Slotte, J. P. (1992) *Biochim. Biophys. Acta 1123*, 326–333.
Slotte, J. P., & Bierman, E. L. (1988) *Biochem. J. 250*, 653–658.
Slotte, J. P., & Grönberg, L. (1990) *J. Lipid Res. 31*, 2235–2242.
Slotte, J. P., Hedström, G., Rannström, S., & Ekman, S. (1989) *Biochim. Biophys. Acta 985*, 90–96.
Slotte, J. P., Härmälä, A.-S., Jansson, C., & Pörn, M. I. (1990) *Biochim. Biophys. Acta 1030*, 251–257.
Verger, R., & De Haas, G. H. (1973) *Chem. Phys. Lipids 10*, 127–136.
Yeagle, P. L. (1985) *Biochim. Biophys. Acta 822*, 267–287.
Yeagle, P. L., Martin, R. B., Lala, A. K., & Bloch, K. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4924–4926.

# On the Mechanism of a Mammalian Neuronal Type Nicotinic Acetylcholine Receptor Investigated by a Rapid Chemical Kinetic Technique. Detection and Characterization of a Short-Lived, Previously Unobserved, Main Receptor Form in PC12 Cells†

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ABSTRACT: The mammalian nicotinic acetylcholine receptor in PC12 cells has many properties characteristic of the neuronal receptors involved in key chemical reactions that are responsible for signal transmission between cells of the nervous system. This report describes initial investigations of the mechanism of this receptor using a rapid chemical kinetic technique with a time resolution of 20 ms, which represents a 250-fold improvement over the best time resolution (5 s) employed in previous studies. Carbamoylcholine, a stable analogue of the neurotransmitter acetylcholine, was the activating ligand used, and the concentration of open transmembrane receptor–channels in PC12 cells was measured by recording whole-cell currents at pH 7.4, 21–23 °C, and a transmembrane voltage of −60 mV. Two receptor forms that account for 80% and 20% of the receptor-controlled current were detected; the main receptor form, accounting for 80% of the whole-cell current, desensitized completely before the first measurements had been made in previous studies. Only the main receptor form has been investigated so far using the new method. The constants of a mechanism that accounts for the concentration of the open transmembrane receptor–channel over a 100-fold range of carbamoylcholine concentration were evaluated: the dissociation constant of the site controlling channel opening ($K_1$ = 2.0 mM), the channel-opening equilibrium constant ($\Phi^{-1}$ = 5.0), and the dissociation constant of an inhibitory site to which carbamoylcholine binds ($K_R$ = 6.5 mM). These evaluated constants allow one to calculate $P_o$, the conditional probability that at a given concentration of carbamoylcholine the receptor–channel is open. $P_o$ was also determined in the presence of 2 mM carbamoylcholine by an independent method, the single-channel current-recording technique, and the agreement between the $P_o$ values obtained in two independent ways is within experimental error. This result indicates that the time resolution of the chemical kinetic technique employed was sufficient to evaluate the constants pertaining to the active state of the receptor, which forms a transmembrane channel, before its conversion to desensitized receptor forms with different properties. Previous kinetic measurements with a time resolution of 5 s showed that many compounds, such as anesthetic-like molecules, nerve growth factor, and substance P, modify the function of the neuronal receptor in PC12 cells or react specifically with the neuronal but not with the muscle receptor, for example, some toxins. The results presented here indicate, however, that in previous experiments the properties of only inactive (desensitized) receptor forms were observed; these forms have ligand-binding properties quite different from those of the active receptor forms. The effects all these compounds have on the active receptor form that leads to channel opening and signal transmission are, therefore, still unanswered and interesting questions for future research.

The neuronal nicotinic acetylcholine receptor is one of many membrane-bound proteins involved in the key reactions that determine whether signals are transmitted between cells of the nervous system (Kandel & Schwartz, 1985). Ultimately signal transmission depends on the concentration of open transmembrane channels formed in these reactions. Understanding the mechanism that allows the concentration of open receptor–channels to be adjusted by a variety of factors, and thus whether or not a signal is transmitted between nerve cells, continues to be a major challenge for all the neurotransmitter receptors so far identified (for example, those for acetylcholine, glycine, glutamate, and γ-aminobutyric acid). Our aim was to determine what one can learn about a receptor mechanism

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using a chemical kinetic technique with a 20-ms time resolution that we had developed (Udgaonkar & Hess, 1987a) for studying neurotransmitter receptors in single cells of the nervous system. The kinetic measurements described here are an extension of our previous studies in which a quench-flow technique with a 5-ms time resolution was used to investigate acetylcholine receptor-controlled cation flux in membrane vesicles prepared from the electric organ of *Electrophorus electricus* (Hess et al., 1979, 1983) in which the receptor concentration is high. The chemical reaction leading to the open-channel in the *E. electricus* receptor, based on quench-flow measurements (Hess et al., 1979; Aoshima et al., 1980), was formulated (Cash & Hess, 1980) as

mechanism I

$$\begin{array}{ccccccc} A + L & \overset{K_1}{\rightleftharpoons} & AL & \overset{K_1}{\rightleftharpoons} & AL_2 & \overset{\Phi}{\rightleftharpoons} & \overline{AL_2} \\ & & k_{21}\uparrow\downarrow k_{12} & \alpha & k_{43}\uparrow\downarrow k_{34} & & \\ & & IL & \overset{K_2}{\rightleftharpoons} & IL_2 & & \end{array}$$

A represents the receptor in its active form, L represents acetylcholine (or another activating ligand, for instance, carbamoylcholine), and the subscript indicates the number of ligand molecules that are bound to the receptor molecule. The binding of two ligand molecules to the frog muscle receptor prior to channel opening has been indicated previously (Katz & Thesleff, 1957). $\overline{AL_2}$ represents the open-channel form of the receptor and mediates the exchange of inorganic ions across the membrane, thus initiating an electrical signal and signal transmission. $K_1$ is the intrinsic dissociation constant of the receptor site that controls channel opening, and $\Phi^{-1}$ is the channel-opening equilibrium constant (Cash & Hess, 1980). Two first-order transitions to inactive (desensitized) receptor forms have been observed: a rapid process (milliseconds) was first discovered for the receptor from the electroplax of *E. electricus* (Hess et al., 1979), and a slow process (seconds to minutes), which is not shown in mechanism I, was first discovered in investigations of the frog muscle receptor (Katz & Thesleff, 1957). For the receptor of *E. electricus*, the slow process has a time constant of hours (Aoshima, 1984). $\alpha$ is the rate coefficient for the interconversion between active and inactive receptor forms. The inactive, desensitized receptor forms (IL and $IL_2$) have ligand-binding properties, characterized by the dissociation constant $K_2$, that are different from those of the receptor form involved in signal transmission (Katz & Thesleff, 1957; Cash & Hess, 1980; Hess et al., 1983; Changeux et al., 1984) and dominate the measurements once the receptor has desensitized (Cash & Hess, 1980; Hess et al., 1983). The activating ligand not only induces desensitization but also binds to an inhibitory site, which is characterized by the dissociation constant $K_R$; this process is not shown in mechanism I.

A quench-flow technique (Hess et al., 1979; Cash & Hess, 1981) allowed us to evaluate the constants relevant to channel opening and signal transmission before receptor desensitization occurred (Hess et al., 1983), and to calculate the concentration of the open receptor-channels, in *E. electricus* electroplax over wide (5000-fold) concentration ranges of acetylcholine and its analogues, in the presence and the absence of receptor inhibitors (Hess et al., 1983, 1987; Udgaonkar & Hess, 1986). The existence of a desensitized form of the receptor in absence of activating ligand, the formation of open transmembrane channels by the desensitized receptor form, or the direct conversion of the open-channel form to a desensitized receptor are neither required nor excluded by the quench-flow measurements. These steps have, therefore, been omitted from mechanism I. The quench-flow technique that we used in investigations of the *E. electricus* receptor (Hess et al., 1979) has also been used to study the acetylcholine receptor from the electric organ of *Torpedo* spp (Neubig & Cohen, 1980; Walker et al., 1981a,b, 1982; Ochoa et al., 1989) and the $\gamma$-aminobutyric acid receptor in membrane vesicles prepared from rat brain (Cash & Subbarao, 1987a,b, 1988).

