0026-895X/86/010006-07$02.00/0

MOLECULAR PHARMACOLOGY, 30:6-12

Two Components of Carbamylcholine-Induced Loss of Nicotinic Acetylcholine Receptor Function in the Neuronal Cell Line PC12

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Received November 26, 1985; Accepted April 10, 1986

SUMMARY

Loss of responsiveness of the neuronal-type nicotinic acetylcholine receptor (nAChR) on PC12 cells, a cell line derived from a rat pheochromocytoma, was induced by exposure to carbamylcholine (carbachol). Nicotinic receptor function was assessed by carbachol-induced $^{22}Na^{+}$ uptake. We found that, in addition to classically described desensitization, a second process occurs which results in a nonrecoverable loss of nAChR activity. This second process, which we have labeled inactivation, has a slower onset than the classically described desensitization ($t_{½}$ = 14.7 min for inactivation and 0.78 min for desensitization at 1 mM carbachol). Inactivation could not be explained by inadequate washing, a loss of electrochemical driving force, or a loss of cell viability. The onset of inactivation is dependent on the concentration of desensitizing ligand and is blocked by nicotinic antagonists. No recovery of the loss of activity from inactivation was observed even after 2 hr of incubation in recovery buffer. Inactivation does not appear to require formation of a desensitized state since desensitization was reduced in the absence of Ca^{2+} whereas inactivation was not affected by the absence of Ca^{2+}. The mechanism which underlies inactivation remains to be determined; however, it is possible that inactivation is the first step in nAChR down-regulation and it may also explain previous observations of rapid and prolonged tolerance to the effects of nicotinic agonists.

Desensitization is a process where repeated or prolonged exposure of a receptor to an agonist leads to a reduction in the magnitude of response to subsequent exposure to an agonist. This process has been described for the nicotinic acetylcholine receptor (nAChR) and a cyclical model for desensitization has been suggested (1, 2). This model consists of the receptor in a resting state which rapidly binds acetylcholine to form an active complex (i.e., open ion channel) which then slowly converts to a desensitized state (i.e., acetylcholine still bound but the channel non-conducting). After removal of free acetylcholine from the medium the agonist dissociates from the receptor and the receptor returns to the resting state. The conversion from the active state to the desensitized state has been shown to be accompanied by a change in the binding site for the agonist molecule from a low affinity state to a high affinity state (3–5). Thus, the continued presence of an agonist molecule will eventually shift nAChR from a low affinity resting state through an active state and then to a high affinity desensitized state.

Although this model has served well to explain many observations regarding desensitization, numerous investigators have reported that the process of desensitization is more complicated than this cyclical model. When investigators have looked at desensitization of nAChR from *Torpedo californica* in the millisecond-to-second time scale, using quench-flow techniques to measure ion flux (6–8) or by single-channel patch-clamp recording techniques (9), two phases of desensitization have been observed, one with a half-time of onset of 100–300 msec, and a second with a half-time of onset of 1–10 sec. These two phases of desensitization have also been observed by single-channel patch-clamp recordings of denervated cutaneous pectorius muscle of the frog (10) and bovine adrenal chromaffin cells (11), and by high frequency iontophoretic application of acetylcholine on denervated rat soleus muscle (12). When investigators have examined desensitization of nAChR in the second-to-minute time scale by measurement of acetylcholine-induced currents in voltage-clamped muscle fibers from frog (13, 14) and voltage-clamped neurons from *Aplysia* (15), the onset of desensitization was best fit by two exponential processes. Half-times of onset were found to be in the seconds and tens of seconds range. In addition to these observations, several investigators have noted that prolonged exposure to acetylcholine causes a slowing or incomplete recovery of receptor function and have concluded that this may represent yet another phase of desensitization (1, 12, 16).

The investigators who have reported incomplete recovery have only mentioned it as an anecdotal observation and, as yet,

This work was supported in part by National Science Foundation Grant BNS-8215572.

ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TPP, tetraphenylphosphonium; LDH, lactate dehydrogenase.

no one has made a systematic investigation into this phenomenon. We have examined desensitization of nAChRs on PC12 cells, a rat pheochromocytoma cell line (17), to determine whether these neuronal cells exhibit a nonrecoverable component as reported in other systems (1, 12, 16). $^{22}Na^+$ uptake measurements were used as the method to quantitate nAChR function. The nAChRs on these cells resemble mammalian neuronal nAChRs in that they are α-bungarotoxin insensitive (18). In this communication we demonstrate that the neuronal-type nAChRs found on these cells exhibit a nonrecoverable component to desensitization, and we have termed this process inactivation.

