Electrophysiological and biochemical studies on enhancement of desensitization by phenothiazine neuroleptics
(neuromuscular synapse/chlorpromazine/trifluoperazine/prochlorperazine/endplate currents)

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ABSTRACT The actions of the phenothiazines chlorpromazine, prochlorperazine, and trifluoperazine were studied on the acetylcholine receptor–ionic channel complex of frog and rat skeletal muscle and of Torpedo californica to determine their role in pharmacological desensitization and their interactions with different states of the receptor–ionic channel complex. The phenothiazines depressed the peak amplitude of spontaneous and evoked endplate currents while having negligible effect on the decay time constants. Mean channel lifetime and single channel conductance were not altered by these drugs. They also produced a frequency-dependent depression of the peak amplitude of endplate potentials evoked by repetitive microiontophoresis at the extrajunctional region. In addition, these drugs enhanced the ability of carbamoylcholine to displace 125I-labeled α-bungarotoxin from receptor-rich membrane preparations of T. californica when used in concentrations that had no effect on 125I-labeled α-bungarotoxin binding alone (10 μM). Similarly, the phenothiazines inhibited the binding of tritiated ionic channel ligands, such as phenycyclidine and perhydrohistrionicotoxin, a process also enhanced by the presence of carbamoylcholine. These data suggest that the phenothiazines augment agonist-induced desensitization primarily by interacting with the receptor–ionic channel complex prior to channel opening.

Desensitization of the nicotinic receptor at the neuromuscular junction has been described as a gradual decrease in depolarization upon continued application of agonist (1). The progressive decrease in endplate responsiveness has been suggested to be associated with a concomitant increase in receptor affinity for the agonist (2, 3). In light of these observations, desensitization has been proposed to result from an agonist-induced conformational change, generating a nonconducting species of the acetylcholine (AcCho) receptor–ionic channel complex (4–6).

Drugs such as meproalen (6, 7) and chlorpromazine (CIPZ) (8–11) have been shown to produce a phenomenon resembling desensitization. These drugs produce a progressive decrease in endplate responsiveness to AcCho under conditions in which the agonist alone did not produce desensitization. The similarity of the pharmacologically enhanced desensitization to the phenomenon initially described by Thesleff (1) and Katz and Thesleff (4) is further borne out by the observation that these agents increase the affinity of the agonist for its binding site. Despite these similarities, it remains unclear whether these two phenomena proceed by identical processes. One point of controversy is whether the interaction between these agents and the receptor–ionic channel complex that produces these effects occurs before (8) or after (9) opening of the ionic channel.

The objective of the present investigation is to provide an electrophysiological and biochemical analysis of the action of the phenothiazines CIPZ, trifluoperazine (TFP), and prochlorperazine (PCIP) on the nicotinic receptor–ionic channel complex of the frog and rat neuromuscular synapse as well as of the electric organ of Torpedo californica and to establish the mechanism(s) by which the phenothiazines and other agents that enhance desensitization act.

METHODS AND MATERIALS

Electrophysiology. Endplate currents (EPCs), miniature endplate currents (MEPCs), and AcCho-induced noise were recorded from glycerol-treated (12, 13), voltage-clamped superficial fibers of cutaneous pectoris muscles of the frog Rana pipiens as described (14–16). MEPCs and AcCho-induced noise were recorded in the presence of 0.3 μM tetrodotoxin (Sigma). Endplate potentials (EPPs) evoked by iontophoretic application of AcCho were recorded from superficial fibers of denervated rat soleus and extensor digitorum longus muscles as described (17). Ten consecutive EPPs were evoked by iontophoretic application of brief pulses (each of 0.1- to 0.5-msec duration) of AcCho at frequencies of 0.5, 1.0, 2.0, and 5.0 Hz. In all electrophysiological experiments, the following drugs were dissolved in the appropriate physiological solutions: CIPZ hydrochloride, PCIP edisylate, and TFP dihydrochloride (Smith Kline & French).

