activation and/or muscle activity are prerequisites for the occurrence of this type of regulation in muscle cells.

In contrast to the extensive investigations into the regulatory mechanisms involved in the neuromuscular

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[1] The abbreviations used are: ACh, acetylcholine; TPP, methyltriphenylphosphonium; carbachol, carbamoylcholine; DMEM, Dulbecco's modified Eagle's medium; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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junction, little information has emerged concerning regulation of neuronal nicotinic ACh receptors. This is due in part to the absence of a good analytical tool for studying neuronal nicotinic ACh receptors, such as α-bungarotoxin, which binds irreversibly to the muscle ACh receptor, blocking receptor function. The use of α-bungarotoxin as a ligand for assaying the neuronal nicotinic ACh receptor has been questioned [for review, see Oswald and Freeman (7)]. The toxin does not appear to inhibit nicotinic receptor function in chick sympathetic ganglia, chick ciliary ganglia, rat superior cervical ganglia, and a rat sympathetic neuronal cell line (7). Hunt and Schmidt (8) showed that the embryologic development of nicotinic receptors does not correspond with the timing of development of toxin-binding sites in the rat hippocampus. Furthermore, septal lesions in the rat, which abolished cholinergic input to the hippocampus, had no effect on the development of α-bungarotoxin-binding sites (8). Finally, antisera that inhibit ACh receptor function do not precipitate the α-bungarotoxin-binding component in the sympathetic neuronal cell line PC12, suggesting that the functional ACh receptor and the α-bungarotoxin receptor are two separate entities (9). Thus, at present, α-bungarotoxin does not appear to be useful as a marker for neuronal nicotinic ACh receptors.

To investigate some of the factors influencing the expression of a neuronal nicotinic receptor, we have chosen the pheochromocytoma cell line PC12, which possesses functional ACh receptors. The PC12 cell line was originally isolated from a rat adrenal medullary tumor, but the properties of PC12 cells more closely resemble those of undifferentiated sympathetic neurons than adrenal chromaffin cells (10). The cells have a depolarizing ACh receptor which appears to be similar or identical to other neuronal nicotinic receptors. Activation of the receptor causes an increased permeability to cations, which is relatively nonspecific for the various cations, although under normal conditions most of the current is carried by Na^+ (11). The receptor-induced depolarization leads to activation of voltage-dependent Ca^{2+} channels and subsequent neurosecretion from the cells. The ACh receptor of the PC12 cells also appears neuronal, in that the cells have saturable binding sites for α-bungarotoxin, but the toxin does not inhibit the ion flux response to receptor activation (12). Based on the above properties, the PC12 cell appears to be a good model system for studying regulation of a neuronal nicotinic ACh receptor.

MATERIALS AND METHODS

Tissue culture. The rat pheochromocytoma cell line, clone PC12, was obtained from Dr. Lloyd Greene, Department of Pharmacology, New York University Medical Center. The cells were routinely grown in plastic tissue culture flasks in DMEM with 5% fetal bovine serum and 10% heat-inactivated horse serum at 37° in a humidified atmosphere containing 90% air and 10% CO_2. Animal sera were from K. C. Biologicals.

Ion flux measurements. The direct flux of cations through the ACh receptor channel was measured using $^{86}Rb^+$ in a modification (13) of the procedure described by Huang *et al.* (14) and Stallcup (11). The ACh-activated channel of both muscle and PC12 cells transports cations in a relatively nonselective manner, and it has been shown that the ion channel will allow passage of Rb^+ as effectively as Na^+ and K^+ (14). We have used $^{86}Rb^+$ rather than $^{22}Na^+$ since it is a less hazardous isotope.

Experiments were performed on cells that were replated from flasks onto poly-L-lysine-coated 24-well culture plates and used within 24 hr. Each well, which had a surface diameter of 16 mm^2, contained between 150 and 350 μg of cellular protein. Plates were first removed from the incubator and growth medium was replaced by 1 ml of a balanced salt solution consisting of 150 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 0.8 mM NaH_2PO_4, 10 mM glucose, 1.8 mM $CaCl_2$, and 15 mM HEPES (adjusted to pH 7.4 with NaOH). Cells were equilibrated with this buffer medium for 15 min at 22°. Ion flux measurements were carried out at 22° instead of 37° because desensitization is sufficiently slowed at this temperature to allow more accurate measurement of ion uptake. The actual ion uptake was performed in a modified assay medium in which the NaCl was replaced by sucrose to maintain osmolarity. The sodium-depleted conditions allowed enhanced influx of the tracer quantities of $^{86}Rb^+$. Initial experiments confirmed that qualitatively identical results were obtained if the assay was performed in the balanced salt solution, but the apparent uptake of $^{86}Rb^+$ was much lower and less accurately measured. Ouabain (Sigma) was also included in all flux assays at a concentration of 2 mM to inhibit (Na^+,K^+)-ATPase. This was necessary because Rb^+ behaves like K^+ due to the similarity of the ionic radii and thus will be pumped into the cell by the ATPase. Following a 1-min ouabain pretreatment, each well was washed twice with 1 ml of Na^+-depleted medium and uptake was initiated by exposing the cells to 0.2 ml of assay medium containing ouabain, 5 μCi/ml of $^{86}Rb^+$ (Amersham), and indicated concentrations of carbachol. Uptake was terminated after 20 sec (unless indicated) by aspirating the radioactive solution and rapidly washing four times with 1.0 ml of the balanced salt solution at room temperature. The washed cells were solubilized in 1 M NaOH overnight to permit determination of $^{86}Rb^+$ uptake and protein content. Radioactivity was measured by liquid scintillation counting of an aliquot of the solubilized cells. Uptake of $^{86}Rb^+$ was always determined in the presence and absence of agonist, and the difference was taken as receptor-mediated ion flux.