Materials and Methods

PC12 cell culture. PC12 cells (17) were obtained from Dr. George Hess, Cornell University (Ithaca, NY) and grown on 100-mm plastic tissue culture dishes in Dulbecco's modified Eagle's medium with 10% (v/v) horse serum, 10% (v/v) fetal calf serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and maintained as described by Stallcup and Patrick (19). Culture dishes were kept at 37° in a humidified atmosphere of 10% CO_2 and 90% air. For $^{22}Na^+$ flux assays, cells were triturated and replated with fresh medium/serum onto 35-mm plastic tissue culture plates coated with polylysine (100 μg of polylysine in 2 ml of phosphate buffered saline for 1 hr, then washed twice with phosphate-buffered saline). Plating density was approximately 10×10^6 cells/35-mm plate. Measurements were made 2–6 hr after replating.

$^{22}Na^+$ flux measurements. Flux experiments on 35-mm culture dishes were performed as follows. Medium was removed by aspiration and replaced with low Na^+ buffer (280 mM sucrose, 2 mM $CaCl_2$, 5 mM KCl, 5 mM glucose and 10 mM HEPES adjusted to pH 7.4 with NaOH), and the cells were allowed to equilibrate at room temperature for 2–12 min. Following the equilibration period the buffer was changed to the desensitization buffer (low Na^+ buffer with 0.1 mg/ml of bovine serum albumin plus various drug concentrations and mixtures) and the cells were incubated for various lengths of time (see figure legends for details). When the influence of various ions was tested, the sucrose concentration was reduced to maintain iso-osmotic buffers. After the appropriate desensitization time, the desensitization buffer was removed, the plates were washed twice with low Na^+ buffer, and the cells were allowed to recover for various lengths of time in the low Na^+ buffer. Each aspiration and replacement of buffer (2 ml volume) took approximately 6 sec. As evident from washing $^{22}Na^+$ from plates, two washes succeeded in removing greater than 99% of the desensitization buffer. After the appropriate recovery time, the recovery buffer was aspirated and replaced with high Na^+ buffer (140 mM NaCl, 2mM $CaCl_2$, 5 mM KCl, 5 mM glucose, and 10 mM HEPES adjusted to pH 7.4 with NaOH) which contained 0.5–1.0 μCi/ml of $^{22}Na^+$, 0.5 mM ouabain, 0.1 mg/ml of bovine serum albumin, and 3 mM carbachol. Control plates with no carbachol were always run in parallel and the uptake on these plates (generally less than 20% of carbachol-stimulated uptake) was subtracted from all values determined in the presence of carbachol. After 30 sec ($^{22}Na^+$ uptake is linear over this time period; see Ref. 20), the flux buffer was removed and the plates were immediately washed five times with 2 ml of wash buffer (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 25 mM Tris-HCl, pH 7.4). After washing, the cells were dissolved overnight in 2.0 ml of 0.4 N NaOH and the amount of radioactivity remaining with the cells was determined in a Beckman gamma counter (68% efficiency). All results are expressed as the percentage of control. Control consisted of plates run parallel in the assay but not exposed to the desensitization buffer. All points were done in duplicate. In high Na^+ buffers in our laboratory, 3 mM carbachol stimulated a specific $^{22}Na^+$ uptake for PC12 cells of 116 nmol/min/mg of cell protein (20).

TPP uptake experiments. To monitor the effects of various desensitization conditions on the membrane potential of the cells, we examined the distribution of [^{3}H]TPP, a charged molecule which will cross cell membranes and distribute according to the electrical gradient (21). Since we were only concerned with changes in the membrane potential relative to a control condition, we did not quantitate the membrane potential with [^{3}H]TPP but only monitored changes in the intracellular concentration. After the appropriate desensitization condition, 2 ml of low Na^+ buffer containing 2 μM TPP (spiked with 250,000 dpm of [^{3}H]TPP) were added to the plates for 16 min. The [^{3}H]TPP solution was then aspirated and the plates were rapidly washed three times with wash buffer (see flux assay). Plates were then treated as in the flux assay except that cell-associated tritium was determined by scintillation counting in a Beckman LS1800 liquid scintillation counter (46% efficiency). Results are expressed as percentage of control. Control uptake of [^{3}H]TPP was when cells were exposed to low Na^+ buffer during desensitization and during [^{3}H]TPP uptake.

