Acetylcholine-Releasing Effects of Some Nicotinic Agents on Chick Biventer Cervicis Nerve Muscle Preparation[1] (34299)

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There are three basic mechanisms by which a drug may activate cholinergic receptors: (i) combining with the receptors similar to acetylcholine (ACh), (ii) inhibiting cholinesterases to preserve ACh, and (iii) releasing ACh from the storage sites of the nerve terminals. It is classically believed that cholinergic agents act primarily at the receptor cites to induce cholinergic effects. However, some investigations indicated that the real mechanisms of cholinergic responses may not be so simple. It has been suggested that choline derivatives induce vasodepression probably by releasing endogenous ACh (1) and that carbachol may act on the presynaptic terminals to release ACh (2). It has also been reported that nicotine, choline, and tetramethylammonium (TMA) may contract the guinea pig ileum *via* the indirect effect of releasing ACh from the nerve tissues (3–5). The present communication reports the ACh-releasing effects of nicotinic agents on the neuromuscular junction of baby chick biventer cervicis. The ACh-releasing effects of the nicotinic agents on the guinea pig ileum, synaptic vesicles, and synaptosomes will be reported shortly.

Methods. Baby chicks, weighing 150–250 g, were sacrificed with ether. The biventer cervicis nerve muscle preparation was isolated according to the methods described by Ginsborg and Warriner (6). A loop was tied around the lower (caudal) belly of the muscle and hooked on the electrode assembly. The upper end of the tendon was passed through the electrode and attached to a Statham transducer (Type GT-03). The electrode assembly was similar to the one described by Ginsborg and Warriner (6) except a small cup with a hole for superfusion was attached above the electrode. The small cup over the electrode was essential for superfusion of the biventer cervicis muscle because it eliminated the mechanical stimuli of the drops of superfusion fluid on the motor nerve, which caused irregular twitching of the muscle.

The preparation was superfused with Tyrode solution (NaCl, 8.0; KCl, 0.2; $CaCl_2$, 0.2; $MgCl_2$, 0.1; NaH_2PO_4, 0.05; $NaHCO_3$, 1.0; and dextrose, 2.0 g/l). The Tyrode solution was oxygenated with 95% O_2–5% CO_2 at 37°. The rate of flow of superfusion fluid was 3–4 ml/min and was maintained by a Holter motor pump (Type RD 45). Drug solutions were injected into the small cup attached above the electrode in volumes of not more than 0.1 ml. The initial tension placed on the biventer cervicis was 1 g. The tension developed by contractions of the muscle was measured in grams and recorded on an Offner Dynograph recorder (Type RS). Interrupted supramaximal tetanic stimulation was applied for 0.2 sec every 10 sec with a Grass stimulator (model S-4C) with appropriate circuit interrupter. The stimulation parameters used were 250-cps frequency, 1-msec duration and 100–150-V voltage. The supramaximal voltage used was rather high because the motor nerve was surrounded by the tendon (6). Solutions of the compounds were prepared in distilled water. The dose–response curves were determined by injecting drugs in 2-fold logarithmically increasing doses from that producing the low-

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est response to that producing the maximum response. The percentage response of drugs was calculated from the maximum response of ACh as 100% and was plotted against the log dose of drugs injected. The dose–response curves of the nicotinic agents were redetermined after triethylcholine (TEC) treatment (1.2×10^{-2} *M* TEC) or after neuromuscular blockade. The latter treatment was achieved by treating the preparation with 1.2×10^{-2} *M* TEC along with the interrupted tetanic nerve stimulation. The drug dose which induced maximal control response was termed maximum dose. The concentrations of TEC tested ranged from 3.75×10^{-4} to 2.4×10^{-2} *M*. The concentration of 1.2×10^{-2} *M* was selected to block nicotinic agents throughout the experiments because at this concentration level TEC produced maximum blockade of the effects of nicotinic agents, whereas the ACh response was not altered.