Normalization of $^{86}Rb^+$ uptake to the amount of protein in each well was done by prelabeling cells with [^{3}H]leucine while they were growing in culture flasks. Routinely, [^{3}H]leucine (0.075 μCi/ml) was added to the DMEM at least 48 hr prior to the time that cells were to be transferred to multiwell plates. At the time of transfer, the growth medium was switched to DMEM without [^{3}H]leucine. At the end of each experiment, four wells were randomly selected from each group of 24 for determination of both protein [by the method of Lowry *et al.* (15) using bovine serum albumin as a standard] and [^{3}H]leucine content. The amount of protein in each well was then calculated from the amount of ^{3}H in each well. This method was shown to give an accurate reflection of the amount of protein per well for a large range of cell densities.

In addition to normalizing uptake to the amount of protein in each well, it was necessary to normalize for the concentration of $^{86}Rb^+$ in the uptake medium to account for small day to day variations in the isotope concentration. Initial experiments demonstrated that carbachol-stimulated uptake of $^{86}Rb^+$ was linearly proportional to the concentration of $^{86}Rb^+$ over at least a 2-fold variation in concentration in the region of 5 μCi/ml. Therefore, all uptake values have been normalized to what they would have been if the concentration of $^{86}Rb^+$ was 5 μCi/ml.

For experiments utilizing chronic drug treatments, the drugs were dissolved in distilled H_2O and sterilized, and small volumes were added to the culture flasks. Each group of experiments was conducted on cells that came from a common parent culture to control for potential differences among cultures. Appropriate control flasks containing no treatment were also run in parallel for each chronic experiment to control for growth conditions. As described above, the cells were removed from the flasks and placed into the multiwell plates in the

presence of fresh drug or vehicle in serum-containing DMEM 12 to 24 hr prior to the uptake experiment. At the time of the experiment, the multiwell plate was removed from the incubator and the drug-containing growth medium was aspirated. Each well was then washed 4 times with 1 ml of serum-free DMEM and a final 1 ml of serum-containing DMEM was placed on each well. The plate was then replaced in the incubator for a recovery period of 30 to 40 min. Acute effects of the chronic drug treatments were reversed by this procedure while allowing the chronic effects to be seen. After this recovery period, the $^{86}Rb^+$ uptake procedure outlined above was performed on each well.

Measurement of membrane potential and intracellular volume. Membrane potential of the PC12 cells was determined by measuring the uptake of the lipophilic cation [^{3}H]TPP, which has been shown to distribute across biological membranes according to membrane potential (16). Cells were grown as described above in the presence and absence of carbachol and plated in 24-well plates as for determination of $^{86}Rb^+$ uptake. After transfer to a 37° water bath and removal of growth medium, cells were exposed to [^{3}H]TPP (1 μCi/ml, New England Nuclear) for 60 min in HEPES-buffered balanced salt solution. Excess [^{3}H]TPP then was removed by aspiration and washing. By varying the extracellular K^+ concentration from 5.4 (normal) to 105 mM (by isosmotic substitution of KCl for NaCl), the membrane potential dependence of [^{3}H]TPP uptake was determined. Although actual estimation of a numerical value for membrane potential with this technique can be complicated and must be interpreted cautiously (16), the technique does give a reliable measure of whether or not a treatment has altered the membrane potential. Intracellular volume was measured using ^3H_2O and [^{14}C]inulin (16).

RESULTS

Characterization of agonist-stimulated $^{86}Rb^+$ uptake. The time course of uptake of $^{86}Rb^+$ into the PC12 cells between 10 sec and 1 min (Fig. 1) was essentially linear through approximately 20 sec in both the presence and absence of carbachol stimulation. The net uptake is the difference between basal (unstimulated) and 1 mM carbachol-stimulated uptake. At stimulation times of greater than 20 sec, the response to carbachol began to plateau, which is indicative of receptor desensitization (17). From these experiments, an uptake time of 20 sec was chosen as the standard condition for the assay.

The concentration dependence for stimulation of $^{86}Rb^+$ uptake by carbachol was biphasic in nature (Fig. 2). The net uptake increased between the concentrations of 10 μM and approximately 1 mM carbachol, beyond which it diminished. The biphasic response to stimulation of the ACh receptor was seen for other agonists (data not shown) and thus was not a phenomenon restricted to carbachol. A similar biphasic response has been observed upon nicotinic receptor stimulation in other systems (18). The mechanism responsible for the biphasic dose-response curve is not known. However, there are several possibilities. The simplest one is that high agonist concentrations may lead to more desensitization (19), resulting in a decrease in the rate of ion flux into the cells. Alternatively, blockade of the ACh receptor ion channel by carbachol at higher concentrations may result in a decreased flux of ions during receptor activation.

