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INTERACTION OF IMIPRAMINE WITH THE IONIC CHANNEL OF THE ACETYLCHOLINE RECEPTOR OF MOTOR ENDPLATE AND ELECTRIC ORGAN

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Imipramine (IMIP), a tricyclic antidepressant, has been shown to block biogenic amine uptake (1) and to inhibit muscarinic cholinergic (2), histaminergic (3,4), α-adrenergic (5), and serotonergic (6) receptors. On neuromuscular preparations the drug has complex effects which suggest that it interferes with Na^+ and K^+ channels of both nerve and muscle (7-11) and may inhibit neuromuscular transmission noncompetitively (11). Our studies on the pharmacology of the nicotinic acetylcholine (ACh) receptor of motor endplates and electric organs have indicated that some drugs which possess cholinolytic properties, and IMIP does, are usually good blockers of the nicotinic receptor-regulated ionic channel (12). Furthermore, quinacrine, which is an effective channel blocker (13), has a tricyclic ring structure. This raised the possibility that earlier observations of noncompetitive inhibition of neuromuscular transmission (11) might be due to interaction of IMIP with the ionic channel site of the nicotinic ACh-receptor.

The ACh-receptor/channel complex is identified by binding of [^{3}H]ACh and [^{3}H]α-bungarotoxin ([^{3}H]α-BGT) to the receptor sites and by binding of [^{3}H]perhydrohistrionicotoxin ([^{3}H]-H_{12}-HTX) (14) and [^{3}H]phencyclidine ([^{3}H]PCP) (15) to the ionic channel sites. Binding of drugs to this receptor/channel complex is affected by the conformational state of the protein, thus the presence of agonists increases dramatically the apparent rate of binding of the channel probe, an increase that is inhibited by α-BGT, and by conditions that desensitize the protein (e.g. high concentrations of agonist or preincubation with it). The present study was initiated to investigate the interactions of IMIP with the receptor/channel system of electric organs of Torpedo sp., frog motor endplates and denervated rat muscles, using both biochemical and biophysical methods, and to evaluate [^{3}H]IMIP as a probe for these channel sites.

Materials and Methods. Receptor-enriched membranes were prepared from frozen electric organs of Torpedo ocellata (obtained from the Mediterranean and stored at -90°) by differential centrifugation as described (16). All binding measurements were done at 23°, using 50 mM Tris-HCl buffer, pH 7.4. Binding of [^{3}H]ACh (90 mCi/mmole, New England Nuclear Corp., Boston, MA) was done by equilibrium dialysis after inhibition of ACh-esterase with 0.1 mM diisopropylfluorophosphate (DFP) (16), and binding of [^{3}H]α-BGT (48 Ci/mmole, Amersham, Arlington Heights, IL) was determined by a filter assay using Whatman GF/C filters as described previously (17). Binding of [^{3}H]H_{12}-HTX (21 Ci/mmole (16)) and [^{3}H]IMIP (29.8 Ci/mmole, New England Nuclear) was studied by filtering the mixture of membranes and radiolabeled drug on Whatman GF/B filters that had been dipped in 1% organosilane concentrate (Prosil-28) to reduce nonspecific binding (18). The filters were washed with 7 ml of Tris buffer, and their radioactivity was counted in a toluene-based solution containing 4% BioSolv (16). Binding was determined after different times of incubation and in the absence as well as the presence of receptor agonist. When the effect of drugs on this binding was determined, the drugs were added to the incubation medium. Specific binding to the ionic channel sites of either [^{3}H]H_{12}-HTX or [^{3}H]IMIP was the difference between total binding in the absence and presence of 5 mM amantadine, which blocks the ionic channel site (19). In addition, specific [^{3}H]IMIP binding was obtained by including 100 μM nonradiolabeled IMIP.

Endplate currents (EPCs) were recorded from endplates of glycerol-treated frog sartorius muscles as described (20). The voltage clamp circuitry was similar to that of Takeuchi and Takeuchi as modified by Kuba et al. (21). Muscle preparations were pinned under slight tension to a paraffin block having a plano-convex lens in the center and placed in a 20 ml capacity bath. ACh sensitivity was measured on soleus muscles removed from Wistar rats that had been denervated for at least 7 days. The muscles were mounted as above, and the ACh sensitivity was assessed in surface fibers by a method similar to that described by others (22).

Results. IMIP (up to 100 μM) had no effect on the binding of [^{3}H]ACh (0.5 μM) or [^{3}H]α-BGT (1 nM) to the ACh-receptor sites of Torpedo membranes. However, it was a strong inhibitor of [^{3}H]H_{12}-HTX binding to the ionic channel site (Fig. 1A), though the displacement of [^{3}H]-H_{12}-HTX was noncompetitive as shown by the intercept of the two lines of the Dixon plot at the abscissa (Fig. 1B), suggesting that the two drugs may be binding to different sites.