LDH measurements. To verify cell viability during our experimental procedures, we monitored LDH activity in the supernatants from both the desensitization period and the recovery period. The assay procedure for LDH has been described previously (22). Maximum LDH activity was determined by treating plates with 1% Triton for 30 min followed by repeated sonications (three times for 30 sec). The supernatant from this treatment was kept on ice and assayed in parallel with the supernatants from the desensitization and recovery periods. Results are expressed as percentage of maximal activity.

Calculations and statistics. Data from recovery and onset time courses were computer fitted by the nonlinear least squares method of Marquardt and Levenberg adapted from Bevington (23). We first tried to fit recovery data to a single exponential assuming that recovery was complete. This was found to be inadequate since recovery was never complete in the time course of our observations. However, the recovery that was observable in the time course of our observations was well fit by a single exponential (see Results). To simplify discussion of the data, we have termed the nonrecoverable component inactivation and the recoverable component desensitization. Recovery from desensitization was fit to the equation:

$$Y = (Yo^*e^{-kt}) + I \quad (1)$$

where Yo is the maximum percentage inhibition from desensitization, I is the extent of inactivation (nonrecoverable activity), Y is the percentage inhibition of control flux at any time t, and k is the rate constant for recovery from desensitization. The values for Yo, I, and k were allowed to float in the fitting routine. Curves were fit to individual experiments and the resulting values were then averaged.

Onset of desensitization was determined by subtraction of the time course of inactivation from the time course of the total loss of receptor activity. Each component was then analyzed separately. The time course of inactivation was measured by incubation of the cells in desensitization buffer for various periods of time and then removal of the buffer and measurement of nAChR responses after 16 min of recovery (at this time recovery from desensitization was virtually complete; see Results). The values determined for the onset of inactivation were then subtracted from the corresponding values determined when no time was allowed for recovery (these values represent reduction in nAChR responses due to both desensitization and inactivation). The resulting values were considered representative of the desensitization component. Each component was then fit to the single exponential equation:

$$Y = (Yo^*e^{-kt}) + R \quad (2)$$

where Yo is the maximum activity lost due to desensitization or inactivation, R is the amount of activity remaining at equilibrium, Y is the activity at time t, and k is the rate constant of onset of desensitization or inactivation. The values for Yo, R, and k were allowed to float in the fitting routine. Curves were fitted to the averaged data from three to seven experiments.

The data were statistically analyzed as listed in each legend. A paired Student's t test was used when two conditions were compared to each

other and both conditions were run in parallel; $p < 0.05$ was considered statistically significant. Statistical methods can be found in Zar (24).

Materials. $^{22}Na^+$ was obtained from Amersham (Arlington Heights, IL) or New England Nuclear (Boston, MA). [^{3}H]TPP was purchased from New England Nuclear. Tissue culture supplies were purchased from Gibco (Grand Island, NY). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Computer analysis was performed on a Digital Equipment Corp. PDP 11/24 processor.

Results

Incubation of cells with carbachol produced a time-dependent loss in subsequent carbachol-induced $^{22}Na^+$ uptake (Fig. 1A, *open circles*). This has been previously described by many investigators and termed desensitization. If cells were exposed to carbachol for 16 min and then allowed to recover in carbachol-free buffer, the recovery of activity proceeded in a single exponential manner. However, only a portion of the activity returned even when recovery was allowed to proceed for up to 128 min (Fig. 1*B*).

To dissect the individual components of loss of activity, hereafter termed desensitization (recoverable) and inactivation (nonrecoverable), we performed a series of experiments to examine the onset of each component. If cells are incubated with carbachol for various periods of time and then washed free of drug and allowed to recover for 16 min, a time when recovery from desensitization is complete (Fig. 1B), the time course of the onset of inactivation can be examined (Fig. 1A, *open squares*). The onset of loss of activity measured when no recovery is allowed (Fig. 1A, *open circles*) is thus the sum of desensitization and inactivation. Close examination of this curve indicates a biphasic nature (Fig. 1A, *inset*). To isolate the onset of desensitization from the onset of inactivation we subtracted the amount of activity lost due to inactivation from the values determined when no recovery was allowed. The resulting values are a measure of the onset of desensitization, and we found these values to be well described by a single exponential (Fig. 1A, *solid circles*). The half-time for onset at 1.0 mM carbachol was found to be 0.78 min for desensitization and 14.7 min for inactivation. Recovery from desensitization was found to be independent of the desensitizing concentration of carbachol ($t_{1/2}$ = 1.39 ± 0.14 min for 0.1 mM carbachol and 1.54 ± 0.07 min for 1.0 mM carbachol) (Fig. 1B). Inactivation was essentially nonrecoverable since, even if recovery was allowed to proceed for 128 min, there was no further increase in activity over that found after 16 min of recovery (Fig. 1B).