Biochemical Techniques. Membranes were prepared from the electric organs of T. californica (Pacific Bio-Marine, Venice, CA) and were stored in liquid nitrogen. Tissue samples were homogenized in 5 vol of 50 mM Tris·HCl (pH 7.4) containing 0.1 mM phenylmethylsulfonic acid to prevent proteolysis by using a glass Waring blender. The homogenate was filtered through four layers of cheesecloth and was centrifuged at 20,000 × g for 20 min. The resulting pellet was suspended in 50 mM Tris·HCl (pH 7.4) at a concentration of 1–2 mg of protein per ml. Sodium azide (0.02%) was added and the tissue was kept on ice for up to 3 days before use.

125I-Labeled α-bungarotoxin (125I-BGT; 10–20 Ci/μg, New England Nuclear; 1 Ci = 3.7 × 1010 becquerels) binding to the nicotinic AcCho receptor was measured at room temperature by using a filtration procedure as described (18, 19).

RESULTS

Effects of Phenothiazines on Nerve-Evoked EPCs. At a membrane potential of −90 mV, CIPZ in concentrations of 3, 5, and 7.5 μM depressed significantly the peak amplitude of the EPC to 63%, 51%, and 30% of control, whereas the decay

Abbreviations: AcCho, acetylcholine; BGT, α-bungarotoxin; CIPZ, chlorpromazine; EPC, endplate current; EPP, endplate potential; Hg·HTX, perhydrohistrionicotoxin; MEPC, miniature endplate current; PCIP, prochlorperazine; PCP, phenycyclidine; TFP, trifluoperazine.

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time constant of the EPC ($\tau_{EPC}$) was only 95%, 87%, and 82% of control, respectively (Fig. 1). PCIP in concentrations of 5 and 10 $\mu$M depressed the peak amplitude of the EPC to 88% and 32% of control, whereas the $\tau_{EPC}$ was 103% and 101% of control, respectively. TFP in concentrations of 5, 7, 10, and 20 $\mu$M depressed the peak amplitude to 62%, 31%, 18%, and 3% of control, whereas the $\tau_{EPC}$ was 100%, 82%, 74%, and 51% of control, respectively. For each of these drugs, the current–voltage relationships remained linear at all concentrations used. No differences in the peak amplitude of the EPC were found at any membrane potential between −150 and −50 mV when the membrane potential was changed in either the hyperpolarizing or the depolarizing direction (i.e., no hysteresis was observed) (20). Thus, CIPZ, PCIP, and TFP do not affect the voltage- and time-dependent nature of the EPC.

**Effects of Phenothiazines on MEPCs.** Fig. 2 shows the effects of the phenothiazines on the peak amplitude (Fig. 2A–C) and the decay time constant of the MEPC ($\tau_{MEPC}$) (Fig. 2D–F). CIPZ in concentrations of 2 and 5 $\mu$M depressed ($P < 0.05$) the peak amplitude of the MEPC at membrane potentials of −60, −90, and −120 mV, whereas the $\tau_{MEPC}$ was depressed ($P < 0.05$) only by 5 $\mu$M at membrane potentials of −90 and −120 mV, respectively. After 5 $\mu$M CIPZ, the peak amplitude was 36% and 31% of control, whereas the $\tau_{MEPC}$ was 70% and 65% of control at membrane potentials of −90 and −120 mV, respectively. At 2 $\mu$M, PCIP depressed ($P < 0.05$) the peak amplitude at a membrane potential of −90 mV and, at 5 $\mu$M, it depressed the peak amplitude at membrane potentials of −60, −90, and −120 mV. $\tau_{MEPC}$ was not affected by PCIP at any of the concentrations or membrane potentials studied. TFP at a concentration of 5 $\mu$M depressed ($P < 0.05$) the peak amplitude at membrane potentials of −90 and −120 mV, whereas $\tau_{MEPC}$ was depressed ($P < 0.05$) by concentrations of 2 and 5 $\mu$M at a membrane potential of −60 mV. In the presence of 5 $\mu$M TFP, the peak amplitude was 55% and 52% of control at membrane potentials of −90 and −120 mV, respectively. At a membrane potential of −60 mV, the $\tau_{MEPC}$ was 80% and 86% of control in the presence of 2 and 5 $\mu$M TFP, respectively.