The $^{86}Rb^+$ uptake elicited by carbachol was due to actual activation of nicotinic ACh receptors. Nicotine was more potent than carbachol in stimulating $^{86}Rb^+$ uptake, whereas methacholine, a muscarinic agonist, had no effect on $^{86}Rb^+$ uptake even at concentrations as high as 10 mM. In addition, simultaneous exposure to carbachol and the cholinergic antagonist mecamylamine resulted in a concentration-dependent inhibition of the $^{86}Rb^+$ uptake as shown in Table 1. Mecamylamine itself had no effect on basal uptake of $^{86}Rb^+$. A number of other cholinergic antagonists inhibited carbachol-induced $^{86}Rb^+$ influx with relative potencies consistent

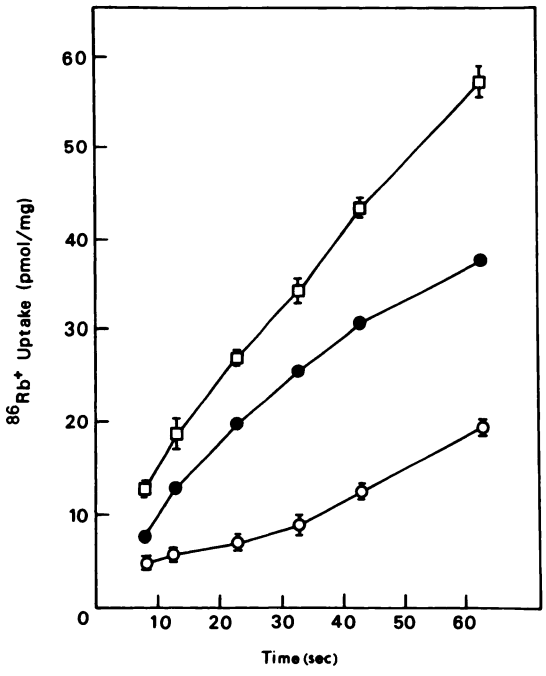

FIG. 1. *Time-dependence for uptake of $^{86}Rb^+$ into PC12 cells*
Basal and total $^{86}Rb^+$ uptake (stimulated with 1 mM carbachol) was measured at the indicated times as described in Materials and Methods. Net uptake is the difference between carbachol-stimulated and basal uptake. Each basal and carbachol-stimulated value is the mean ± standard error for four to six replicates. Basal uptake (○); 1 mM carbachol-stimulated uptake (□); net uptake (●).

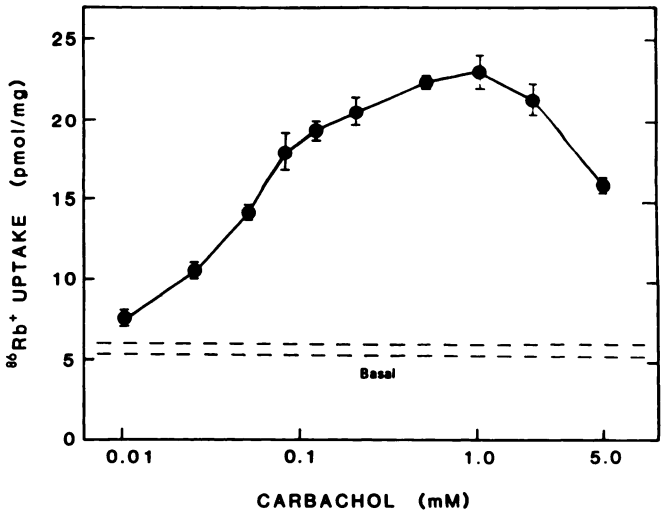

FIG. 2. *Concentration dependence for carbachol-induced $^{86}Rb^+$ uptake into PC12 cells*
Uptake of $^{86}Rb^+$ was measured at the indicated concentrations of carbachol after 20 sec of exposure as described in Materials and Methods. Each value is the mean ± the standard error for four to five replicates. The dashed lines bracket the range of basal uptake.

TABLE 1

Inhibition of carbachol-stimulated $^{86}Rb^+$ uptake into PC12 cells by mecamylamine

$^{86}Rb^+$ uptake was determined in cells exposed to the indicated concentrations of mecamylamine along with 500 μM carbachol as described in Materials and Methods. Basal uptake was taken as $^{86}Rb^+$ uptake in the absence of carbachol or mecamylamine. Each value is the mean ± the standard errors for four to five replicate cultures. Inhibition refers to inhibition of carbachol-stimulated uptake, i.e., after subtraction of basal uptake.

[Mecamylamine]	$^{86}Rb^+$ uptake	Inhibition[a]
μM	*pmol/mg protein*	%
0.0	51.3 ± 1.6	
0.01	47.7 ± 2.3	7
0.1	35.0 ± 1.3	37
1.0	16.5 ± 0.8	80
10.0	10.5 ± 0.5	93
Basal	7.6 ± 0.3	

[a] Based on uptake values after subtraction of basal uptake.

with ion influx being mediated by nicotinic rather than muscarinic receptors (data not shown). A detailed description of the pharmacologic characterization of these receptors will appear elsewhere.[2]

Characterization of the effects of chronic treatment with carbachol on $^{86}Rb^+$ uptake. Exposure of PC12 cells to agonists for periods of time greater than 1 min has been shown to cause receptor desensitization and loss of receptor responsiveness to subsequent stimulation (13, 17). This acute desensitization was reversed upon removal of agonist (17). In order to eliminate this acute desensitization from more prolonged adaptive responses, cells exposed to carbachol for prolonged periods of time were washed free of carbachol and allowed to recover for 40 min before assessment of agonist-induced uptake of $^{86}Rb^+$. As expected, initial experiments demonstrated that this procedure totally reversed receptor desensitization. For each condition of chronic treatment with other agents, an acute reversal protocol was tested to ensure that the effects being measured after chronic treatment were real and not artifacts of an altered acute response.