Like H_{12}-HTX and other drugs that compete for binding sites on the ionic channel (13,19, 23,24), IMIP had a voltage-dependent inhibitory effect on neuromuscular transmission. Under voltage clamp conditions, the peak EPC was voltage dependent such that the amplitude was linearly related to membrane potentials over a wide range (+50 to -150 mV). IMIP decreased the peak EPC amplitude in a concentration-dependent manner and induced nonlinearity in the current-voltage relationship (Fig. 2A). The decrease in peak EPC amplitude produced by 1 hr of treatment with 10 μM IMIP could not be reversed by washing for 1 hr. However, IMIP (5 and 10 μM) had no significant effect on EPC rise or decay times (Fig. 2B). The ACh sensitivity of the denervated rat soleus muscle was reduced in a dose-dependent manner by IMIP above 5 μM (Fig. 2C), though at 1 μM it produced potentiation. All concentrations of IMIP tested produced a consistent rundown of ACh sensitivity over the course of the train, with steady level of blockade reached after ten responses. This stimulation-dependent blockade produced by IMIP was short-lived, the ACh sensitivity returning virtually to the initial level 20 sec after the last ACh application.

The specific binding of [^{3}H]IMIP (2 nM) to Torpedo membranes was linear with protein concentration up to at least 100 μg. Binding of 2 nM [^{3}H]IMIP reached equilibrium rapidly (within 1 min) (Fig. 3A), and its dissociation by dilution or excess nonradioactive IMIP was equally fast (data not shown). In the absence of tissue, the filters bound 0.7% of the

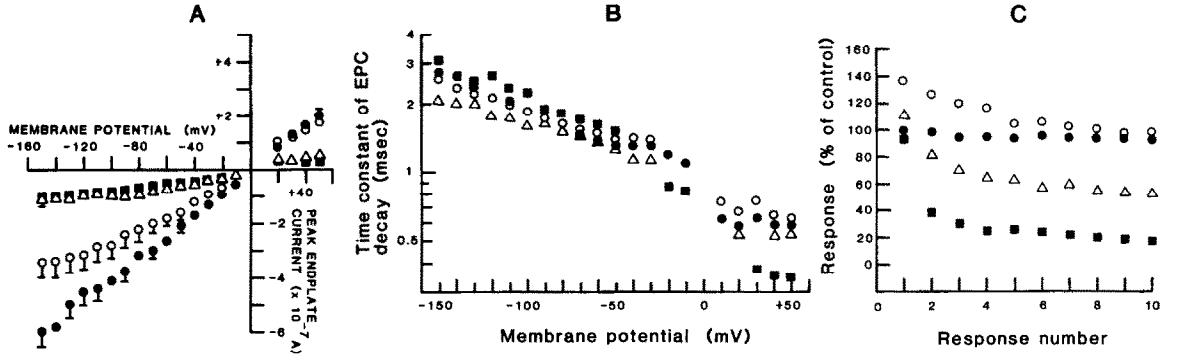

Fig. 1. Effect of IMIP on the binding of receptor and ionic channel ligands to Torpedo membranes. Each symbol is the mean of three experiments; standard deviations were less than 10%. (A) Dose-response curve of the effect of various concentrations of IMIP (10 nM - 100 μM) on the binding of [^{3}H]ACh (0.5 μM) (o), [^{3}H]α-BGT (1 nM) (Δ), and [^{3}H]H_{12}-HTX (2 nM) (●). (B) Dixon plot of the effect of IMIP (0.25 to 2 μM) on the binding of 2 nM (●) and 4 nM (o) [^{3}H] H_{12}-HTX.

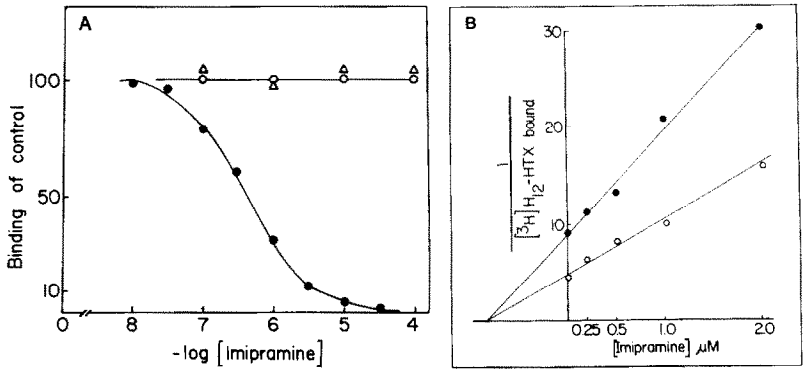

Fig. 2. Effect of IMIP on endplate current and ACh sensitivity. (A) The relationship between membrane potential and peak EPC amplitude in frog sartorius muscle in the absence and presence of IMIP. (B) The relationship between membrane potential and the time constant of EPC decay in frog sartorius muscle in the absence and presence of IMIP. The concentrations of IMIP used were: (●) control; (o) 5 μM; (Δ) 10 μM; and (■) 60 min wash with normal physiological solution after 10 μM IMIP. Each symbol is the mean of at least twelve fibers in at least three muscles; the washout is the mean of three fibers from one muscle. (C) Effect of trains of ten ACh potentials (elicited at 1.0 Hz) on ACh sensitivity of surface fibers of denervated rat soleus muscles. The concentrations of IMIP used were: (●) control; (o) 1 μM; (Δ) 5 μM; and (■) 10 μM.