**Effect of Phenothiazines on EPCs Evoked by Microiontophoretic AcCho Application.** CIPZ at a concentration of 1 $\mu$M did not affect single channel conductance ($\gamma$) and channel lifetime ($\tau_I$). The value (mean ± SEM for six fibers) recorded at −80 mV for $\gamma$ under control conditions was 25 ± 2 pS and, after 30–60 min of exposure to CIPZ (1 $\mu$M), the value was 20 ± 1 pS (n = 4). Similarly, $\tau_I$ was 1.34 ± 0.19 msec (n = 6) and 1.04 ± 0.12 msec (n = 4) for control and after the CIPZ exposure, respectively. Under similar conditions neither PCIP nor TFP had any statistically significant effect ($P > 0.05$) on either the $\gamma$ or $\tau_I$.

**Effect of Phenothiazines on EPPs Evoked by Repetitive Application of AcCho.** As shown in Fig. 3, there was no time dependence under control conditions in the peak amplitude of consecutive EPPs evoked by iontophoretic application of AcCho. The mean peak amplitudes of the second to the tenth EPPs in a train of 10 EPPs did not vary by >10% from the amplitude of the first EPP when elicited at frequencies from 0.5 to 5.0 Hz. However, all three phenothiazines induced a time-dependent depression of the peak amplitude of consecutively evoked EPPs. In the presence of 1 and 5 $\mu$M CIPZ, the mean (± SEM) peak amplitude of the tenth EPP of a train of 10 EPPs evoked at 0.5 Hz was 96% ± 5% and 69% ± 1% of control, respectively (Fig. 3A). The time-dependent depression of the peak amplitude of the EPP was more pronounced at higher frequencies. At 1 and 5 $\mu$M CIPZ, the mean (± SEM) amplitude of the tenth EPP of a train of 10 EPPs evoked at 5.0 Hz was 68% ± 3% and 22% ± 2% of control, respectively. Similar effects were observed with PCIP (Fig. 3B) and TFP (Fig. 3C). At concentrations of 1 and 5 $\mu$M PCIP, the mean (± SEM) peak amplitude of the tenth EPP of a train of 10 EPPs evoked at 0.5 Hz was 96% ± 2% and 71% ± 2%, and at 5.0 Hz it was 96% ± 2% and 59% ± 1% of control, respectively. At concentrations of 1 and 5 $\mu$M TFP, the mean (± SEM) peak amplitude of the tenth

![Image](image_url)
EPP of a train of 10 EPPs evoked at 0.5 Hz was 81% ± 8% and 33% ± 3%, and at 5.0 Hz it was 81% ± 3% and 13% ± 1% of control, respectively.

Effects of Phenothiazines on Ionic Channel and AcCho Receptor Binding. As summarized in Table 1, the phenothiazines blocked the specific (i.e., amantadine-sensitive) binding of [3H]-labeled perhydrohistrionicotoxin ([3H]H12-HTX) and [3H]-labeled phencyclidine ([3H]PCP) to sites associated with the ionic channel. In the absence of receptor ligands, all drugs inhibited [3H]H12-HTX and [3H]PCP binding with EC50 values between 1 and 4 μM. In the presence of 1 μM carbamoylcholine, phenothiazine affinity was increased 2- to 8-fold so that the EC50 values were in the range of 0.3 to 1.4 μM. In contrast, the phenothiazines were weak inhibitors of [3H]-labeled AcCho and 125I-BGT binding to the AcCho receptor. None of the three compounds inhibited receptor binding at 0.1 nM. However, the phenothiazines increased carbamoylcholine’s affinity for the receptor, as identified by the increased ability of the agonist to inhibit the binding of 125I-BGT (Fig. 4). A carbamoylcholine concentration of 4.3 μM inhibited the binding of 125I-BGT (1 nM) by 50%. In the presence of 10 μM of CIPZ, PCIP, and TFP, the concentration of carbamoylcholine needed to inhibit 125I-BGT binding by 50% was decreased to 1.6, 1.8, and 2.3 μM, respectively. 125I-BGT binding (1 and 5 nM) in the absence of carbamoylcholine was not affected by any of the phenothiazines at this concentration.