When PC12 cells were exposed to carbachol for prolonged periods of time, their subsequent responsiveness to carbachol stimulation was decreased and could not be recovered by the resensitization procedure. The concentration dependence for the effects of chronic carbachol treatment for 1 week is shown in Fig. 3. The dashed lines bracket the range of $^{86}Rb^+$ uptake under control conditions without carbachol treatment. Carbachol concentrations of 50 μM or less caused no consistent changes in carbachol-stimulated uptake. At carbachol concentrations of 100 μM and higher, however, net $^{86}Rb^+$ uptake declined in a concentration-dependent manner. It thus appeared that there was a threshold concentration for eliciting effects of chronic carbachol treatment. Beyond this threshold, the ion flux response of the ACh receptor progressively decreased as the concentration of carbachol in the growth medium was increased.

The extent of decreased responsiveness also was found

[2] D. Robinson and R. McGee, Jr., in preparation.

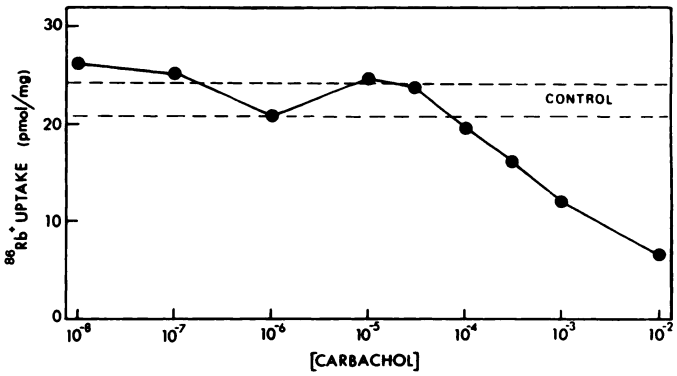

FIG. 3. *Concentration dependence for effects of chronic treatment with carbachol in PC12 cells*

Cells were exposed to the indicated concentrations of carbachol for 7 days. Carbachol was then removed from the growth medium, the cells were allowed to recover for 40 min at 37°, and $^{86}Rb^+$ uptake was measured as described in Materials and Methods. Net uptake is the difference between 1 mM carbachol-stimulated and basal uptake. Basal uptake was not different from control at any carbachol concentration. Each value is the mean of four to six replicates. The dashed lines bracket the range of net $^{86}Rb^+$ uptake under control conditions without carbachol treatment.

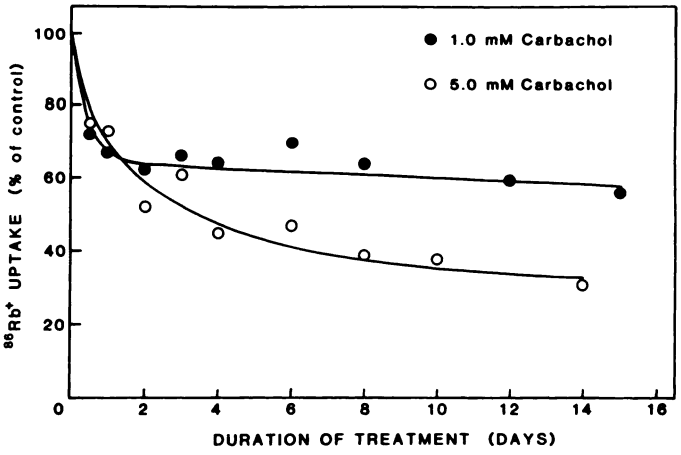

FIG. 4. *Time dependence for effects of chronic treatment with carbachol in PC12 cells*

Cells were exposed to 1 or 5 mM carbachol for the indicated times. At the end of the treatment time, the carbachol was removed from the growth medium, the cells were allowed to recover for 40 min, and $^{86}Rb^+$ uptake was measured as described in Materials and Methods. Net uptake is plotted as the percentage of control uptake (no carbachol exposure). Each value represents the mean of five to six replicates.

to be dependent upon the duration of carbachol exposure (Fig. 4). The net uptake of $^{86}Rb^+$ is plotted as a percentage of control uptake versus days of carbachol treatment for two different concentrations of carbachol (1.0 and 5.0 mM). For both concentrations, the net ion flux in response to acute receptor activation was decreased by 25% as early as 12 hr after addition of carbachol. This chronic effect appeared to plateau by 2 days of treatment with 1 mM carbachol, while cells grown with 5 mM carbachol continued to decline in their responsiveness until approximately 8 days of treatment. The maximal effect of the chronic treatment with 5 mM carbachol was approximately double the maximal effect of 1 mM carbachol

treatment. It can be seen that the extent of this "functional down-regulation" of the ACh receptor was dependent upon the exposure time and the concentration of agonist in the growth medium. In addition, the time required for a maximal chronic effect was dependent upon the agonist concentration; the peak effects of lower concentrations were reached more quickly than the peak effects of higher concentrations.