Use of the quench-flow technique is restricted, however, to only a few receptors that occur in sufficient abundance in nature to enable one to prepare membrane vesicles. Direct measurements of receptor function in cells overcome the problem of preparing and purifying membrane vesicles. However, in ion flux measurements with cells, the time interval between the mixing of cells with a ligand and tracer ions and the first measurement, the time resolution of the technique, has been many seconds (Catterall, 1975; Stallcup & Cohen, 1976), during which the receptors are likely to be converted to desensitized forms whose properties then dominate the measurements. To overcome the problem of receptor desensitization in kinetic investigations with single cells, the rapid flow of solutions containing receptor-activating ligands over a cell surface was introduced (Krishtal & Pidoplichko, 1980). In those experiments, the concentration of open receptor–channels was measured by a whole-cell current-recording method (Hamill et al., 1981; Marty & Neher, 1983). The seal between the recording electrode and the cell membrane is unstable at high solution flow rates. We modified the flow method by allowing solutions to flow at a moderate rate over a cell surface so that many measurements using different concentrations of ligand can be made with the same cell (Hess et al., 1987; Udgaonkar & Hess, 1987a). We then used the hydrodynamic theory for solution flowing over submerged spherical objects (Landau & Lifshitz, 1959; Levich, 1962) to correct the observed concentration of open receptor–channels for desensitization that occurs during the time the receptors equilibrate with the activating ligand (Hess et al., 1987; Udgaonkar & Hess, 1987a). In the chemical kinetic investigations with PC12 cells reported here, this approach, which we call cell-flow by analogy with the quench- and stopped-flow techniques, has a time resolution of 20 ms, whereas in previous investigations of the receptor in PC12 cells the first measurements were made at least 5 s after mixing the cells with tracer ions and an activating ligand (Stallcup & Patrick, 1980), and hence a 250-fold improvement over previous investigations was achieved in the present approach. Carbamoylcholine, a stable analogue of acetylcholine, was used as the activating ligand.

The nicotinic acetylcholine receptor in PC12 cells is of interest because it is a mammalian receptor with properties that are common to neuronal receptors (Deneris et al., 1989). In contrast to the muscle type of acetylcholine receptor, the PC12 receptor and other neuronal acetylcholine receptors are not inhibited by a highly specific snake neurotoxin, $\alpha$-bungarotoxin (Patrick & Stallcup, 1977a; Clark, 1987), although they are inhibited by other specific toxins (Ravdin & Berg, 1979; Yamada et al., 1985; Loring & Zigmond, 1988; Lukas, 1989). Valuable and interesting information about the mechanism of mammalian neuronal receptors came from a previously developed method that allowed one to measure the uptake of inorganic ions by cells in culture (Catterall, 1975; Stallcup & Cohen, 1976). The effects of various acetylcholine or carbamoylcholine concentrations on the flux of inorganic tracer ions (Patrick & Stallcup, 1977b; Simasko et al., 1985; McHugh & McGee, 1986; Boyd, 1987; Lukas, 1989), of

compounds that are known to modify the function of nicotinic acetylcholine receptors (Stallcup & Patrick, 1980; Karpen et al., 1982; Amy & Bennett, 1983; Sachs et al., 1983; Mitsuka & Hatanaka, 1984; Simasko et al., 1985; McHugh & McGee, 1986; Boyd & Leeman, 1987; Whiting et al., 1987), and of chemical modification of the receptor (Leprince, 1983) on receptor function in PC12 cells were all studied in this way. The time resolution in these studies was between 5 s and several minutes (Patrick & Stallcup, 1977b; Simasko et al., 1985; McHugh & McGee, 1986; Boyd, 1987; Lukas, 1989). The results obtained here show that the conversion of the active receptor, responsible for signal transmission, to inactive (desensitized) receptor forms, which have different properties, occurs before the mixing of reagents with PC12 cells occurred in the tracer flux experiments. Therefore, the effects of various compounds or chemical modification on the receptor forms that desensitize before the first measurement of tracer ion flux was made were not observed.

The questions we address are, What information about receptor mechanisms can one obtain using single neuronal cells and the cell-flow technique with a 20-ms time resolution? Are the mechanism and constants describing the function of mammalian neuronal receptors different from those pertaining to the receptor of the *E. electricus* electric organ, which is of the muscle type? Are tracer flux measurements with a time resolution of ≥5 s adequate to evaluate the constants that determine the concentration of open receptor–channels, and do they allow one to evaluate the effects of compounds and chemical modification of the receptor on the function of the active form of the receptor in PC12 cells prior to desensitization?

## Experimental Procedures

*Cell Culture.* The PC12 cell line was isolated by Greene and Tischler (1976) from rat pheochromacytoma cells. When the cells are exposed to nerve growth factor (NGF), they differentiate to become mature sympathetic cells (Greene & Tischler, 1982) containing an increased number of nicotinic acetylcholine receptors (Dichter et al., 1977; Amy & Bennett, 1983; Mitsuka & Hatanaka, 1984; Whiting et al., 1987). The PC12 cells we used were provided by Drs. J. Patrick and J. M. Boulter (Salk Institute, CA) and Dr. E. Racker (Cornell University, NY); unless otherwise noted, the cells from the Salk Institute were used in the experiments described here. Cells were cultured at 37 °C in Dulbecco's Modified Eagle's Medium with 1000 mg/L glucose (DMEM-1000) (GIBCO, NY), supplemented with 10% heat-inactivated fetal bovine serum (GIBCO, NY) and 10% heat-inactivated horse serum (GIBCO, NY), in a humidified atmosphere of 6–8% $CO_2$ and 92–94% air, in 250-mL T-flasks at a density of $2 \times 10^5$ to $10^6$ cells per flask, and were passed twice a week. For single-channel current recordings, the cells were plated on 35-mm plastic dishes (Corning, NY), which had been coated with 5 μg of rat tail collagen (type VII, Sigma) dissolved in 100 μL of 60% ethanol–40% water and dried in a sterile air stream in a laminar flow hood. The stock solution of collagen was made by dissolving 5 mg of collagen in 5 mL of 0.2% acetic acid. For differentiation induced by 2.5S NGF (Collaborative Research, MA, or Boehringer Mannheim, IN), cells were cultured in DMEM-1000 supplemented with 5–10% fetal bovine serum, 5–10% horse serum, and 50–100 ng/mL NGF. The medium was exchanged after 3 days, and the cells were cultured for 2 more days. By this time, most cells had long processes. In the absence of NGF treatment, the cells contained only a few receptor molecules per cell; NGF treatment increased the receptor density (Dichter et al., 1977; Amy & Bennett, 1983; Mitsuka & Hatanaka, 1984; Whiting et al., 1987). Except for one experiment (Figure 1C), only NGF-treated cells were used in our measurements.

For cell-flow experiments, the cells were replated on day 5 at a density of 20–100 cells/mm$^2$ on 35-mm collagen-coated plastic dishes in RPMI 1640 medium (GIBCO, NY) supplemented with 5% heat-inactivated fetal bovine serum and 10% heat-inactivated horse serum. When used within 24 h after removing the cells from this medium, the cells could be suspended from a recording electrode in a stream of solution emerging from the cell-flow device. This procedure resulted in PC12 cells that were round and without processes. Single-channel measurements were made after the cells had been cultured in this medium for 1–5 days. The cells were fed with fresh medium every 2 or 3 days.

*Whole-Cell Recording.* An amplifier (List L/M-EPC7) was used for the current-recording measurements. The solution in the pipet was 145 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, and 25 mM HEPES (pH 7.4), and the bath solution was 150 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 5 mM glucose, and 25 mM HEPES (pH 7.4). In some experiments 145 mM KCl was replaced by 145 mM CsCl, which gave the same results as KCl. Glass pipets (electrodes) were pulled on a home-made puller and fire-polished; the electrode resistance was typically 3–6 MΩ when filled with the pipet solution described above. The series resistance was typically 5–20 MΩ. The transmembrane voltage of −60 mV in the experiments was held constant (±6 mV) as described in detail by Sigworth (1983). In whole-cell current measurements (Hamill et al., 1981), the current signal from the amplifier was passed through a low-pass filter (Krohn-Hite, MA, model 3322) with a cut-off frequency of 0.1–1 kHz (−3 dB point), amplified, and digitized at a 100–200-Hz sampling frequency, and then stored on a hard disk of a PDP 11/23 minicomputer for later analysis. The fitting of a decay phase and the calculation of the current amplitude corrected for receptor desensitization were done on a Prime 750 computer using a data analysis program LTPLOT, a Convex C210 computer using a data analysis program PLOT (Material Science Center, Cornell University), or an IBM-PC compatible computer (Northgate Elegance 386, 33 MHz) using GENPLOT version 0.91 or 1.01 (Computer Graphics Service, Ithaca, NY). All experiments were performed at a transmembrane voltage ($V_m$) of −60 mV and at room temperature (21–23 °C) and pH 7.4.

*Rapid Application of Ligand Solutions.* The flow method used to apply a ligand solution rapidly to cells at constant transmembrane voltage has been described (Krishtal & Pidoplichko, 1980; Udgaonkar & Hess, 1987a). In brief, a U-shaped stainless steel capillary tube (250-μm i.d.) with a circular porthole, approximately 150 μm in diameter, at the base of the U was connected to pumps at both ends so that the solution containing ligand could be driven into the tube at one end at a certain flow rate and withdrawn from the other end of the U-tube at twice that flow rate. The porthole of the U-tube was placed about 50–100 μm away from a cell attached to a whole-cell current-recording electrode. A solenoid valve between the suction pump and the U-tube was normally open but could be closed by an electrical pulse so that flow through the porthole was inverted. The flow rate of the solution emerging from the porthole was about 1 cm/s. The time required for the concentration of the ligand in the emerging solution to change from zero to its final value was on average about 20 ms and depended on the distance between the porthole of the flow device and the PC12 cell. This time interval was determined by changing the conductance of the solution

emerging from the flow device and using an electrode to measure the change of conductance (Fenwick et al., 1982) in the area normally occupied by a PC12 cell in a cell-flow measurement.