Further examination of these two components revealed that the rates of onset of both desensitization and inactivation were dependent on the concentration of agonist (Fig. 2). Interestingly, when 10 mM carbachol was used to induce loss of receptor function, the loss of activity that was attributable to desensitization actually decreased from that observed with 1.0 mM carbachol (Fig. 2A). The decreased desensitization can be explained by the observation that the amount of inactivation with 10 mM carbachol is greatly accelerated such that nearly 50% of the activity is nonrecoverable within 2 min (Fig. 2B). This would suggest that the inactivation process is occurring in parallel with desensitization and, at high concentrations of

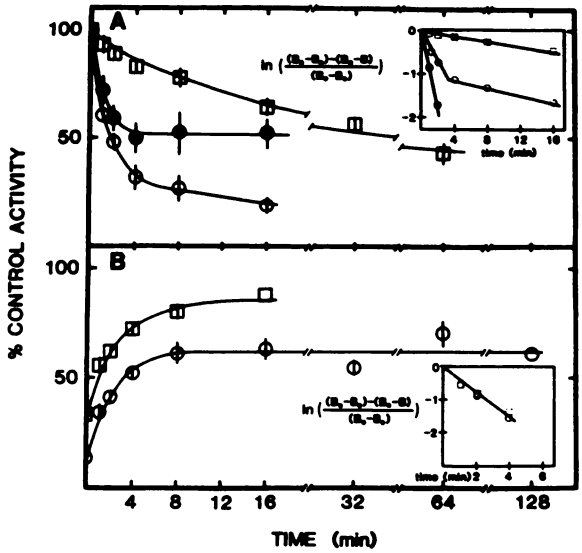

Fig. 1. Time courses of onset (A) and recovery (B) of desensitization and inactivation. A, Cells were incubated in the desensitization buffer (1 mM carbachol) for the time indicated. ○, carbamylcholine-stimulated $^{22}Na^+$ flux measured immediately after the desensitization period; □, cells allowed to recover for 16 min before the flux measurement. The loss of activity shown (□) is inactivation (nonrecoverable). ●, difference between the two sets of data; this loss of activity reflects desensitization (recoverable). *Curves* (□, ●) are from the fit of the data as described in Materials and Methods. *Inset*, semilog plot of the data. B, Cells were desensitized for 16 min and then allowed to recover for the time indicated. ○, cells incubated with 1.0 mM carbamylcholine; □, cells incubated with 0.1 mM carbamylcholine. *Curves* shown are from the nonlinear fit to the data as described in Materials and Methods. *Inset*, semilog plot of the data. *Points* in A and B with *error bars* (±SE) are the mean of four to seven independent determinations each done in duplicate. See Materials and Methods for experimental details.

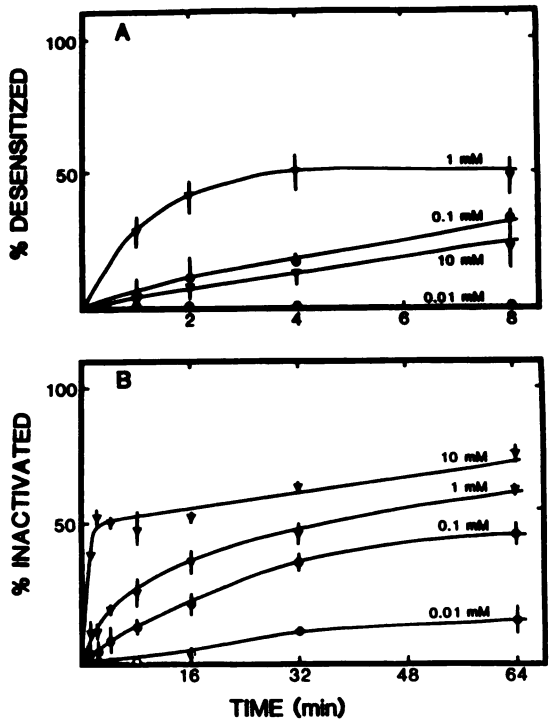

Fig. 2. Time courses of onset of desensitization (A) and inactivation (B) with different concentrations of agonist. *Points* in A were generated as described in Fig. 1 for desensitization (concentrations of carbamylcholine are indicated on the figure). *Points* in B were generated as described in Fig. 1 for inactivation (concentrations of carbamylcholine are indicated on the figure). Each *data point* is the average of two to seven independent determinations each done in duplicate (*error bars* are ±SE).

carbachol, proceeds at such a rapid rate that the number of receptors available to be desensitized has been reduced.