If chronic exposure to carbachol were activating a normal regulatory mechanism to cause a decrease in cellular responsiveness, then this change should be reversible upon cessation of receptor occupation. After 21 days of treatment, the cells were washed extensively to remove the carbachol-containing growth medium. In addition, a parallel group of cells was also washed to control for possible effects of the washing procedure. The data in Fig. 5 show that the cells did return to normal upon removal of carbachol and that recovery from the effects of 5 mM carbachol was complete within 5–8 days. Of importance is the fact that the time dependence was on the scale of days as opposed to minutes or hours, thus distinguishing this phenomenon from receptor desensitization.

Other effects of chronic exposure to carbachol. Growth of PC12 cells in the presence of carbachol caused no morphologic differences in the cells and no changes in the relative adhesiveness of the cells to either plastic tissue culture flasks or poly-L-lysine-coated surfaces. Also, no gross differences in rate of cell division could be detected on the basis of visual inspection of growing cells. To look more closely for less dramatic differences in cell growth, the effects of carbachol treatment on protein synthesis were determined by measuring the incorporation of [^{3}H]leucine into trichloroacetic acid-precipitable material. Even with prolonged exposure to high concentrations of carbachol, no effects on protein synthesis were observed (data not shown). Thus, apparent down-regulation of the ACh receptor was not the result of any overall adverse effect on cell growth and viability.

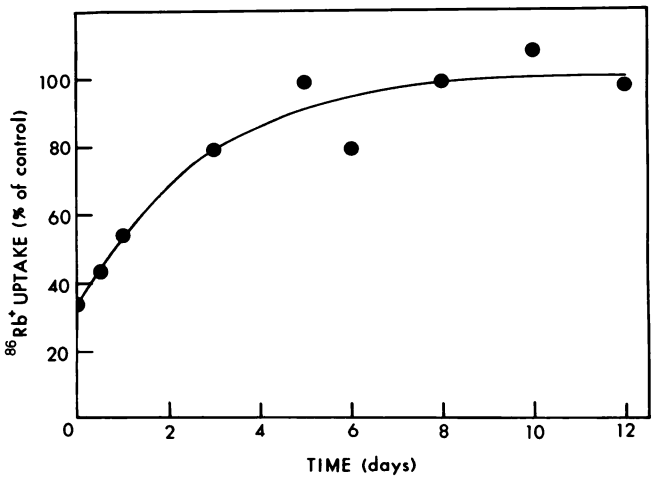

FIG. 5. *Recovery from effects of chronic treatment with carbachol in PC12 cells*

Cells were grown in the presence or absence of 5 mM carbachol for 21 days. At this time, the carbachol was removed from the growth medium and the cells were allowed to recover for the indicated times. ^{86}Rb$^+$ uptake was determined as described in Materials and Methods at each of the time points, with the data point at zero representing no reversal. Net uptake of ^{86}Rb$^+$ is plotted as percentage of control uptake (100% uptake occurring in control cultures which had never been exposed to carbachol). Each value represents the mean of five to six replicates.

Two other changes in the cells that could have led to decreased ^{86}Rb$^+$ uptake were a decrease in the resting membrane potential, which would reduce the driving force for ^{86}Rb$^+$ uptake upon receptor activation, or a decrease in cell volume. Indirect evidence against changes in either of these parameters was the absence of alteration in basal (unstimulated) uptake of ^{86}Rb$^+$ by carbachol-treated cells. Direct evidence for the absence of change in membrane potential in carbachol-treated cells was obtained by measuring the membrane potential-dependent distribution of [^{3}H]TPP into the cells. In addition to untreated cells, cells that had been chronically treated with carbachol were examined both in the continued presence of the drug and after a recovery period like that used to resensitize the receptors (Table 2). For all of the cells, increasing extracellular K^+ caused a progressive decrease in [^{3}H]TPP uptake. At 105 mM K^+, the cells will be almost completely depolarized, and the [^{3}H]TPP accumulated by the cells will primarily represent membrane potential-independent uptake. At no concentration of K^+ was any difference in uptake observed for any group of cells. Thus, chronic treatment with carbachol caused no change in resting membrane potential, and the decrease in agonist-induced uptake of ^{86}Rb$^+$ could not reflect a decreased driving force for uptake.

Cell volume also was unaffected by growth with carbachol. Control cells had volumes of 4.12 ± 0.15 and 4.25 ± 0.06 μl/mg of protein under resting and K^+-depolarized conditions, respectively, whereas cells exposed to 10 mM carbachol for 5 days had volumes of 4.13 ± 0.04 and 4.39 ± 0.09 μl/mg of protein under the same conditions. Therefore, decreased uptake of ^{86}Rb$^+$ did not result from a decrease in cell volume.

TABLE 2

Effects of chronic exposure to carbachol on membrane potential as determined by uptake of [^{3}H]methyltriphenylphosphonium

Cells were grown in the presence or absence of 5 mM carbachol for 4 days. Immediately prior to assessment of uptake of methyltriphenylphosphonium, half of the carbachol-treated cells were washed free of carbachol and allowed to resensitize as for ^{86}Rb$^+$ uptake measurements. The other carbachol-treated cells were continuously exposed to carbachol throughout the remainder of the experiment. Uptake of [^{3}H] methyltriphenylphosphonium was determined as described in Materials and Methods. Values represent the mean ± standard errors for four to six replicate cultures.