Usually the ligand solution flowed over the cell, and the resulting current flowing through the open receptor–channel was recorded, for 5–10 s, at a sampling interval of 5 or 10 ms. At the end of the experiment, the flow was reversed for a period of 7–8 min to remove the solution containing the ligand. The measurements were then repeated using a different ligand concentration, 6–15 experiments being done with the same cell. Carbamoylcholine, a stable analogue of acetylcholine, was the activating ligand.

*Calculation of Corrected Current Amplitude.* Because some receptor molecules begin to desensitize before the current reaches a maximum amplitude, an effect that is not always negligible, the amplitude corrected for desensitization was calculated using (Udgaonkar & Hess, 1987a; Hess et al., 1987):

$$I_A = (e^{\alpha\Delta t} - 1)\sum_{i=1}^{n}(I_{obs})_{\Delta ti} + (I_{obs})_{tn} \quad (1)$$

$I_A$ is the current amplitude corrected for receptor desensitization, $(I_{obs})_{\Delta ti}$ is the observed current during the $i$th time interval, and $(I_{obs})_{tn}$ the observed current during a time interval greater than the time to reach the maximal current. $\alpha$ is the rate coefficient for receptor desensitization, and $\Delta t$ is the sampling time interval.

*Single-Channel Recording.* The composition of the pipet solution was the same as that of the bath solution in the whole-cell recording experiments except that carbamoylcholine was added. Cells that had long processes were chosen, and the current was recorded from the soma membrane in a cell-attached mode (Hamill et al., 1981). After each single-channel measurement, resting membrane potentials were measured in a whole-cell recording configuration as the voltage required to minimize the flow of currents. Data were stored on an FM analogue tape using a Racal storage 4DS recorder. For analysis, data were first low-pass-filtered with a cut-off frequency of 0.5–1.5 kHz (−3 dB point), adjusted so that the average base line deviation was 8–10 times less than the unit amplitude of the single-channel current. After filtering, the data were digitized at a sampling frequency (5 or 10 kHz) at least three times the cut-off frequency of the low-pass filter, using a PDP 11/23 minicomputer, and were stored on a hard disk. The data were then transferred to a Prime 750 or a Convex C210 computer for the actual analysis. An automated analysis program based on and modified from the IPROC program developed by Sachs et al. (1982) was used to detect events.

## RESULTS

Figure 1 shows the results of a cell-flow experiment in which 2 mM carbamoylcholine flowed from the porthole of the U tube over a PC12 cell suspended in the solution stream, and the resulting whole-cell current was measured. The current reached a maximal value within 40 ms (Figure 1A); the time-dependent decay of the current, considered to be due to receptor desensitization, in two different time regions is shown in parts a and b of the figure. Two exponentials are required to fit the decaying phase, corresponding to rate coefficients for desensitization, $\alpha$ and $\beta$, of 2.1 and 0.4 $s^{-1}$, respectively. The percent of the current, corrected for receptor desensitization, associated with the rate coefficients $\alpha$ and $\beta$ is 80% and 20%, respectively. It should be noticed that in kinetic measurements with a time resolution of 1 s or greater, only the second, minor receptor component will be observed.

In the experiment shown in Figure 1B, a commonly used rapid perfusion technique, in which the cell remains attached to the culture dish during the measurements, was employed. The upper trace was obtained in the presence of 1 mM carbamoylcholine. Even though the flow rate of the solution was somewhat larger than the one used in the experiment illustrated in Figure 1A, the time for the current to reach its maximal value was considerably (~30 times) longer than in the experiment in Figure 1A. In the type of experiment illustrated in Figure 1B, the rate-limiting step in the equilibration of receptors with ligand is expected (Landau & Lifshiftz, 1959; Levich, 1962) to be the rate of transfer of the ligand molecules through the diffusion layer to the cell surface; if it is slow compared to receptor desensitization, as it appears to be from the current rise time (Figure 1B), the receptor will desensitize during the equilibration process. In contrast, it has been calculated (Hess et al., 1987) that in the cell-flow technique (Figure 1A) the transfer of ligand from the flowing solution to the cell surface occurs within 2 ms, a time interval that is short compared to receptor desensitization.

To ascertain that the major component of the carbamoylcholine-induced cell current in PC12 cells, a component that has not been observed previously, reflected the properties of the nicotinic acetylcholine receptor, we tested the effect of a specific inhibitor, *d*-tubocurarine, on the current. The upper trace (Figure 1B) was obtained in the presence of 1 mM carbamoylcholine and the lower trace in the presence of 1 mM carbamoylcholine and 50 $\mu$M *d*-tubocurarine. A 50% decrease in the whole-cell current was observed; the minor, more slowly desensitizing receptor form was completely inhibited by 50 $\mu$M *d*-tubocurarine, and the rapidly desensitizing form was 35% inhibited. The rapidly desensitizing receptor form was further inhibited at higher concentrations of *d*-tubocurarine.

The same perfusion technique was used in the experiments in Figure 1, panels B and C. However, the upper trace in Figure 1C was obtained in the presence 1 mM carbamoylcholine and the lower trace in the presence of 1 mM carbamoylcholine and 100 $\mu$M suberyldicholine. Suberyldicholine activates nicotinic acetylcholine receptor–channels in muscle cells (Adams, 1977; Koblin & Lester, 1978; Neher & Steinbach, 1978) and in *E. electricus* electric organ (Pasquale et al., 1983). In contrast, suberyldicholine is an inhibitor of the neuronal receptor in PC12 cells (Figure 1C); in the experiments described here, both receptor forms were inhibited between 60% and 70% by 100 $\mu$M suberyldicholine.

The effect of carbamoylcholine concentration on the main receptor species present, characterized by a rate coefficient for desensitization, $\alpha$, of 2.1 $s^{-1}$ (Figure 1A), was analyzed in order to evaluate the parameters that control the formation of transmembrane channels. The current due to the second, minor component, which desensitizes to the extent of only ~1% during the rising phase of the current, was subtracted from the main receptor component. The remaining current was corrected for receptor desensitization using eq 1 (Udgaonkar & Hess, 1987a) and designated as $I_A$, with $I$ representing the current and A the active, undesensitized receptor form. The value of $I_A$ as a function of carbamoylcholine concentration was determined from experiments such as the one illustrated in Figure 1A. The effect on $I_A$ of a 100-fold change in carbamoylcholine concentration is demonstrated in Figure 2A. Because the concentration of receptors, as measured by the whole-cell current, differs considerably from