Since the activity lost by inactivation appeared to be nonrecoverable, we proceeded with a series of investigations to demonstrate that the loss in activity was not an artifact of the protocol or the assay system. The loss of activity was shown to be due to a decrease in maximum activity and not a decrease in receptor sensitivity to carbachol since the EC_{50} for carbachol stimulation of flux was unchanged following inactivation (data not shown). Increasing the number of washes between the desensitization period and the recovery period from two to four increased the amount of activity recovered by only a few per cent (data not shown). Cell viability in our assay system was examined by measurement of LDH activity in the supernatants from both the desensitization period and the recovery period. In all situations (0, 1, 5, or 15 min in desensitization buffer and 15 min in recovery buffer) and at both concentrations of carbachol tested (0.1 and 1.0 mM), the amount of LDH activity in the supernatant was within 3–5% of control (data not shown; see Materials and Methods for control conditions).

To test whether changes in intracellular ion concentrations occurring during the experimental manipulations were responsible for the nonrecoverable loss of activity, we examined the effects of various ionic compositions of the desensitization buffer. It was found that depolarization of the cells by high K^+ (140 mM) caused a slight reduction in $^{22}Na^+$ uptake if the uptake was measured immediately after the depolarization buffer was removed from the cells; however, this loss of activity was recovered after 16 min in recovery buffer (Fig. 3, *bars* labeled high K^+,$-CC$). This observation would indicate that a small portion of what we have labeled desensitization could be due to depolarization during the desensitization period. Effects of depolarization were further examined by performing desensitization experiments in a depolarizing buffer. It was found that the extent of desensitization and inactivation was not significantly altered by the presence of 140 mM KCl in the desensitizing buffer (Fig. 3, compare *bars* labeled *low* Na^+,$+CC$ to those labeled *high* K^+,$+CC$). The possibility that Ca^{2+} played a role in either of these two processes was examined by excluding Ca^{2+} from the desensitization buffer. Absence of Ca^{2+} in control conditions (no exposure to carbachol) had no effect on subsequent flux measurements (data not shown). When Ca^{2+} was left out of the desensitizing buffer in the presence of carbachol, the amount of desensitization was significantly reduced, whereas inactivation was not affected (Fig. 3, *bars* labeled *high* K^+,$+CC$,$-Ca^{2+}$). Finally, experiments with [^{3}H]TPP showed that there was no change in the electrical gradient at the time when inactivation was assessed (Table 1).

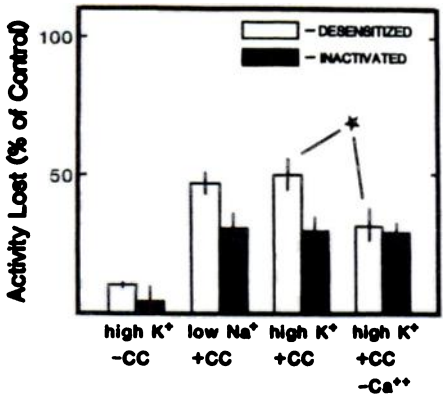

Fig. 3. Effects of changes in the ionic composition of the buffer applied during the desensitization period. Cells were first equilibrated in low Na^+ buffer for 5–10 min and then exposed to the desensitization buffer for 16 min. At the end of the desensitization period cells were washed twice with low Na^+ buffer, and $^{22}Na^+$ uptake was determined either immediately or 16 min after recovery in low Na^+ buffer. □, amount of activity that returned during the recovery period (i.e., desensitized activity); ■, amount of activity that did not recover (i.e., inactivated activity). The 100% value was when cells were exposed to only low Na^+ buffer throughout the protocol. High K^+ buffer was 140 mM KCl. *+CC* or *−CC* indicates the presence or absence of 1.0 mM carbachol during the desensitization period. The *bars* labeled $-Ca^{2+}$ had Ca^{2+} omitted from the buffer. (See Materials and Methods for buffer composition.) Each condition was tested three to five times with each determination done in duplicate (*error bars* are ±SE). ★, significant difference determined by paired Student's *t* test, $p < 0.005$.