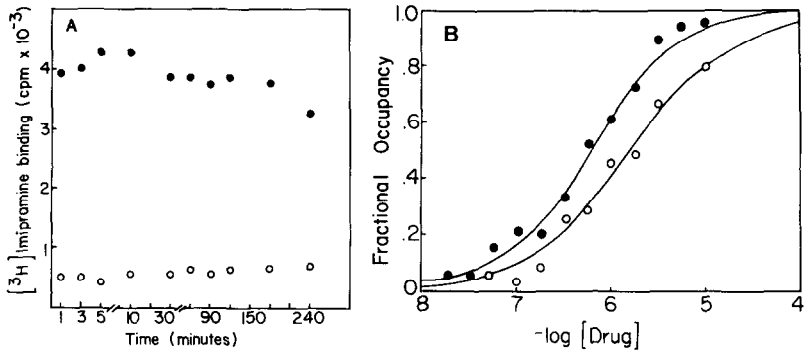

Fig. 3. Binding of [^{3}H]IMIP to Torpedo membranes in the absence (o) and presence (•) of 20 μM carbamylcholine. Each point is the mean of three experiments; standard deviations are <10%. (A) Time course of binding of [^{3}H]IMIP (2 nM) to Torpedo membranes. (B) Specific binding of [^{3}H]IMIP (presented as fractional occupancy) as a function of IMIP concentration (M). Maximum number of binding sites in the absence and presence of carbamylcholine were 49.7 and 247 pmoles/g tissue, respectively. The curves were drawn from a least squares fit of the data using the MLAB system of NIH (25).

[^{3}H]IMIP (at 2 nM) added in the assay. The presence of 20 μM carbamylcholine increased the apparent rate of [^{3}H]IMIP binding dramatically (5- to 10-fold) (Fig. 3A), but total binding in the absence of carbamylcholine did not reach the level in its presence even after 4 hr of incubation. The saturation isotherms of [^{3}H]IMIP binding gave a K_d value of 1.6 μM in the absence of carbamylcholine and a K_d of 0.64 μM in the presence of 20 μM carbamylcholine, with respective total numbers of binding sites of 49.7 ± 3.2 and 247 ± 18 pmoles/g tissue (Fig. 3B). Comparable maximum number of binding sites for [^{3}H]H_{12}-HTX was 750 ± 53 pmoles/g tissue in the presence of 20 μM carbamylcholine.

Discussion. The data suggest that the tricyclic antidepressant IMIP interacts with the ionic channel sites of the nicotinic receptor. It inhibited binding of the channel probe [^{3}H]-H_{12}-HTX without affecting [^{3}H]ACh or [^{3}H]α-BGT binding (Fig. 1A) and caused nonlinearity in the voltage-current relationship (Fig. 2A). In addition, [^{3}H]IMIP binding was accelerated in the presence of carbamylcholine (Fig. 3A), which reflects binding to site(s) that is/are coupled to the ACh-receptor site. Therefore, like [^{3}H]H_{12}-HTX and [^{3}H]PCP (15), binding of IMIP was enhanced by receptor activation, and its inhibition of ACh sensitivity in denervated muscle was stimulation dependent (Fig. 2C), suggesting that the three drugs prefer binding to the activated conformation of the receptor/channel molecule. Also, similarly, the increased binding due to agonist stimulation was partly due to increased affinity (Fig. 3B).

The action of IMIP on the ACh-receptor/channel complex is different from that of H_{12}-HTX in several aspects. Unlike H_{12}-HTX, IMIP did not affect the time constant of EPC decay (Fig. 2B), and the total number of binding sites for IMIP in Torpedo electroplax was 247 pmoles/g tissue compared to 750 pmoles/g tissue for H_{12}-HTX in presence of 20 μM carbamylcholine. Like H_{12}-HTX, receptor stimulation by carbamylcholine increased greatly the initial rate and affinity of binding of [^{3}H]IMIP (Fig. 3A,B), but unlike H_{12}-HTX, [^{3}H]IMIP maximal binding in the absence of carbamylcholine reached only 20% of that reached in its presence, while maximal binding of [^{3}H]H_{12}-HTX was not significantly affected by carbamylcholine (14). The increased binding of 2 nM [^{3}H]IMIP due to carbamylcholine (Fig. 3A) was due to both increased binding affinity (K_d from 1.6 μM to 0.64 μM) as well as binding sites (maximum sites increasing from 49.7 to 247 pmoles/g tissue), and not to a decreased apparent rate of dissociation of [^{3}H]IMIP which was unaffected by carbamylcholine (data not shown). In addition, IMIP inhibition of [^{3}H]H_{12}-HTX binding was noncompetitive (Fig. 1B), which is contrary to a very recent report of competitive inhibition (26). This suggests that the binding sites for IMIP on the ionic channel are different from those for H_{12}-HTX (14) and PCP (23). On the other hand, IMIP is unique among the channel drugs studied so far in the absence of effect on EPC decay phase. It is suggested that it binds preferentially to the activated but nonconducting conformation of the receptor/channel complex, thus preventing opening of the channel.

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