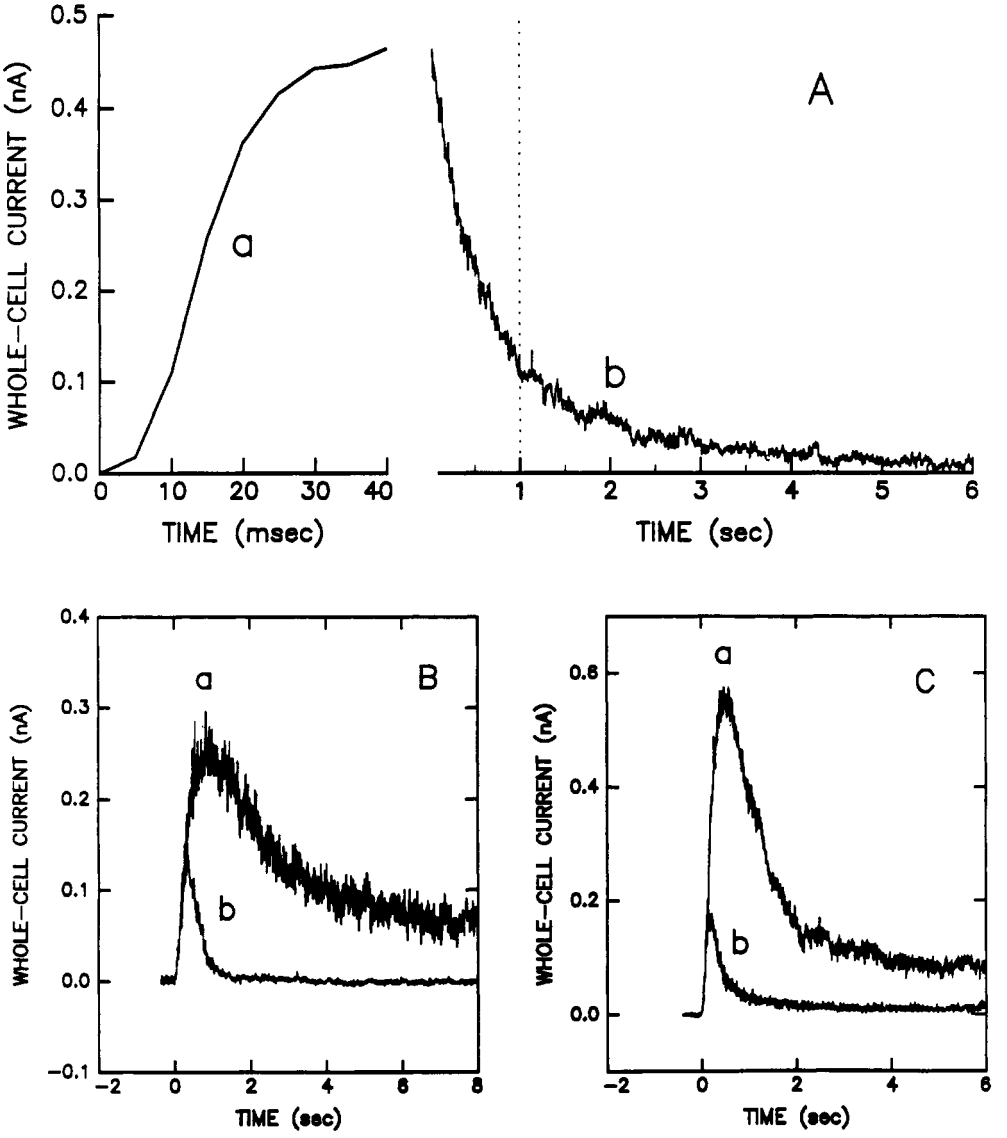


FIGURE 1: Examples of flow experiments with PC12 cells. The whole-cell current is a measure of the number of receptors in the cell membrane in the open-channel form. The whole-cell transmembrane voltage was maintained at −60 mV at 21–23 °C, pH 7.4. In panel A, a cell-flow technique was used (see Experimental Procedures); in panels B and C a rapid perfusion technique was used, in which solutions from the flow device were allowed to flow over a PC12 cell attached to the tissue culture dish. All cells came from the Salk Institute. The PC12 cells used in the experiments shown in Figures 1A and 2A,B were received in September, 1987, and those used in the experiments shown in Figure 1B,C were received in July, 1986. The cells used for the experiments in Figure 1C were not treated with NGF. (A) The flow rate of the solution emerging from the flow device was approximately 1 cm $s^{-1}$, and the carbamoylcholine concentration was 2 mM. The current reached its maximum value within 40 ms. Approximately 80% of the current decayed with a rate coefficent $\alpha$ of 2.1 $s^{-1}$, and approximately 20% of the current decayed with a rate coefficent $\beta$ of 0.4 $s^{-1}$. (B) The flow rate of the solution was approximately 1.5 cm $s^{-1}$, and the carbamoylcholine concentration (upper curve, a) was 1 mM. The rise time of the current was approximately 800 ms. Approximately 80% of the current decayed with a rate coefficient $\alpha$ of 0.5 $s^{-1}$. The decay of the second component was too slow to be measured during the time period of observation, 8 s. The lower trace (b) was obtained with the same cell, but *d*-tubocurarine (50 $\mu$M) was added to the carbamoylcholine solution flowing over the cell. Only a single exponential decay of the current was observed. The whole-cell current decreased by 50% (compared to that observed without *d*-tubocurarine), and $\alpha$ increased by a factor of about 6 to a value of 3.0 $s^{-1}$. (C) The flow rate of the solution was approximately 1.5 cm $s^{-1}$, and the carbamoylcholine concentration (upper curve, a) was 1 mM. The rise time of the current was approximately 475 ms; approximately 85% of the current decayed with a rate coefficient $\alpha$ of 1.2 $s^{-1}$. The decay of the second component, characterized by the rate coefficient $\beta$, was too slow to evaluate. The lower trace (b) was obtained with the same cell, but suberyldicholine (100 $\mu$M) was added to the carbamoylcholine solution flowing over the cell. The current due to the rapidly desensitizing receptor form was decreased by approximately 70%, and that due to the slowly desensitizing form was decreased by approximately 60%. The values of $\alpha$ and $\beta$ are 4.0 and 0.4 $s^{-1}$, respectively.

cell to cell, all $I_A$ values were normalized to the value (1.0 nA) obtained at 1 mM carbamoylcholine. The value of $I_A$ first increased with increasing carbamoylcholine concentration and then decreased at carbamoylcholine concentrations 4 mM and larger. However, the value of the desensitization rate coefficient, $\alpha$, increased with increasing carbamoylcholine concentration (Figure 2B), at least to a carbamoylcholine concentration of 10 mM, even though $I_A$ decreased by 33% when 10 mM carbamoylcholine was used. A decrease in the fraction of receptors in the open-channel form, represented by a decrease in $I_A$ without a concomitant decrease in $\alpha$ at high concentrations of activating ligands, was previously observed in studies of the acetylcholine receptors from the electroplax of *E. electricus* and *T. californica* (Pasquale et al., 1983; Shiono et al., 1984; Takeyasu et al., 1983, 1986).

The relationship between $I_A$, the concentration of open receptor–channels, and the constants pertaining to the mechanism has been derived in terms of the mechanism for the receptor from the electric organ of *E. electricus* (Cash & Hess, 1980; Pasquale et al., 1983; Udgaonkar & Hess, 1987a). When the initial concentration of the activating ligand is much larger than the moles of receptor molecules in the membrane

$$I_A = I_M R_M (\overline{AL_2})_o \frac{K_R}{K_R + L} \quad [2(i)]$$

$$(\overline{AL_2})_o = L^2[L^2(1 + \Phi) + 2K_1 L\Phi + K_1^2\Phi]^{-1} \quad [2(ii)]$$

$I_M$ is the current due to 1 mole of open receptor–channels, $R_M$ represents the moles of receptors in the cell membrane, $K_1$ is the dissociation constant of the receptor site controlling channel opening, $K_R$ is the dissociation constant of the regulatory (inhibitory) site, $\Phi^{-1}$ is the channel-opening equilibrium constant, and $(\overline{AL_2})_o$ is the fraction of receptor molecules in the open-channel form. All five constants in eq 2 can be determined, starting with $K_R$. When the ligand concentration $L$ is much larger than the dissociation constant $K_1$, and the two receptor sites controlling channel opening are saturated with activating ligand, $(\overline{AL_2})_o$ is given by $I_M R_M(1 + \Phi)^{-1}$ (Cash & Hess, 1980), and eq 2(i) becomes eq 1A(i) (the letter A after the equation number means the equation is given in the Appendix). Equation 1A(i) can be transformed to give eq 1A(ii). We evaluated $K_R$ from the slope and $I_{A(max)} = I_M R_M/(1 + \Phi)$ from the intercept of a linear plot of the experimental data, $1/I_A$ versus $L$, according to eq 1A(ii). The value of $K_R$ (6.5 mM) thus obtained was then used to correct the observed $I_A$ values for the inhibition due to carbamoylcholine binding to the regulatory site that is observed at carbamoylcholine concentrations greater than 4 mM in Figure 2A. We can now evaluate $K_1$, $\Phi$, and $I_M R_M$ using eq 2A, which is a linear form of eq 2(ii) above, and a nonlinear least-squares computer program. The values of all the parameters obtained from the measurements shown in Figure 2A are given in Table I and were used to calculate the coordinates of the solid line in Figure 2A.

The measurements of $I_A$, and the interpretation of the dependence of $I_A$ on carbamoylcholine concentration, can be compared with results obtained by an entirely different approach and methodology, namely, measurements of single-channel currents (Neher & Sakmann, 1976). These allow one to determine the conditional probability ($P_o$) that the channel is open while the receptor is in a nondesensitized state (Sakmann et al., 1980). An example of such measurements made in the presence of 2 mM carbamoylcholine is shown in Figure 3A, where bursts of channel activity are underlined; it can be seen that the bursts are separated by periods of no channel activity (Figure 3A). The long silent periods represent the lifetimes of desensitized receptor states (Sakmann et al., 1980). One of the challenges in those measurements was the definition of the beginning and ending of individual bursts (Sakmann et al., 1980). In the experiments with PC12 cells and carbamoylcholine, we were able to separate bursts of channel activity from silent periods only in the presence of 2 mM carbamoylcholine. The lifetime of a burst corresponds to the lifetime of the nondesensitized state and is a measure of the rate coefficient for receptor desensitization (Sakmann et al., 1980). From our measurements, we calculated a value of $\sim 5$ $s^{-1}$ for this rate coefficient.

The fraction of time the channel is open during a burst of channel activity is a measure of $P_o$ (Neher, 1983; Ogden & Colquhoun, 1983). Determination of $P_o$ and $I_A$ at a given concentration of activating ligand allows evaluation of $I_M R_M$ [eqs 2(i) and 3A]. At 2 mM carbamoylcholine and a transmembrane potential of −60 mV, $P_o$ has a value of 0.5 and $I_A$ has a value of 1.6 nA; using these two values and eq 3A, an $I_M R_M$ value of 3.2 nA was calculated from single-channel measurements and of 3.5 nA (Table I) from chemical kinetic measurements, demonstrating agreement between results obtained by the two techniques.