To verify that the effects we were observing were specifically mediated by the nAChR, we examined whether the nicotinic antagonists *d*-tubocurare and gallamine could block the activity loss. Both compounds were found to inhibit carbachol-stimulated $^{22}Na^+$ uptake into the cells when added simultaneously with carbachol (Fig. 4A). Gallamine was found to be of extremely low potency. When examined for their effects on desensitization, we found that both *d*-tubocurare and gallamine by themselves appeared to induce some desensitization (i.e., slowly recoverable loss of activity; Fig. 4B). Whether this is true desensitization or reflects a slow dissociation of the antagonist from the receptor cannot be ascertained by our experiments. The fact that the flux recovered to 100% of control after gallamine alone would indicate that the wash procedure was adequate to remove all of the gallamine applied. This conclusion, however, cannot be made for *d*-tubocurare. The lack of full recovery of the flux after *d*-tubocurare could be due to it remaining bound to the receptor after the recovery period, or *d*-tubocurare may have agonistic properties with respect to inducing inactivation. It has previously been reported that *d*-tubocurare is able to stabilize a conformation of the nAChR that is similar to the desensitized state (25). Thus, neither compound proved to be ideal in its ability to block all of the effects we were observing. However, we did find that both compounds were able to attenuate carbachol-induced inactivation (Fig. 5). Gallamine (5 mM) reduced carbachol-induced inactivation by approximately 50%. This is in reasonably close agreement with the amount of inhibition of carbachol-induced $^{22}Na^+$ uptake produced when gallamine is added to the flux

TABLE 1

Effect of desensitization on membrane potential

Membrane potential was assessed by the uptake of [^{3}H]TPP over the 16 min of recovery. See Materials and Methods for experimental details and buffer composition. CC is 1.0 mM carbachol.

Desensitization condition	Recovery condition	N	Percentage of control
Low Na^+ buffer	Low Na^+ buffer	3	100[a]
Low Na^+ buffer + CC	Low Na^+ buffer	3	106 ± 1
High K^+ buffer + CC	Low Na^+ buffer	3	97 ± 1
High K^+ buffer + CC − Ca^{2+}	Low Na^+ buffer	3	98 ± 2
Low Na^+ buffer	High K^+ buffer	2	34 ± 5[b]

[a] This condition is defined as control.
[b] Significantly different from control ($p < 0.001$, Dunnett's test).

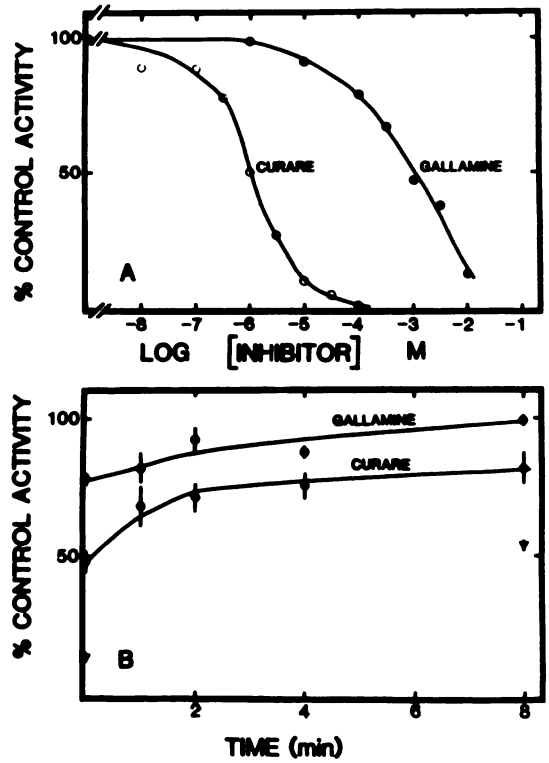

Fig. 4. Effects of nicotinic antagonists on receptor activation and recovery from desensitization. A, Concentration-inhibition curves for *d*-tubocurare (○) and gallamine (●) when added simultaneously with flux buffer ($^{22}Na^+$ plus 1.0 mM carbamylcholine). B, Time course of recovery of carbamylcholine-induced flux after cells have been incubated for 16 min with 10 μM *d*-tubocurare (○), 5 mM gallamine (●), or 1.0 mM carbamylcholine (▽). Each *point* is the mean of three to four independent determinations each done in duplicate (*error bars* are ±SE).