DISCUSSION

Both the electrophysiological and biochemical evidence suggest that the phenothiazines produce a phenomenon that may resemble desensitization at the neuromuscular junction. First, CIPZ, TFP, and PCIP all cause a frequency-dependent depression of consecutive EPPs evoked by microiontophoretic application of AcCho. The similarities between frequency-dependent depression of EPPs caused by CIPZ and agonist-induced desensitization observed in denervated rat muscle have been demonstrated previously (8, 9). Second, the phenothiazines, in concentrations that have no effect on BGT binding alone, enhance the ability of carbamoylcholine to displace BGT from the AcCho receptor. This suggests that the phenothiazines may produce a relative increase in the affinity of the AcCho receptor for the agonist compared with that for the antagonist. Enhanced affinity for the AcCho receptor for agonist coupled with a depression of ion flux have been shown to occur upon exposure of receptor-rich Torpedo membrane preparations to agonist, suggesting that formation of a high affinity state of the AcCho receptor is a characteristic phenomenon of desensitization (3, 4). Because phenothiazines produced both a frequency-dependent decrease in endplate responsiveness and an apparent relative increase in receptor affinity for agonist, it seems likely that these drugs enhance agonist-induced desensitization at the neuromuscular junction.

The effects of the phenothiazines on EPCs and spontaneous MEPCs may explain how these drugs interact with the receptor-ionic channel complex and cause the pharmacological desensitization. All three phenothiazines depressed the peak amplitude of the EPC and MEPC at concentrations that had little or no effect on the τEPC and τMEPC. At higher concentrations τEPC and τMEPC were decreased, but always to a lesser degree than were the peak amplitudes of EPCs and MEPCs. Inasmuch as the τEPC and τMEPC reflect τ1 (21–23), the phenothiazines would appear to be only weak blockers of the ionic channel in its "open" conformation. The minimal effect of the phenothiazines on the τEPC and τMEPC is consistent with the observation that these drugs had no significant effect on τ1 or γ, as determined by noise analysis. However, other drugs that produce pharmacological desensitization do not share this profile of effects. Although meprofen and also produces a decrease of the peak amplitude of the EPC with little or no effect on the τEPC (6), histrionicotoxin causes a marked reduction in both peak amplitude and τEPC (24). Thus, contrary to the hypothesis of Anwyl and Narahashi (9), these data suggest that a drug interaction with the receptor-ionic channel complex in its open conformation may not be essential for the expression of pharmacological desensitization (see step 3 in the reaction scheme below, in which A represents the agonist, D represents the drug, and RI, RI*, and RI represent the receptor-ionic channel
complex in the "closed," open, and "desensitized" conformations, respectively; the $k_n$ values refer to micro-rate constants).

These data suggest that the phenothiazines exert their effects

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\begin{align*}
A + RI & \xrightarrow{k_1} ARI & \xrightarrow{k_4} ARI^* & \xrightarrow{k_5} ARID \\
& \xrightarrow{k_{-1}} & \xrightarrow{k_{-4}} & \xrightarrow{k_{-5}} \\
& \xrightarrow{k_2} & \xrightarrow{k_3} & \\
& \xrightarrow{D} & \xrightarrow{D+} & \\
R'I'D & \xrightarrow{k_{-2}} & \xrightarrow{k_3} & \xrightarrow{D} \\
(1) & \xrightarrow{D} & \xrightarrow{D} & \xrightarrow{D} \\
\end{align*}
\]

primarily through an interaction with the receptor–ionic channel complex prior to channel opening. It has been proposed that, even in the absence of agonist, CIPZ and meproprazine stabilize a species of the receptor–ionic channel complex that has a high affinity for agonist (7, 10, 25). If this high affinity species were similar to the high affinity species that is incapable of activation and is produced by exposure to agonist alone, then the phenothiazines could be producing a shift in the equilibrium concentrations of receptor–ionic channel complexes in favor of a species that is incapable of activation (see steps 1 and 2 in the reaction scheme above). The depression in the peak amplitude of EPCs elicited at low frequencies could be explained by the phenothiazine–induced increase in the fraction of receptor–ionic channel complexes in the state that is incapable of activation (step 1 in the reaction scheme). To explain the frequency–dependent depression of the peak amplitude of EPPs, one has to assume that $k_3 > k_{-3}$. Under this condition, the species ARID that is incapable of activation could accumulate over the course of consecutive stimulations when the time interval between successive stimuli was sufficiently brief that equilibrium concentrations of the species that are capable and incapable of activation could not be reestablished.