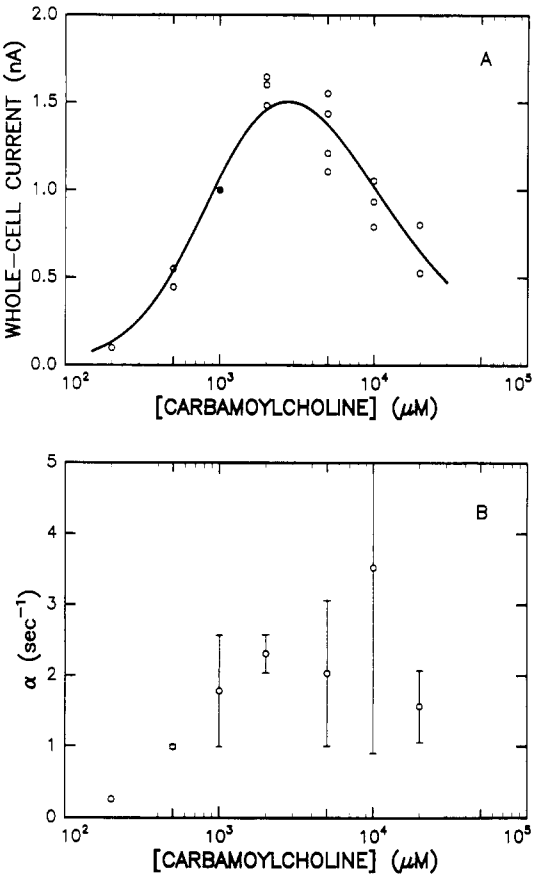


FIGURE 2: Relationship between $I_A$, $\alpha$, and carbamoylcholine concentration at −60 mV and 21–23 °C. (A) The current associated with the minor, slowly desensitizing, receptor form was subtracted from the whole-cell current, which was then corrected for receptor desensitization (see Experimental Procedures). The data were normalized to the $I_A$ values obtained in the presence of 1 mM carbamoylcholine (●). Each point represents a different cell; 6–15 measurements with different carbamoylcholine concentrations were made with each cell. The precision of measurements made with the same cell was ±15%. The parameters used for computing the solid line ($I_M R_M$, $K_1$, $\Phi$) are listed in Table I and were computed as described under Results. (B) The same cells as were used in panel A above were also used to determine the effect of carbamoylcholine concentration on the rate coefficient for receptor desensitization, $\alpha$. The error bars represent plus or minus standard deviations from the mean.

Table I: Constants Determining the Formation of Acetylcholine Receptor Transmembrane Channels in PC12 Cells and the Electric Organ of *E. electricus*

| | PC12 cells (21–23 °C, pH 7.4, −60 mV transmembrane voltage) | | *E. electricus* (pH 7.0, 0 mV transmembrane voltage) | |
|---|---|---|---|---|
| | chemical kinetics[a] | single channel | chemical kinetics[a] | single channel |
| $K_1$ (mM) | 2 | | 1.7[b], 1.0[c] | 0.6[c] |
| $K_R$ (mM) | 6.5 | | 0.8[d] | |
| $\Phi^{-1}$ | 5.0 | | 0.3[b], 0.3[c] | 0.4[c] |
| $\alpha$ ($s^{-1}$) | ~2.4[e] | ~5[e] | 4[f] | |
| $I_M R_M$ (nA) | 3.5 | 3.2 | | |

[a] Average error in constants ±50%. [b] Cash and Hess (1980). Value obtained at 1 °C. [c] Udgaonkar and Hess (1987). Value obtained at 12 °C. [d] Takeyasu et al. (1986). Value obtained at 1 °C, −45 mV transmembrane voltage. [e] 2 mM carbamoylcholine. Value obtained at 21–23 °C. [f] Aoshima et al. (1981), 2 mM carbamoylcholine. Value obtained at 1 °C.

Since $I_M R_M$ correponds to the whole-cell current that would be observed if all the receptor–channels opened in the presence of a saturating concentration of activating ligand, the number of receptor–channels present in the cell membrane can be

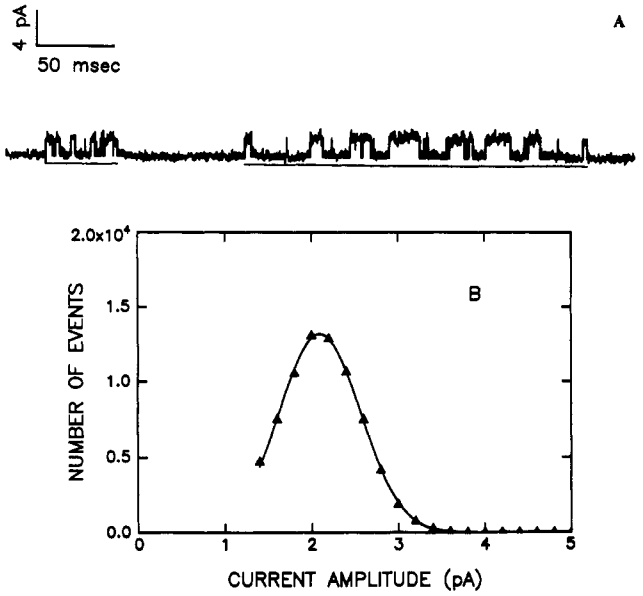


FIGURE 3: Single-channel current recordings. (A) An example of single-channel currents recorded in the presence of 2 mM carbamoylcholine at −70 mV, 21–23 °C. Two bursts of channel activity are shown; a line has been drawn under each burst to denote the duration of the burst. The scales correspond to 50 ms and 4 pA. At −70 mV $P_o$ is 0.57, and at −105 mV it is 0.81; the $P_o$ value of 0.5 at −60 mV was obtained by linear extrapolation of the data. (B) The distribution of the amplitudes of single-channel currents in the presence of 2 mM carbamoylcholine at −70 mV, 23 °C. The baseline fluctuations of the current were fitted to a Gaussian distribution, and the mean value was adjusted to 0 pA.

determined by dividing $I_M R_M$ by the current that passes through a single open receptor–channel. This value can be determined by the single-channel current-recording technique (Neher & Sakmann, 1976) (Figure 3B and below). When one divides the number of receptor–channels in the membrane by Avogadro's number, one obtains the moles of receptor–channels in the membrane, i.e., the value of $R_M$, and, therefore, the value of $I_M$ can also be calculated. The distribution of single-channel current amplitudes is shown in Figure 3B. The distribution is consistent with a receptor form having a mean current amplitude of 2 pA at −60 mV. But, as we have already seen (Figure 1), there appear to be two receptor forms in PC12 cells; one form contributes about 80% to the current measurements and desensitizes approximately five times faster than the form that contributes 20%. Which of the two forms did we observe in the traces shown in Figure 3A? Or do both receptor forms have the same current amplitude? In the presence of 2 mM carbamoylcholine, the rate coefficient for desensitization of the main receptor form in PC12 cells is in the 2.1–2.7 $s^{-1}$ range and of the minor component is about 0.4 $s^{-1}$. The value of the rate coefficient for receptor desensitization, estimated from the single-channel current measurements shown in Figure 3A, is approximately 5 $s^{-1}$ or approximately 25 times that of the minor receptor form in PC12 cells. This suggests, therefore, that we observed the main receptor form in the single-channel current measurements.

It can be seen from eq 4A(ii) in the appendix that the relation between the rate coefficient for receptor desensitization, $\alpha$, and carbamoylcholine concentration is complex and contains a number of constants that we can not yet determine. No attempt has, therefore, been made to draw a theoretical line through the data points in Figure 2B. The results in Figure 2B are treated in more detail under Discussion. A summary of the results obtained, and a comparison with those obtained with the acetylcholine receptor from the *E. electricus* electric organ, appears in Table I.