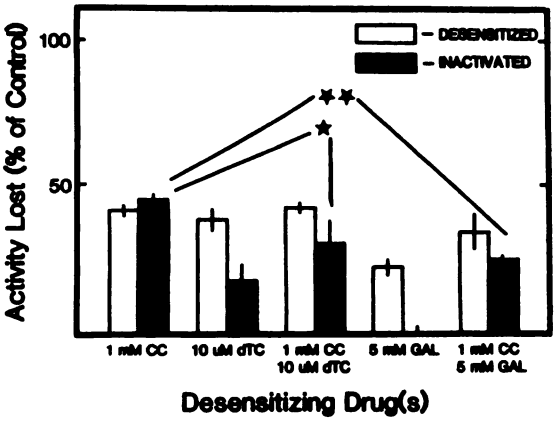

Fig. 5. Effects of nicotinic antagonists on the extent of desensitization and inactivation. □, amount of desensitization (i.e., recoverable loss of activity) caused by the indicated drug(s); ■, amount of inactivation (i.e., nonrecoverable loss of activity) caused by the indicated drug(s). *CC*, carbamylcholine; *dTC*, *d*-tubocurare; *GAL*, gallamine. Desensitized and inactivated activity was determined as outlined in Fig. 3. Each condition was determined in duplicate in three or four independent experiments (*error bars* are ±SE). ★, significantly different (Student's *t* test, $p < 0.05$); ★★, significantly different (Student's *t* test, $p < 0.001$).

medium (Fig. 4A). Unlike gallamine, which caused no inactivation by itself, 10 μM *d*-tubocurare did cause some loss of activity that did not recover in 8 min (Fig. 4B). However, the loss of activity caused by *d*-tubocurare was not as great as that caused by 1.0 mM carbachol (17% versus 45%, Fig. 5). When both compounds were present during the desensitization period, 29% of the receptors were inactivated (Fig. 5). This value is significantly lower than that with carbachol alone ($p < 0.05$) and, if one assumes that all of the *d*-tubocurare has been washed from the flux plate, this suggests that *d*-tubocurare is a partial agonist with respect to inactivation. Regardless of the complications in interpreting the *d*-tubocurare results, these experiments indicate that inactivation is mediated by a specific binding site for carbachol.

Discussion

The data presented in this communication would indicate that, in the time period we have examined, two agonist-induced processes cause a loss of nAChR function. One of these processes leads to a recoverable loss of activity, which we have labeled desensitization, and the other process leads to a nonrecoverable loss of activity, which we have labeled inactivation. The apparent rate of onset and the extent of desensitization are dependent on the concentration of desensitizing ligand and are not affected by depolarization of the cell. The apparent rate constant of recovery from desensitization is independent of the concentration of desensitizing ligand. In addition, we found that desensitization was decreased in the absence of Ca^{2+}. The loss of activity we call desensitization is undoubtedly analogous to desensitization as reported by numerous other investigators in various other systems determined by many different protocols (1, 3, 4, 5, 12, 16, 19). The rate of onset of desensitization has been shown to be dependent on the concentration of desensitizing ligand (1, 3, 4, 16) and to be affected by Ca^{2+} (26), although this last observation is not universally agreed upon (1, 12). Scubon-Mulieri and Parsons (27) have reported that desensitization is sensitive to membrane potential, an effect we did not observe. However, they compared desensitization rates in voltage-clamped neuromuscular junctions at potentials of −40 mV and +40 mV. Our protocol would only depolarize the cells to 0 mV. The difference between our observation and that of Scubon-Mulieri and Parsons (27) could be that the change in desensitization rate they observed occurs primarily when the membrane potential is greater than 0 mV.

The apparent rate of onset we have observed in our system agrees well with rates reported for desensitization when bath application is used to administer the cholinergic agonist (4, 14, 19). We would not expect to detect a desensitization occurring in the millisecond time range since an event this rapid would be obscured during the 30-sec uptake period used in our assay. In addition, since the first time point we examined after desensitization was 1 min, any process that occurs in shorter time scales would also be obscured. We have previously reported that $^{22}Na^+$ uptake is linear over the first 30 sec of measurement (20). This observations would indicate that either there is not a desensitization process that occurs in the seconds time range or, if there is, the amount of uptake that occurs before this desensitization is complete is insignificant compared to the flux that occurs over 30 sec (if there had been significant uptake, the linear portion of the curve should have intersected the *y* axis at a value greater than 0). Chestnut (14) has previously demonstrated that a bath application of desensitizing ligand tends to obscure the delineation of the two components of desensitization measured by iontophoretic applications of acetylcholine (seconds and tens of seconds time scales). It is reasonable that our assay system is obscuring the desensitiza-

tion process which occurs in the milliseconds to seconds time range, but our measurements would indicate that the loss of flux activity due to this desensitization is relatively insignificant compared to the activity remaining.