[K^+]	Control cells	Carbachol-treated cells	
		Resensitized	Not resensitized
mM		*cpm/μg protein*	
5.4	387 ± 10	381 ± 11	362 ± 11
20	316 ± 11	285 ± 8	313 ± 7
40	272 ± 2	260 ± 17	264 ± 9
70	229 ± 5	218 ± 6	231 ± 6
105	202 ± 5	203 ± 2	203 ± 4

Effects of chronic exposure to an antagonist on $^{86}Rb^+$ uptake. If the long term effects of carbachol represented changes induced by receptor activation, then the effects should not be seen upon exposure of the cells to a receptor antagonist. Furthermore, an antagonist should be able to prevent carbachol-induced changes. A number of antagonists were investigated but many were toxic to the cells, possibly due to nonspecific membrane-disrupting effects at the concentrations that adequately antagonized receptor activation. Mecamylamine, which was previously shown to antagonize receptor activation by carbachol, did not produce any toxic effects on the cells, even with prolonged exposure. Exposure of the cells to 100 μM mecamylamine for 2 days had no effect on carbachol-induced uptake of $^{86}Rb^+$ (Table 3). This verified that actual receptor activation, not simple receptor occupancy, was required to induce adaptation. Furthermore, this concentration of mecamylamine was able to prevent completely the cellular response to 5 mM carbachol in the growth medium for 2 days. These observations confirmed the hypothesis that carbachol was acting through a receptor-mediated mechanism which involved binding to the recognition site and subsequent receptor activation.

Properties of the acetylcholine receptors after adaptation. One explanation for the apparent functional "down-regulation" of the ACh receptors was that the receptor had altered kinetic or ion-conducting properties after chronic exposure to carbachol. To test this possibility, the time dependence for carbachol-stimulated uptake of $^{86}Rb^+$ in control and carbachol-treated cells was compared as shown in Fig. 6. After 1 week of exposure to 1 mM carbachol, net $^{86}Rb^+$ uptake was decreased from control at all stimulation times. The extent of the decrease was essentially the same at all time points, suggesting that the kinetic characteristics of the remaining ACh receptors had not been altered by the chronic agonist exposure. The concentration dependence of carbachol-stimulated $^{86}Rb^+$ uptake before and after 1 week of chronic carbachol treatment also was examined (Fig. 7). Net uptake in the carbachol-treated group of cells was decreased from control at all stimulation concentrations, but the EC_{50} for activation of the receptor was not different after chronic exposure to carbachol.

TABLE 3

Effects of mecamylamine treatment on $^{86}Rb^+$ uptake

Cells were grown under the four conditions indicated for 2 days and placed into multiwell plates. The drugs were washed out and the cells were allowed to recover for 40 min; then basal and carbachol-stimulated (1 mM carbachol) uptake of $^{86}Rb^+$ was determined as described in Materials and Methods. Basal uptake was the same for all treatment groups, 6.4 ± 0.2 pmol/mg of protein when all values were averaged. The uptake values are means ± standard errors for four to six replicates.

Growth condition	$^{86}Rb^+$ uptake	
	Total	Carbachol-stimulated
	pmol/mg protein	
Control	19.6 ± 0.4	13.2
100 μM mecamylamine	18.9 ± 0.4	12.3
5 mM carbachol	15.9 ± 0.4[a]	9.6
5 mM carbachol + 100 μM mecamylamine	21.1 ± 0.9	14.7

[a] Significantly different from control ($p < 0.05$) by Duncan's new multiple range test.

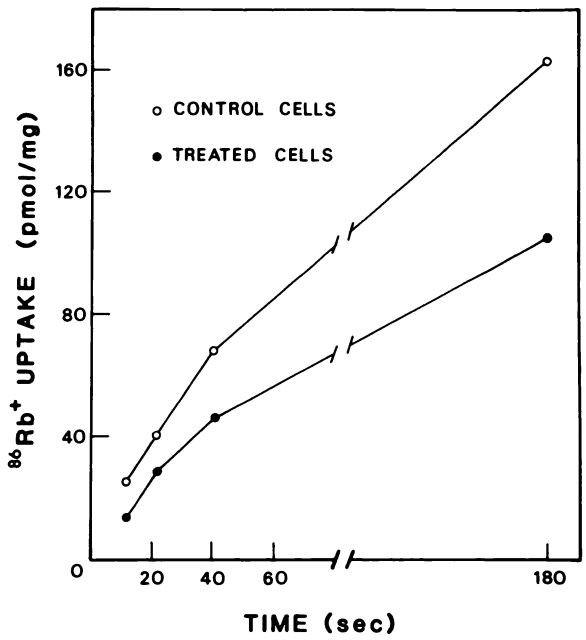

FIG. 6. *Time course for $^{86}Rb^+$ uptake in control and carbachol-treated PC12 cells*

Cells were grown in the presence or absence of 500 μM carbachol for 9 days. The carbachol was then removed from the growth medium, the cells were allowed to recover for 40 min, and $^{86}Rb^+$ uptake was determined for the indicated times as described in Materials and Methods. Net uptake of $^{86}Rb^+$ is the difference between stimulated (1 mM carbachol) and basal uptake at each of the time points. Basal uptake was not different between control and carbachol-treated cells at any time point. Values are the means of four to six replicates.