## Discussion

The chemical kinetic approach with a 20-ms time resolution allowed us to evaluate the constants of a mechanism that accounts for the concentration of the open receptor–channel in PC12 cells. Many techniques exist for flowing solutions over small patches of membrane (Brett et al., 1986) or whole cells (Krishtal & Pidoplichko, 1980). The advantages of the cell-flow technique are that the properties of a relatively large number of receptors are measured and that with the slow rates of solution flow used, at which the seal between the recording electrode and the membrane is stable, many measurements can be made with the same cell. This approach decreases the extensive data collection and analysis needed with other approaches and reduces the statistical error of the measurements, which is proportional to the square root of the number of measurements (Bevington, 1969). The applicability of the theory used in the cell-flow technique (Landau & Lifshitz, 1959; Levich, 1962; Udgaonkar & Hess, 1987a; Hess et al., 1987) has been tested by (i) observing that at different solution flow rates used, which result in different current rise times, the same calculated value of $I_A$ is obtained and (ii) showing that similar results are obtained whether $(\overline{AL_2})_o$ (eq 2) is determined by measuring $I_A$ in the cell-flow method or by using an independent technique, the single-channel current recording technique. These comparisons have so far been made in experiments with the muscle acetylcholine receptor in $BC_3H1$ cells (Udgaonkar & Hess, 1987a), in experiments with the γ-aminobutyric acid (GABA) receptor in cerebral cortical cells (Geetha & Hess, 1992), and in the experiments with the neuronal type of acetylcholine receptor in PC12 cells described here. In these experiments, $I_A$ was measured (Figure 2A) over a 100-fold range of carbamoylcholine concentration; the pertinent constants are given in Table I. The agreement between the results obtained by two independent methods, chemical kinetics and the single-channel current-recording technique (Neher & Sakmann, 1976), which makes different assumptions, is one indication that the time resolution of the cell-flow technique is sufficient to measure the properties of the active receptor form before desensitization occurs. The comparison between the results obtained previously by a quench-flow method with a 5-ms time resolution and by the cell-flow method used here (Table I) also indicates the adequacy of the cell-flow method. The quench-flow measurements were made with the nicotinic acetylcholine receptor from the electric organ of *E. electricus*, and some species and tissue differences are to be expected. The results described, however, indicate that apparently the same mechanism (mechanism I) accounts for the formation of open receptor–channels both in the electric organ of *E. electricus* and in PC12 cells: (1) The effect of carbamoylcholine concentration on $I_A$ (eq 2) indicates that at least two ligand molecules must bind to the receptor before the channel opens. (2) At first, the value of $I_A$ increased with increasing carbamoylcholine concentration, but then, at concentrations greater than 4 mM, it decreased (Figure 2A), although the rate coefficient for receptor desensitization did not increase (Figure 2B). (3) The values of the dissociation constant of the site controlling channel opening and the rate coefficient for receptor desensitization are similar to those of the *E. electricus* receptor (Table I). In experiments with the mammalian muscle acetylcholine receptor in $BC_3H1$ cells (Udgaonkar & Hess, 1987a) and one form of the GABA receptor in mouse cerebral cortical cells (Geetha & Hess, 1992), we could assume that $k_{34}$, the rate constant for the conversion of $AL_2$ to $IL_2$ in mechanism I, is much larger than the other rate constants associated with receptor desensitization

($k_{12}$, $k_{21}$, and $k_{43}$ in mechanism I) and, therefore, dominates the kinetic measurements. This assumption allows one to simplify eq 4A(ii) to give eq 4A(iii) and requires that the constants that account for the concentration dependence of $I_A$, namely, $K_1$ and $\Phi$, also account for the concentration dependence of $\alpha$ (Udgaonkar & Hess, 1987a). Equation 4A(iii) accounts for the effect of carbamoylcholine concentration on the rate coefficient of desensitization of the muscle acetylcholine receptor in $BC_3H1$ cells (Udgaonkar & Hess, 1987a) and one of the two forms of the GABA receptor in mouse cerebral cortical cells (Geetha & Hess, 1992). This simplifying assumption could not be made in quench-flow measurements with the *E. electricus* receptor. In investigations of that receptor, rate constants for the conversion of desensitized receptor forms (IL and $IL_2$ in mechanism I) to active forms had to be measured to establish the relationship between $\alpha$ and carbamoylcholine concentration indicated by the model (model I) and eq 4A(ii), which is based on this model (Aoshima et al., 1981). The measurement of the rate of receptor recovery from desensitization reqires consecutive mixing events: (i) receptor with carbamoylcholine to obtain desensitization, (ii) receptor with buffer for various periods of time to allow the receptors to recover from desensitization, and (iii) receptors with carbamoylcholine to determine the rate at which the receptor activity reappears. We are not yet able to make such measurements with the cell-flow technique. Therefore, we do not yet know whether the minimum model (mechanism I) for the desensitization reaction of the *E. electricus* receptor also applies to the neuronal receptor.

An interesting difference between the muscle-type receptor from *E. electricus* and the mammalian neuronal receptor in PC12 cells lies in the values of $\Phi^{-1}$, 0.4 and 5.0, respectively (Table I). $\Phi^{-1}$ characterizes the equilibrium between the closed- and open-channel forms of the receptor. It was introduced (Cash & Hess, 1980) to account for the cooperativity observed in the effect of carbamoylcholine concentration on the *E. electricus* receptor-controlled flux of inorganic ions into membrane vesicles, and because the maximal influx rates observed in the experiments depended on the activating ligand used (Hess et al., 1983), even though the number of inorganic ions that pass through the open receptor–channel are reported to be independent of the activating ligand (Neher & Steinbach, 1978; Gardner et al., 1984). The demonstration that different activating ligands affect the equilibrium between closed and open receptor–channel forms differently explains the dependence of the maximal influx rates on the activating ligand used, and is consistent with the well-known effect of the binding affinity of a ligand, and, therefore, of its structure, on the equilibrium between protein conformations (Cantor & Schimmel, 1980). Different values of $\Phi^{-1}$, and perhaps a different desensitization mechanism, may reflect the documented structural differences between the receptors in *E. electricus* and in PC12 cells (Deneris et al., 1989).

Both $\Phi$ and $K_R$ can have important regulatory functions. Analysis of the data in Figure 2A (Table I) indicates that the values of $\Phi$ and $K_R$ account for the difference between the maximal cell current observed on saturating the receptor with carbamoylcholine (1.5 nA, Figure 2A) and the current, $I_M R_M$ (3.5 nA, Table I), that would be observed if all the receptor molecules saturated with carbamoylcholine were to form transmembrane channels. In the case of the receptor from the *E. electricus* electric organ (Takeyasu et al., 1986), changes in transmembrane voltage have a minor effect on the value of $\Phi^{-1}$ but a dramatic effect on the value of $K_R$, which changes by a factor of over 60 for a 48-mV change in transmembrane potential. The many factors that regulate the transmembrane potential of cell membranes may, therefore, determine whether or not a signal is transmitted, even when the concentration of neurotransmitter arriving at the receptor sites remains unchanged. Differences in the value of $\Phi$, $K_R$, or both can also account for the observation that suberyldicholine activates the acetylcholine receptor in the electric organ of certain fish but inhibits it in PC12 cells (Figure 1C). The explanations are that suberyldicholine binding is associated with a favorable channel-opening equilibrium constant or a relatively high value of $K_R$ in the case of the electric organ receptor and an unfavorable value of $\Phi^{-1}$ and/or a low value of $K_R$ in the case of the neuronal receptor. The ability of suberyldicholine to open receptor–channels in PC12 cells under some conditions but not others has been reported (Lukas, 1989; Lucero & Lukas, 1989).

To date, the interesting kinetic information about the mammalian nicotinic acetylcholine receptor has come from measurements of the receptor-controlled flux of inorganic tracer ions in PC12 cells. The best time resolution obtained in those experiments was 5 s (Stallcup & Patrick, 1980), and time resolutions up to 30 s have been employed (Simasko et al., 1985). The type of information that can be obtained from low time resolution experiments is, however, limited: (1) The properties of only the desensitized main receptor form present in PC12 cells are measured, as can be seen from the experiment in Figure 1A. Even the slowly desensitizing receptor form ($t_{1/2} \sim 1$ s, Figure 2A) is converted to its desensitized form under the conditions used in most tracer ion flux measurements. Whether the ion-flux activity in an equilibrium mixture of active and desensitized receptors is due to a small concentration of receptors that remain in their active form, or due to the ability of the desensitized receptor to form transmembrane channels, or due to both is not known. The assumption that only the active receptor forms open channels has been sufficient to account for the effect of a large concentration range of acetylcholine, carbamoylcholine, or suberyldicholine on the concentration of open acetylcholine receptor channels in the *E. electricus* electric organ (Hess at al., 1983). Since we have no evidence for the formation of transmembrane channels by the desensitized receptor form, this step is omitted from the minimum mechanism (mechanism I). (2) In measurements of receptor-controlled ion flux, the amount of inorganic tracer ions that have crossed the cell membrane is measured after a constant period time, $[M^+]_t$. Inspection of eq 4A (Appendix) shows that the value of $[M^+]_t$ depends not only on $(\overline{AL_2})_o$ (eq 2) but also on the rate coefficient for receptor desensitization, $\alpha$. $\alpha$ also depends on the ligand concentration, but not necessarily in the same manner as $(\overline{AL_2})_o$ [eq 4A(ii)]. Not only do the constants evaluated from such measurements reflect only indirectly the constants pertaining to channel opening, they also depend on the time interval chosen for the measurements [eq 4A(i)]. The apparent dissociation constants for activating ligands measured in ion flux experiments with PC12 cells, defined by the ligand concentration that gave half of the maximal amount of ion influx or efflux, were in the range of 700 (Patrick & Stallcup, 1977b) to 200 (Lukas, 1989) $\mu$M. Similarly, the desensitization rates reported varied, with values of $t_{1/2}$ ranging from 30 s (Boyd, 1987) to 4 min (calculated from the data of Stallcup & Patrick, 1980). An apparently irreversible process with a $t_{1/2}$ value of 14.7 min has also been reported (Simasko et al., 1985). The most rapid desensitization process detected in tracer ion flux experiments ($t_{1/2} = 30$ s; Boyd, 1987) was 17 times slower than the slower of the two desensitization processes detected