The drugs studies included acetylcholine chloride, nicotine tartrate, carbachol chloride, tetramethylammonium bromide (TMA), 1,1-dimethyl-4-phenylpiperazinium iodide (DM PP), decamethonium bromide (C_{10}), choline iodide, 3-trimethylamino *n*-propanol iodide (TMPro), 4-trimethylamino *n*-butanol iodide (TMB), 5-trimethylamino *n*-pentanol chloride (TMPen), 6-trimethylamino *n*-hexanol iodide (TMH), triethylcholine bromide (TE C), and vinylcholine ether chloride (VCE). Physostigmine sulfate was used to inhibit the cholinesterases on the tissue for showing the potentiation of control responses induced by nicotinic agents.

Results. Effects of trimethylamino alcohols and ACh on biventer cervicis nerve muscle preparation. Figure 1 shows the dose–response curves of trimethylamino alcohols determined on the slow muscle of biventer cervicis. The compounds with longer alcoholic chains were more active. Direct comparison of the relative potencies of these compounds with ACh was not possible because the slopes of these dose–response curves were significantly steeper than that of ACh. The dose–response curves of choline and its derivatives were parallel to one another. The dose–response curves of ACh on the slow muscle of biventer cervicis were not altered by 1.2×10^{-2} *M* TEC (Fig. 2A) nor by neuromuscular blockade achieved by TEC

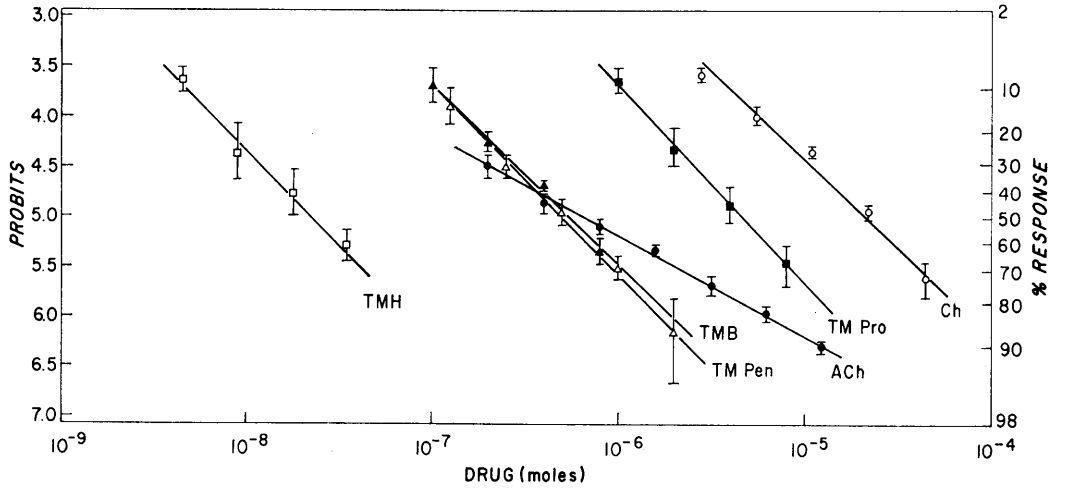

FIG. 1. Dose–response curves of acetylcholine (ACh) and trimethylamino alcohols on baby chick biventer cervicis nerve muscle preparation. Where, Ch = choline; TMPro = trimethylamino propanol; TMB = trimethylamino butanol; TMPen = trimethylamino pentanol; TMH = trimethylamino hexanol. Each point is a mean of 5 values; bars represent standard errors. Note the dose–response curves of trimethylamino alcohols are parallel to one another but not parallel to that of ACh. Also, the compounds with longer alcoholic chains in the molecules produce the higher activity.