The limitations of our assay system in regard to a rapid desensitization do not apply when events of longer time scales are of interest. What is new in this communication is the characterization of a nonrecoverable component of desensitization which we have termed inactivation. The half-time for onset of this effect is much longer than the half-time for the more classical desensitization (14.7 min for inactivation versus 0.78 min for desensitization at 1.0 mM carbachol). This rate of onset of inactivation is dependent on the concentration of inactivating ligand. An observation that gives us particular confidence in the relevance of inactivation is that, with 10 mM carbachol, the amount of desensitization is decreased from that at 1 mM carbachol, an observation that is explained by a large increase in inactivation at this concentration of carbachol. Inactivation does not seem to be affected by the removal of Ca^{2+} and it is blocked by nicotinic antagonists.

There are two explanations for the nonrecoverable decrease in receptor activity which we have termed inactivation. One is that functional nAChRs are being internalized by the cell such that the actual number of cell surface receptors has decreased. The other is that prolonged exposure to agonist induces a conformational change in the receptor to either a nonactivatable state or a state that has a reduced conductance. Recovery from either of these conformations would occur with an extremely long half-time. A desensitization with very slow onset and recovery kinetics has been observed in nAChRs from electroplax (28). These desensitized receptors were shown to recover with a half-time of 46 hr. However, we cannot directly compare our results with these since these experiments were performed on nAChRs in reconstituted membrane vesicles. These receptors could be allowed to recover for days without the complications a living cell would add to the interpretation. It is possible that we would observe full recovery if we carried our experiments for longer periods of time. However, since the turnover time of nAChR is on the same order of magnitude of what we would expect for recovery from inactivation (29–31) any recovery data would be difficult to interpret because the results would not distinguish between recovery from inactivation and synthesis of new receptors. We do not believe that a subconducting state of the receptor has been induced, for a subconducting state has not been observed in patch-clamp experiments which examined desensitized receptors (11). The data we have presented are consistent with either a receptor internalization or a slowly reversible conformational change of the receptor, and we are currently addressing this question with binding studies that allow localization of the receptors during these events.

Our results are similar to those recently reported by Robinson and McGee (30). They found that carbachol down-regulated receptors on PC12 cells when they added carbachol directly to the growth medium. Unfortunately, their first time point (12 hr after addition) is beyond the time we have examined. However, the majority of the down-regulation they observe occurs in the first 12 hr. It is attractive to speculate that the inactivation we observe might be the first step in agonist-induced down-regulation of the receptor. Thus, it is likely that what Robinson and McGee (30) called down-regulation is the same as what we have termed inactivation.

At this time it is premature to propose a model for the transitions of the nAChR between the resting, activated, desensitized, and inactivated states. The fact that nicotinic antagonists attenuate carbachol-induced inactivation would indicate that this is an effect mediated by the acetylcholine-binding site on the nicotinic receptor. It is not clear whether inactivation proceeds from the resting, activated, and/or desensitized state(s). However, two lines of evidence would indicate that desensitization and inactivation are parallel processes. First, removal of Ca^{2+} reduces desensitization without affecting inactivation, and second, at high concentrations of carbachol, desensitization is reduced whereas inactivation is increased, as if the two processes were competing for a single pool of receptors. In addition, modeling the desensitization events of the nAChR is further complicated by the accumulated evidence from numerous laboratories indicating many desensitization processes. Desensitization associated with millisecond kinetics (6–11), second kinetics (1, 6–16, 26, 27), tens of seconds kinetics (3–5, 13–15, 19, and this paper), and slowly reversible or nonreversible desensitization occurring in minutes (1, 5, 16, 28, 30, and this paper) have all been reported. The interconversions between these various states and the resting and activated states and the role of these states in controlling nAChR responsiveness remain to be elucidated.

In conclusion, we have characterized an agonist-induced loss of activity of the nAChR that is different from what has previously been described in the literature as desensitization. This loss of receptor activity would appear to be irreversible given the lifetime of a nicotinic receptor (29). A significant aspect of our observations is that the appearance of this loss is rapid relative to receptor degradation. If this process is the first step in receptor down-regulation, the rate of agonist-induced receptor down-regulation may, at least in PC12 cells, occur faster than previously reported (30). In addition, experiments which examine desensitization in the minutes time range should control for this nonrecoverable component. Inactivation may also explain the rapid and long-lasting tolerance to the effects of nicotine on the stimulation of adrenocorticotropin and prolactin (32).

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