in the cell-flow experiments (Figure 1A). The faster desensitization process (Figure 2B) is characterized by a $t_{1/2}$ value as low as 200 ms, and, in a PC12 cell culture obtained from another laboratory (Professor Racker), the rapid desensitization process was complete within about 150 ms. One consequence of using time resolutions of 5 s and greater is illustrated by the experiment in Figure 1B. It can be seen that, in the presence of 1 mM carbamoylcholine, 50 $\mu$M *d*-tubocurarine completely inhibited the receptor component characterized by the slower desensitization rate coefficient, which is responsible for 20% of the observed current (Figure 1B), but the main receptor form was only 35% inhibited. In contrast, in kinetic experiments with low time resolution, also done with PC12 cells, a *d*-tubocurarine concentration of only 0.3 $\mu$M (less than 1% of the concentration employed in the cell-flow measurements) inhibited 50% of the observed response to 1 mM carbamoylcholine (Lukas, 1989). A simple explanation for this difference in the results obtained, depending on whether the cell-flow technique with a 20-ms time resolution or a method with a low time resolution was used, is that in the latter case the receptor has been completely inactivated in the desensitization process before the first measurement is made. The dissociation constants of acetylcholine and carbamoylcholine are known to be quite different for the active and desensitized acetylcholine receptor forms (Katz & Thesleff, 1957; Cash & Hess, 1980). In equilibrium mixtures of active and desensitized *E. electricus* acetylcholine receptor forms, the dissociation constants pertaining to the desensitized receptor will be measured (Cash & Hess, 1980). Therefore, the dissociation constants of inhibitors may also depend on the presence or absence of desensitized receptor forms and, therefore, on the time resolution of the technique employed in the experiments. Also, changes in the kinetic properties of the rapidly desensitizing receptor form can not be observed when low time resolution techniques are employed because the receptor may have already been desensitized before the first measurement is made. For the same reason, in measurements with a low time resolution it can not be determined whether or not a particular compound, for instance, snake neurotoxins or substance P, inhibits the rapidly desensitizing main receptor component in PC12 cells (Figure 1A). In low time resolution experiments, the main receptor component in PC12 cells is completely inhibited (desensitized) by the activating ligand before the first measurement can be made, and, therefore, the action of a compound that inhibits this receptor form cannot be observed.

The effects of specific toxins that discriminate between muscle and neuronal receptors (Ravdin & Berg, 1979; Patrick & Stallcup, 1977a; Yamada et al., 1985; Clark, 1987; Loring & Zigmond, 1988; Lukas, 1989), compounds that have anesthetic-like effects (Karpen et al., 1982; McHugh & McGee, 1986), the important neuronal peptide substance P (Simasko et al., 1985; Stallcup & Patrick, 1980), NGF (Amy & Bennett, 1983; Mitsuka & Hatanaka, 1984; Boyd & Leeman, 1987), and chemical modification (Leprince, 1983) on the PC12 receptor have all been investigated by low time resolution techniques. However, what effects these and other factors have on the nondesensitized receptor form in PC12 cells, which can only be observed with a 20-ms time resolution technique, remains an interesting problem. Many other, possibly rewarding, investigations of the PC12 receptor are still to be undertaken using fast chemical reaction techniques. For instance, are the two desensitization processes observed in cell-flow experiments due to two independent receptor forms, or does one receptor form desensitize in two consecutive steps? The distribution of the amplitudes of the current flowing through a single channel, measured in the presence of 2 mM carbamoylcholine (Figure 3B), is consistent with a single receptor form. However, comparison of chemical kinetic measurements with the single-channel current-recording technique requires determination of $P_o$ and the lifetime of bursts of channel openings. We succeeded at only one carbamoylcholine concentration (2 mM) in separating bursts of channel activity from silent periods and observed only the rapidly desensitizing receptor forms. The slowly desensitizing receptor form, which is responsible for 20% of the current in the presence of 2 mM carbamoylcholine (Figure 1A), is not observed under these conditions. This form may not be observed if only a small fraction of the receptor remains in its active state in the presence of 2 mM carbamoylcholine (Figure 3A). At low concentrations of acetylcholine, 1–10 $\mu$M, several conductance states of the receptor have been observed in PC12 cells (Bormann & Matthei, 1983; Ifune & Steinbach, 1989), which is consistent with the presence of different receptor forms in the cells. Similarly, the difference in the effect of *d*-tubocurarine on the rapidly and slowly desensitizing receptor forms (Figure 1B) suggests that we may be observing two receptor species. The very slow desensitization processes observed in ion flux experiments with a low time resolution may reflect a second desensitization process of one or both of the receptor forms observed in rapid chemical kinetic measurements (Figure 1A). Now that the experiments with carbamoylcholine have been done, another important and more easily answerable question for the future is, What are the values of the constants that allow one to calculate the relationship between acetylcholine concentration and the concentration of the open receptor–channels, a concentration that determines changes in the transmembrane potential and, therefore, signal transmission?

Many rapid chemical techniques have been used successfully for studying complex and important biological reactions in solution (Eigen, 1967; Hammes, 1982; Fersht, 1985). The introduction of the quench-flow technique in investigations of the acetylcholine receptor in membrane vesicles prepared from the electric organ of certain fish (Hess et al., 1979) made it possible to separate, temporally, the comparatively straightforward reaction leading to formation of the open receptor–channel (mechanism I) from the complex reactions that follow once the receptor starts to desensitize. This approach allowed one to evaluate the constants ($I_M R_M$, $K_1$ $\Phi$) of the individual steps of the channel-opening process and the rate constants for rapid receptor desensitization. Because of the possible presence of multiple receptor forms in PC12 cells, investigation of the mechanism for the neuronal receptor in PC12 cells is more complex than that for the receptor in electric organs. Nevertheless, the fast reaction technique employed appears capable of "separating" along the time axis the main receptor form, not only from the slowly desensitizing receptor form but also from the many receptor species with different ligand-binding properties that appear once the receptor starts to desensitize. The results presented previously (Udgaonkar & Hess, 1987a), here, and in an accompanying paper (Geetha & Hess, 1992) demonstrate that fast chemical reaction techniques may be useful in studying the mammalian neuronal-type acetylcholine receptor and the numerous other membrane-bound receptor proteins that exist in cells from the central nervous system and whose properties are not well known. The effects of acetylcholine itself on the neuronal receptors forms, the mechanism of receptor desensitization, the effects of compounds that modulate receptor function, and

the relation between subunit composition and receptor mechanism are still to be investigated in PC12 cells.

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APPENDIX

*Equation 1A, $L \gg K_I$.*

$$I_A = I_M R_M \frac{K_R}{K_R + L}(1 + \Phi)^{-1} \qquad [1A(i)]$$

When we define $I_M R_M (1 + \Phi)^{-1}$ as $I_{A(max)}$, the highest observable value of $I_A$, and rearrange eq 1A(i) we obtain

$$\frac{1}{I_A} = \frac{1}{I_{A(max)}} + \frac{1}{I_{A(max)}} \frac{L}{K_R} \qquad [1A(ii)]$$

*Equation 2A (Cash & Hess, 1980).*

$$\left[\frac{I_M R_M}{I_A} - 1\right]^{1/2} = \frac{K_1}{L}\Phi^{1/2} + \Phi^{1/2} \qquad (2A)$$

where

$$I_A = I_{A(obs)} \frac{K_R + L}{K_R}$$

*Equation 3A.*

$$P_o = (\overline{AL_2})_o \frac{K_R}{K_R + L} \qquad (3A)$$

*Equation 4A.* For mechanism I, the relationship between inorganic ion flux measured on the millisecond time region and the rate coefficient for receptor-controlled influx $\bar{J}R_o$, the fraction of receptors in the open-channel form $(\overline{AL_2})_o$, and the rate coefficient for receptor desensitization $\alpha$ has been derived (Cash & Hess, 1980):

$$M_t = M_{t\infty}\{1 - \exp[-\bar{J}R_o]\} \times \{[(\overline{AL_2})_{t=0} - (\overline{AL_2})_{t=\infty}]\frac{1 - e^{-\alpha t}}{\alpha} + (\overline{AL_2})_{t=\infty} t\} \qquad [4A(i)]$$

The subscripts refer to the time of measurements; $(\overline{AL_2})_o$ has been defined in terms of the constants $K_1$, $\Phi$, and the ligand concentration $L$ (eq 2).

$$\alpha = \frac{Lk_{43} + 2k_{21}k_2}{L + 2K_2} + \frac{(L^2 k_{34} + 2Lk_{12}K_1)\Phi}{L^2(1 + \Phi) + 2LK_1\Phi + K_1^2\Phi} \qquad [4A(ii)]$$

$k_{12}$ and $k_{34}$ are the rate constants for the conversion of AL and $AL_2$ to the corresponding inactive, desensitized receptor states, respectively, and $k_{21}$ and $k_{43}$ are the reverse rate constants. $K_2$ is the dissociation constant of the ligand from the desensitized receptor.

$$\alpha = \frac{k_{34}\Phi L^2}{L^2(1 + \Phi) + 2K_1 L\Phi + K_1^2\Phi} \qquad [4A(iii)]$$