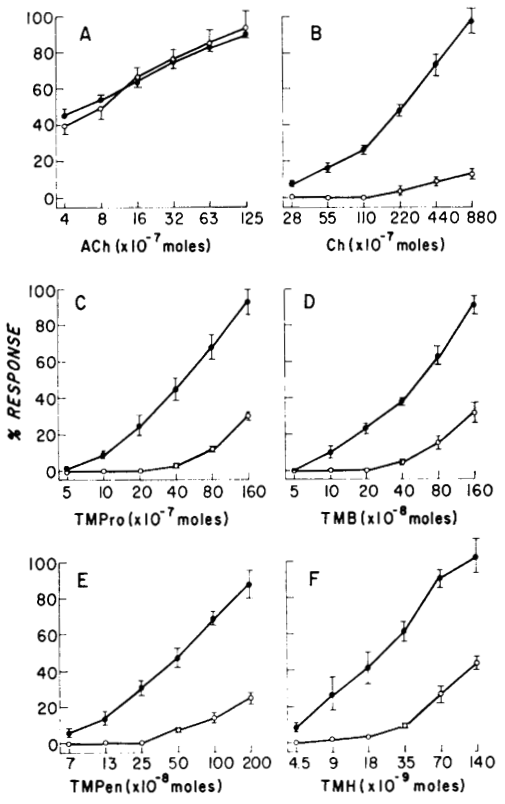

FIG. 2. Effects of triethylcholine (TEC) on the dose–response curves of acetylcholine (ACh) and trimethylamino alcohols on baby chick biventer cervicis nerve muscle preparation. Where, Ch = choline; TMPro = trimethylamino propanol; TMB = trimethylamino butanol; TMPen = trimethylamino pentanol; TMH = trimethylamino hexanol; (●), control responses; (○), responses after 1.2 × 20^{-2} *M* TEC. Each point is a mean of 5 values; bars represent standard errors. Note the dose–response curve of ACh was not altered by TEC, whereas those of trimethylamino alcohols were markedly depressed.

treatment with interrupted tetanic nerve stimulation, indicating that the ACh receptor was not blocked by TEC at this concentration. However, the dose–response curves of choline, TMPro, TMB, TMPen, and TMH were markedly inhibited by the same treatments (Fig. 2B–F), suggesting that these trimethylamino alcohols are probably acting primarily or partially at the nerve terminals to release ACh. After the response of trimethylamino alcohols was blocked by TEC alone, without interrupted tetanic stimulation, the biventer cervicis muscle was still responsive to nerve stimulation, indicating that there was still sufficient ACh available in the nerve terminals for release. Thus, the TEC blockade of trimethylamino alcohols is at the site of the nerve terminals. The responses induced by maximum doses of choline, TMPro, TMB, and TMPen were markedly reduced after TEC treatment, suggesting that they may act primarily through release of ACh from the nerve terminals. However, the response induced by maximum doses of TMH was rather high (43%) after TEC treatment indicating that this agent may act partially through the release of ACh from the nerve terminals and partially at the receptor site on the postjuntional membrane. The responses induced by these agents and ACh were effectively blocked by 1 × 10^{-5} *M* *d*-tubocurarine.

Effects of nicotine, TMA, carbachol, C_{10}, VCE, and DMPP on biventer cervicis nerve muscle preparation. The contractions induced by nicotine, TMA, carbachol, C_{10}, VCE, and DMPP on the slow muscle of biventer cervicis were markedly blocked by 1.2 × 10^{-2} *M* TEC (Fig. 3A–F) as well as by neuromuscular blockade. The responses induced by maximum doses of these drugs were markedly reduced after 1.2 × 10^{-2} *M* TEC, except DMPP which retained 43% of the response. These results indicate that the nicotinic agents tested, except DMPP, act primarily through the release of ACh from the nerve terminals. DMPP may have mixed effects of releasing ACh from the nerve terminals and of acting directly at the receptor sites on the postjunctional membrane. The biventer cervicis muscle was responsive to nerve stimulation after the nicotinic agents were blocked by 1.2 × 10^{-2} *M* TEC. The responses induced by these nicotinic agents were blocked by 1 × 10^{-5} *M* *d*-tubocurarine. The responses of KCl were not significantly altered by 1.2 × 10^{-2} *M* TEC.