DISCUSSION

The neuronal nicotinic ACh receptors of the PC12 cells are subject to long term regulatory influences. Exposure of the cells to a nonhydrolyzable analog of ACh caused a decrease in the ability of the cells to respond to subsequent receptor activation. The extent of this functional down-regulation was dependent both on agonist concentration and time of exposure. No evidence of adaptation was observed until the concentration of carbachol reached at least 100 μM. Beyond this concentration, progressively greater adaptation occurred, until as much as 70% loss of responsiveness was seen. In contrast, direct acute activation of ion influx was elicited by at least a 10-fold lower concentration of carbachol and was maximal by 0.5–1.0 mM carbachol. Above 1 mM carbachol, $^{86}Rb^+$ uptake declined substantially, probably due to an increase in the rate of receptor desensitization. Thus, chronic down-regulation occurred primarily at concentrations beyond those that maximally activated the receptors, but which appeared to greatly facilitate desensitization.

We have referred to the decreased responsiveness of PC12 cells following exposure to carbachol as "functional down-regulation" or "adaptation." The ability of the cells, as model neurons, to respond to cholinergic stim-

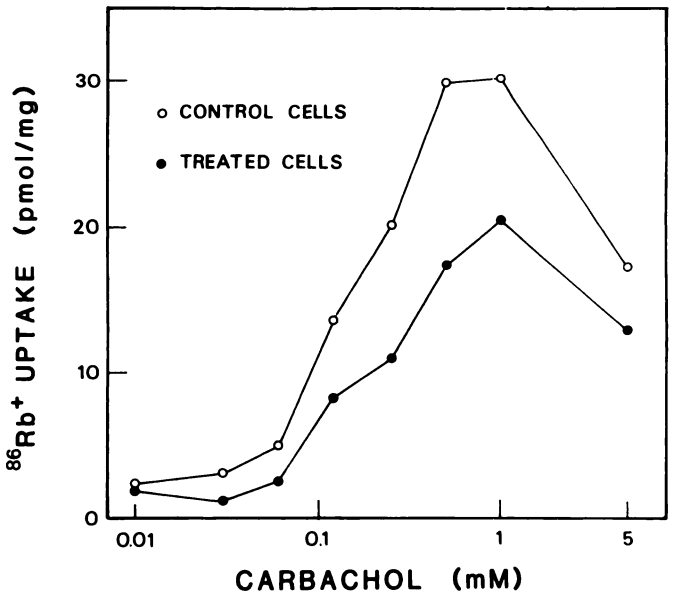

FIG. 7. *Concentration dependence of carbachol-stimulated $^{86}Rb^+$ uptake in control and carbachol-treated PC12 cells*

Cells were grown in the presence or absence of 500 μM carbachol for 7 days. The carbachol was then removed from the growth medium, the cells were allowed to recover for 40 min, and $^{86}Rb^+$ uptake was determined at the indicated concentrations of carbachol as described in Materials and Methods. Data are given as net uptake of $^{86}Rb^+$ which is the difference between stimulated and basal uptake. Basal uptake of $^{86}Rb^+$ was not different between control and carbachol-treated cells. Each value is the mean of three to six replicates.

ulation would most likely be reduced by this adaptation and therefore would be of functional significance to the cells. However, a very important initial question is whether the loss of responsiveness represents a change in receptor number and/or some other property of the receptors. Based on studies with many receptors (both neuronal and non-neuronal), the most likely explanation is simply that the number of receptors per cell has diminished. It is difficult to test this possibility directly by measuring the number of ACh receptors in the PC12 cells using classic binding techniques due to the low numbers of receptors present in the membranes. We have attempted binding measurements using [^{3}H]ACh of high specific activity with little success. In addition, the questionable location and function of the α-bungarotoxin sites in the PC12 cells (9, 12) has ruled out the possibility of using toxin binding as a probe for nicotinic receptor number. Without being able to look directly at receptor number, it was necessary to examine other properties of the ACh receptors in the down-regulated cells. Several pieces of evidence indicated that the properties of the functional receptors that remained in the membrane were unchanged. The EC_{50} for receptor activation was not altered by chronic carbachol treatment. In addition, the time dependence of carbachol-stimulated ion flux through the receptors was unchanged. Therefore, the simplest explanation of the phenomenon is that the number of receptors had decreased upon exposure to carbachol. Small changes in the single channel properties of the receptors, however, would not have been detected by the $^{86}Rb^+$ uptake assay.

Assuming at this point that the adaptation represents a loss of receptors, there appear to be similarities in this process between PC12 and muscle ACh receptors. Incubation of cultured rat myotubes with carbachol for 20 hr produced a decrease in the number of ACh receptors as determined by α-bungarotoxin binding (20). Little change was seen with 10 μM carbachol treatment, but 10 mM carbachol was able to produce a 40% decline in toxin binding (20). This concentration dependence for the chronic agonist-induced changes in toxin binding to muscle receptors was very similar to the change in responsiveness of the PC12 cells produced by chronic carbachol exposure. The EC_{50} for activation of ACh receptors of vertebrate muscle by carbachol (21, 22) also was in the same range as that determined for PC12 cell receptor activation. Thus, adaptive changes in both types of ACh receptors could only be induced by agonist concentrations which caused near maximal activation of the receptors. These concentrations are not excessive or necessarily unphysiologic, however, when one considers the concentrations of ACh achieved during synaptic transmission. Estimates of the ACh concentration in the neuromuscular junction during transmission have been made and generally range between 0.3 and 1.0 mM (23, 24).