REFERENCES

Adams, P. (1977) *J. Physiol. (London) 268*, 291–318.
Amy, C. M., & Bennett, E. L. (1983) *J. Neurosci. 3*, 1547–1553.
Aoshima, H. (1984) *Arch. Biochem. Biophys. 235*, 312–318.
Aoshima, H., Cash, D. J., & Hess, G. P. (1980) *Biochem. Biophys. Res. Commun. 92*, 896–903.
Aoshima, H., Cash, D. J., & Hess, G. P. (1981) *Biochemistry 20*, 3467–3474.
Bevington, P. R. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, McGraw Hill, New York.
Brett, R. S., Dilger, J. P., Adams, P. R., & Lancaster, B. (1986) *Biophys. J. 50*, 987–992.
Bormann, J., & Matthaei, H. (1983) *Neurosci. Lett. 40*, 193–197.
Boyd, N. D. (1987) *J. Physiol. (London) 389*, 45–67.
Boyd, N. D., & Leeman, S. E. (1987) *J. Physiol. (London) 389*, 69–97.
Cantor, C. R., & Schimmel, P. R. (1980) *Biophysical Chemistry*, Vol. 3, W.H. Freeman and Company, San Francisco, CA.
Cash, D. J., & Hess, G. P. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 842–846.
Cash, D. J., & Hess, G. P. (1981) *Anal. Biochem. 112*, 39–51.
Cash, D. J., & Subbarao, K. (1987a) *Biochemistry 26*, 7556–7562.
Cash, D. J., & Subbarao, K. (1987b) *Biochemistry 26*, 7562–7570.
Catterall, W. A. (1975) *J. Biol. Chem. 250*, 1776–1781.
Changeux, J.-P., Devillers-Thiery, A., & Chemouilli, P. (1984) *Science 225*, 1335–1345.
Clark, P. B. S. (1987) *Trends Pharmacol. Sci. 8*, 32–35.
Deneris, E. S., Boulter, J., Connolly, J., Wada, E., Wada, K., Goldman, D., Swanson, L. W., Patrick, J., & Heinemann, S. (1989) *Clin. Chem. (Winston-Salem, NC) 35*, 731–737.
Dichter, M. A., Tischler, A. S., & Greene, L. A. (1977) *Nature 268*, 501–504.
Eigen, M. (1967) *Fast React. Primary Processes Chem. Kinet., Proc. Nobel Symp., 5th, 1967*, 333–369.
Fenwick, E., Marty, A., & Neher, E. (1982) *J. Physiol. (London) 331*, 577–597.
Fersht, A. (1985) *Enzyme Structure and Mechanism*, W. H. Freeman, New York.
Gardner, P., Ogden, D. C., & Colquhoun, D. (1984) *Nature 309*, 160–216.
Geetha, N., & Hess, G. P. (1992) *Biochemistry* (second paper of four in this issue).
Greene, L. A., & Tischler, A. S. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2424–2428.
Greene, L. A., & Tischler, A. S. (1982) *Adv. Cell. Neurobiol. 3*, 373–414.
Hamill, O. P., Marty, A., Neher, E., Sakmann, E., & Sigworth, F. J. (1981) *Pfluegers Arch. 391*, 85–100.
Hammes, G. G. (1978) *Principles of Chemical Kinetics*, Academic Press, New York.
Hess, G. P., Cash, D. J., & Aoshima, H. (1979) *Nature 282*, 329–331.
Hess, G. P., Cash, D. J., & Aoshima, H. (1983) *Annu. Rev. Biophys. Bioeng. 12*, 443–473.
Hess, G. P., Udgaonkar, J. B., & Olbricht, W. L. (1987) *Annu. Rev. Biophys. Biophys. Chem. 16*, 507–534.
Ifune, C. K., & Steinbach, J. H. (1989) *Biophys. J. 55*, 552a.
Kandel, E., & Schwartz, J. H. (1985) *Principles of Neuronal Science*, Second ed., Elsevier, New York.
Karpen, J. W., Aoshima, H., Abood, L. G., & Hess, G. P. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2509–2513.
Katz, B., & Thesleff, S. (1957) *J. Physiol. (London) 138*, 63–86.
Koblin, D. D., & Lester, H. A. (1978) *Mol. Pharmacol. 58*, 559–580.

Krishtal, O. A., & Pidoplichko, V. I. (1980) *Neuroscience 5*, 2325–2327.
Landau, V. G., & Lifshitz, E. M. (1959) *Fluid Mechanics*, Pergamon, Oxford.
Leprince, P. (1983) *Biochemistry 22*, 5551–5556.
Levich, A. G. (1962) *Physicochemical Hydrodynamics*, Prentice-Hall, Englewood Cliffs, NJ.
Loring, R. H., & Zigmond, R. E. (1988) *Trends Neurosci. 11*, 73–78.
Lucero, L., & Lukas, R. J. (1989) *Neurosci. Abstr.*, 678.
Lukas, R. J. (1989) *J. Pharmacol. Exp. Ther. 25*, 175–177.
Marty, A., & Neher, E. (1983) in *Single-Channel Recording* (Sakmann, B., & Neher, E., Eds.) pp 107–122, Plenum, New York.
McHugh, E. M., & McGee, R., Jr. (1986) *J. Biol. Chem. 261*, 3103–3106.
Mitsuka, M., & Hatanaka, H. (1984) *Dev. Brain Res. 12*, 255–260.
Neher, E. (1983) *J. Physiol. (London) 339*, 663–678.
Neher, E., & Sakmann, B. (1976) *Nature 260*, 799–802.
Neher, E., & Steinbach, J. H. (1978) *J. Physiol. 277*, 153–176.
Neubig, R. R., & Cohen, J. B. (1980) *Biochemistry 19*, 2770–2779.
Ochoa, E. L. M., Chattopadhyay, A., & McNamee, M. G. (1989) *Cell. Mol. Neurobiol. 9*, 141–177.
Ogden, D. C., & Colquhoun, D. (1983) *Pfluegers Arch. 399*, 246–248.
Pasquale, E. B., Takeyasu, K. T., Udgaonkar, J. B., Cash, D. J., Severski, M. C., & Hess, G. P. (1983) *Biochemistry 22*, 5967–5973.
Patrick, J., & Stallcup, W. B. (1977a) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4689–4692.
Patrick, J., & Stallcup, W. B. (1977b) *J. Biol. Chem. 252*, 8629–8633.
Ravdin, P. M., & Berg, D. K. (1979) *Proc. Natl. Acad. Sci. U.S.A 76*, 2072–2076.
Sachs, A., Leprince, P., Karpen, J. W., Pasquale, E. B., Abood, L. G., & Hess, G. P. (1983) *Arch. Biochem. Biophys. 225*, 500–504.
Sachs, F., Neil, J., & Barkakati, N. (1982) *Pfluegers Arch. 395*, 331–340.
Sakmann, B., Patlak, J., & Neher, E. (1980) *Nature 286*, 71–73.
Shiono, S., Takeyasu, K., Udgaonkar, J. B., Delcour, A. H., Fujita, N., & Hess, G. P. (1984) *Biochemistry 23*, 6889–6893.
Sigworth, F. J. (1983) in *Single-Channel Recording* (Sakmann, B., & Neher, E., Eds.) pp 3–35, Plenum, New York.
Simasko, S. M., Soares, J. R., & Weiland, G. A. (1985) *J. Pharmacol. Exp. Ther. 235*, 601–605.
Simasko, S. M., Soares, J. R., & Weiland, G. A. (1986) *Mol. Pharmacol. 30*, 6–12.
Stallcup, W. B., & Cohn, M. (1976) *Exp. Cell Res. 98*, 277–284.
Stallcup, W. B., & Patrick, J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 634–638.
Takeyasu, K., Udgaonkar, J. B., & Hess, G. P. (1983) *Biochemistry 22*, 5973–5978.
Takeyasu, K., Shiono, S., Udgaonkar, J. B., Fujita, N., & Hess, G. P. (1986) *Biochemistry 25*, 1770–1776.
Udgaonkar, J. B., & Hess, G. P. (1986) *J. Membr. Biol. 93*, 93–109.
Udgaonkar, J. B., & Hess, G. P. (1987a) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8758–8762.
Udgaonkar, J. B., & Hess, G. P. (1987b) *Biophys. J. 52*, 873–883.
Walker, J. W., Lukas, R. J., & McNamee, M. G. (1981a) *Biochemistry 20*, 2191–2193.
Walker, J. W., McNamee, M. G., Pasquale, E. B., Cash, D. J., & Hess, G. P. (1981b) *Biochem. Biophys. Res. Commun. 100*, 86–90.
Walker, J. W., Takeyasu, K., & McNamee, M. G. (1982) *Biochemistry 21*, 5384–5389.
Whiting, P. J., Schoepher, R., Swanson, L. W., Simmons, D. M., & Lindstrom, J. M. (1987) *Nature 327*, 515–517.
Yamada, S., Isogai, M., Kagawa, Y., Takayanagi, N., Hayashi, E., Tsuji, K., & Kosuge, T. (1985) *Mol. Pharmacol. 28*, 120–127.