Potentiation of nicotinic responses on biventer cervicis nerve muscle preparation by physostigmine. All the responses induced by the nicotinic agents tested were markedly potentiated by 7.7 × 10^{-7} *M* physostigmine as shown in Fig. 4A–F. Since none of these

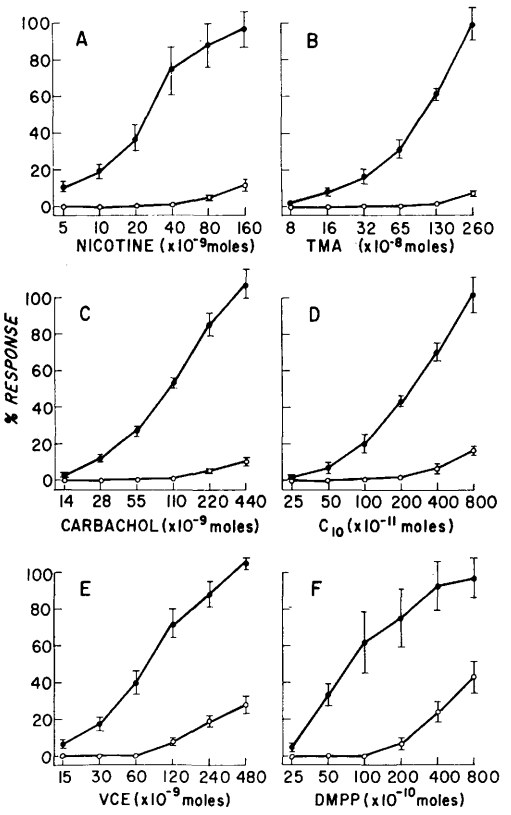

FIG. 3. Effects of triethylcholine (TEC) on the dose–response curves of some nicotinic agents on baby chick biventer cervicis nerve muscle preparation. Where, TMA = tetramethylammonium; C_{10} = decamethonium; VCE = vinylcholine ether; DMPP = 1,1-dimethyl-4-phenylpiperazinium; (●), control responses; (○) responses after 1.2 × 10^{-2} *M* TEC. Each point is a mean of 5 values; bars represent standard errors. Note the dose–response curves of the nicotinic agents tested were markedly depressed by TEC.

nicotinic agents tested were susceptible to enzymatic hydrolysis, their potentiation indicate the release of ACh or the related choline esters from the nerve endings. The biventer cervicis muscle was not contracted by 7.7 × 10^{-7} *M* physostigmine. The responses of KCl were not significantly altered by 7.7 × 10^{-7} *M* of physostigmine (Fig. 4G).

Discussion. Many attempts have been made to determine the actual mechanisms of responses induced by cholinergic agents. It has been reported that TEC has a hemicholinium-like effect of inhibiting ACh synthesis in cholinergic neurons by competing with choline for a carrier mechanism to transport extracellular choline to the intracellular sites (7). Although TEC is considerably less potent than hemicholinum, it has the advantage of possessing relatively weaker postjunctional curare-like action (8). Since the dose–response curve of ACh was not altered by 1.2 × 10^{-2} *M* TEC, the blockade of nicotinic agents by this concentration of TEC must indicate that these nicotinic agents are acting at sites other than that of the ACh receptor on the postjunctional membrane, possibly at the membrane site of the nerve terminals where the TEC is supposed to interact primarily.