In the three-state kinetic model for the nicotinic ACh receptor (25, 26), the binding of two agonist molecules to the inactive or resting state of the receptor-channel complex causes a conformational change to the open configuration and ion flux commences. Within seconds or less, the complex enters the desensitized state in which ion flux ceases. Progression through this sequence of conformational changes requires ligand binding and is dependent upon time and concentration. Therefore, in the presence of a high concentration of agonist during chronic treatment, a greater proportion of the receptor population will exist in the desensitized state at any point in time. A cycling through the various receptor conformations will continually occur, but the equilibrium will favor a shift to the desensitized state. The observation that regulation is only occurring at high concentrations of agonist in both muscle and PC12 cells may indicate that the cells are responding to an increase in the steady state proportion of desensitized receptors. Alternatively, high agonist concentrations could lead to a larger steady state number of open channels, which serves as the signal for down-regulation. It should be recognized that both of these hypotheses are based on the assumption that the neuronal nicotinic receptor undergoes the same state changes as other nicotinic receptors, which at present has not been clearly documented.

In addition to being a concentration-dependent phenomenon in the PC12 cells, down-regulation of the receptor response was also a time-dependent phenomenon. Exposure to 5 mM carbachol caused a greater degree of down-regulation and required a longer period of treatment to produce the full response than did exposure to 1 mM carbachol. A plateau was reached in 2 to 8 days depending on the carbachol concentration being used. With either concentration of carbachol, a large proportion of the change occurred within 12 hr, implying the

existence of a readily adjustable pool of membrane surface receptors. The fact that a plateau is reached rather than a continual decline in responsiveness shows that the cells have the capacity to sense the carbachol concentration and create a new steady state level of sensitivity. This is consistent with *in vivo* studies of many different types of receptors where adaptation proceeds to new steady states which are then stable with time (1, 27).

The changes in ion uptake elicited by chronic carbachol treatment were reversible. Recovery was evident 12–24 hr after removal of the agonist, and responsiveness returned to control levels within 5–8 days. The observation of reversibility is important beyond the simple demonstration of its occurrence. As discussed above, PC12 cells grown in the absence of carbachol can be thought of as representing a "denervated state" and, as such, should be maximally "up-regulated." Cells down-regulated by growth in the presence of carbachol could approximate more closely an "innervated" state. From this innervated state, up-regulation can be achieved by removing the carbachol. Thus, the cells display the full range of adaptations one would hope for in a system that is to model physiologic adaptation.

Our observation of functional down-regulation of the nicotinic response in PC12 cells is in contrast to data obtained by Siman and Klein (28); they observed no down-regulation after a 10-day exposure of the cells to carbachol. Siman and Klein measured agonist-induced uptake in Na^+-containing buffer, but this does not explain the discrepancy between the two studies. Even when assaying receptor function in the same solution as in the previous study, we still observed down-regulation.[3] The most likely reasons for the discrepancy between the two reports include variation in the source or age of the PC12 cells and/or their growth conditions. The PC12 cells used in our studies were obtained from Dr. Lloyd Greene and grown with fetal bovine serum. The cells in the previous report were obtained from Dr. James Patrick, Salk Institute, and grown with newborn calf serum with gentamicin. It is well established that different growth conditions select for different populations of cultured cells. We also have noted that cells grown for an excessive number of generations (6 months or more beyond thawing of frozen stock cultures) have an attenuated adaptive response to carbachol.

The effects of long term increases in receptor occupation obtained in PC12 cells are in agreement with the results reported for *in vivo* studies of chronic cholinesterase inhibitor administration (29, 30). It would be expected that inhibition of ACh hydrolysis would lead to increases in receptor stimulation and subsequent decreases in receptor numbers. The treatment schedule for the inhibitors utilized in these studies inhibited acetylcholinesterase by more than 80% and did cause decreases of 23–29% in ACh receptor numbers after 10 days of exposure. Estimates were not available for the actual synaptic concentrations of agonist attained; therefore, quantitative comparisons with our data are not possible. However, our data are in contrast to *in vivo* studies of chronic nicotine administration (29, 31), where *increases* in receptor numbers after 10 days of nicotine treatment were observed. The data from studies of chronic nicotine administration are difficult to interpret for several reasons. Due to its high lipid solubility, nicotine is able to penetrate biological membranes and thus crosses the blood-brain barrier easily. As a consequence of this property, nicotine may have various membrane-perturbing effects that could contribute to the unanticipated results seen following chronic treatment. In addition, the actual synaptic concentration of active drug obtained in these studies was difficult to estimate. Thus, for now, one must conclude that chronic exposure to nicotine *in vivo* produces receptor changes which cannot be accounted for simply by increased receptor occupancy by an agonist.

Neuronal nicotinic receptors appear to be subject to adaptive influences as with most other receptors that have been studied. It is interesting that continuous agonist exposure induces adaptation even though these receptors become desensitized under these conditions. Since synaptic transmission produces only intermittent periods of receptor activation, it was possible that continuous exposure to the agonist might not have triggered adaptation. Being able to study simple model systems using continuous agonist application as a first approximation to altered synaptic transmission should allow further study of the molecular events associated with adaptation of neuronal nicotinic receptors.

[3] D. Robinson and R. McGee, Jr., unpublished observation.

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Send reprint requests to: Dr. Richard McGee, Department of Pharmacology, Georgetown University, 3900 Reservoir Road, NW, Washington, D. C. 20007.