Meproprazine, which also induces pharmacological desensitization, has been demonstrated to produce hysteresis and nonlinearity in the current–voltage plots for EPCs (7). These irregularities in the current–voltage relationship have been attributed to this drug's interaction with the receptor–ionic channel complex in its closed conformation at a site that is sensitive to membrane potential (20). However, the phenothiazines did not alter the voltage or time dependence of the peak am-

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\begin{align*}
\text{Phenothiazine inhibition of the binding of ion channel blockers to } & \text{Torpedo electricocyte membranes} \\
| & \text{}\begin{array}{|c|c|c|c|c|}
\hline
\text{Drug} & \text{Control} & \text{Carbamoylcholine} & \text{R}^* & \text{Control} \\
\hline
\text{CIPZ} & 3.5 & 1.4 & 2.5 & 1.3 \\
\text{PCIP} & 2.3 & 0.44 & 5.2 & 1.3 \\
\text{TFP} & 2.3 & 0.48 & 4.8 & 3.3 \\
\hline
\end{array}
\end{align*}
\]

* Phenothiazine concentration (μM) that inhibited the specific binding of 2 nM [3H]H12-HTX or 3 nM [3H]PCP by 50%. Binding was measured in the absence (control) and presence of 1 μM carbamoylcholine. Each value is the mean of three determinations that varied by <20%.

The ordinate on the graph represents the fractional binding of $[125I]-BGT$ to the AcCho receptor. The specific binding of $1 \text{ nM } [125I]-BGT$, expressed on the ordinate as the fraction of total specific binding, was measured in the presence of the concentration of carbamoylcholine indicated on the abscissa. Binding was measured in the absence of phenothiazines (×) or in the presence of 10 μM PCIP (○), CIPZ (□), or TFP (Δ). Each point represents the average of three determinations that varied by <15%.
plitude of the EPC, which suggests an interaction with a membrane site that is not voltage-sensitive. Two classes of noncompetitive antagonist binding sites have been proposed for drugs that stabilize the high affinity form of the receptor–ionic channel complex (10, 25). C1PZ has been proposed to bind to one of these sites, whereas meproadifen binds preferentially to the other. Thus, it is possible that the differential binding of these agents could reflect their interactions with voltage-sensitive and voltage-insensitive sites at the receptor–ionic channel complex.

Despite the apparent lack of effect of the phenothiazines on \( \tau \), it is possible that open channel blockade might contribute to decremental postjunctional membrane responsiveness to agonist by a mechanism unrelated to desensitization under certain kinetic conditions. Upon channel activation, if \( k_{-4} > k_{5} \), the phenothiazines would not be expected to be potent in decreasing the \( \tau_{EPC} \) and \( \tau_{MEPC} \). Furthermore, if \( k_{5} > k_{5} \), then the fraction of receptor–ionic channel complexes that is capable of activation during a train of evoked responses would be dependent on the time interval between successive stimulations. Under such kinetic conditions, it is possible that both the frequency-dependent depression of EPPs and the weak depression of \( \tau_{EPC} \) and \( \tau_{MEPC} \) result in part from a slow interaction of the phenothiazines with the ionic channel in its open conformation relative to the channel closure event. In this case, the lack of effect of the phenothiazines on \( \tau \) might result from the rates of association and dissociation of the drug–ionic channel complex being sufficiently slow that a drug-induced effect may be unmeasurable by noise analysis in the frequency range studied (approximately 4–800 Hz). A more rigorous kinetic and single channel analysis of the effects of the phenothiazines must be performed to determine the extent to which the mechanisms discussed in this paper may be involved in the precise actions of these phenothiazines.

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