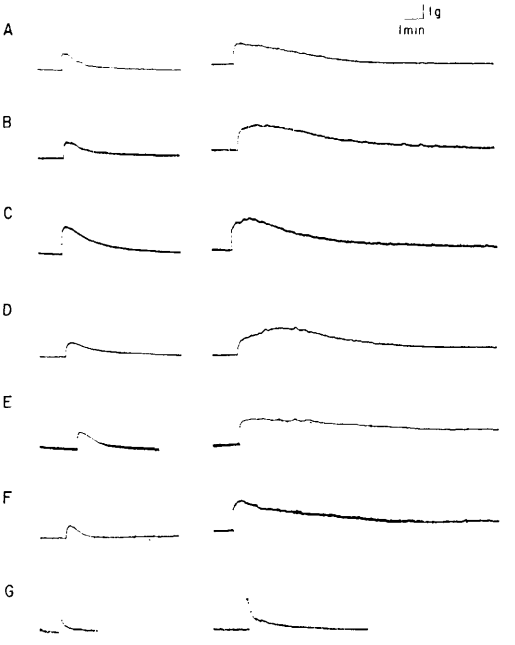

FIG. 4. Potentiation of nicotinic effects by physostigmine on baby chick biventer cervicis muscle. The tracings on the left panel indicate control responses and those on the right panel indicate responses of the same agents at the same dose levels after 7.7 × 10 m*M* physostigime; A, 5.5 × 10^{-6} moles of choline; B, 1.3 × 10^{-7} moles of trimethylamino pentanol; C, 9 × 10^{-9} moles of trimethylamino hexanol; D, 1 × 10^{-8} moles of nicotine; E, 5 × 10^{-10} moles of decamethoinum; F, 2.5 × 10^{-9} moles of 1,1-dimethyl-4-phenylpiperazinium (DMPP); G, 3.3 × 10^{-5} moles of KCl. Note both duration and amplitude of the nicotinic responses were markedly increased after treatment with physostigmine except those of KCl.

After nicotinic responses of the agents tested were blocked by 1.2×10^{-2} *M* TEC alone, the biventer cervicis muscle was still responsive to the nerve stimulation, suggesting that the endogenous ACh is available for release after TEC blockade. Therefore, it seems that the nicotinic agents tested have to be taken up by the nerve terminals from extracellular space to the intracellular sites before they can release the endogenous ACh. Whereas TEC is acting at the membrane site of the nerve terminals to block the uptake of the nicotinic agents and thus to block the release of endogenous ACh by these agents. Recently, it has been shown that nicotine is effectively taken into the intracellular site of the superior cervical ganglia, which is blocked by hexamethonium (9). Therefore, our proposal is not without precedent.

The marked potentiation of nicotinic responses in biventer cervicis muscle by physostigmine provides further evidence that these responses are induced through release of ACh from the nerve terminals, since none of the nicotinic agents studied here are susceptible to the enzymatic hydrolysis of cholinesterases. It is concluded that most of the nicotinic agents are acting primarily through the release of ACh from the nerve terminals but not at the ACh receptors on the postjunctional sites. The TEC probably blocks the responses of nicotinic agents at the membrane site of the nerve terminals through inhibition of the transport of nicotinic agents from the extracellular space to the intracellular sites.

A question might arise as to whether the site of blocking effects of TEC on these nicotinic agents might be at receptors other than the ACh-receptor on the postjunctional membrane. This seems unlikely because the main effects of TEC are known to be at the prejunctional nerve terminals (7). Also, the responses induced by these nicotinic agents as well as by ACh can be blocked by a common blocking agent, *d*-tubocurarine, at the postjunctional membrane. This suggests that all of these agents are acting at the same receptor site, probably directly by ACh and indirectly by nicotinic agents through release of endogenous ACh.

If the interpretations of the above experiments are valid, it would suggest that the mechanisms of action of these drugs are entirely different from those of ACh. Consequently the validity of the descriptions about the conformations of ACh receptors based on the information obtained from the studies of structure activity relationship (SAR) of these nicotinic agents would be doubtful and meaningless. A necessary assumption of all SAR studies is that the compounds being compared act by the same mechanism. This assumption has been applied to many SAR studies of cholinergic agents and many of the conclusions that have been drawn may be in error.

Summary. The mechanism of action of some nicotinic agents has been studied on the baby chick biventer cervicis nerve muscle preparation. The effects of various nicotinic agents on this preparation were blocked by 1.2×10^{-2} *M* triethylcholine (TEC) or by neuromuscular blockade achieved by TEC treatment with interrupted tetanic nerve stimulation, whereas the dose–responsive curve of acetylcholine (ACh) was not altered. These results suggest that the nicotinic agents tested are presumably acting primarily at the nerve terminals rather than at the receptor sites on the postjunctional membrane. After the effects of the nicotinic agents were completely blocked by TEC alone, the muscle was still responsive to nerve stimulation. This suggests that the TEC blockade of the nicotinic effects of the drugs tested may be at the membrane site of the nerve terminal since ACh is still available in the storage site for release after TEC blockade. In the same preparation, the responses to most of the nicotinic agents were markedly enhanced and prolonged by the presence of 7.7×10^{-7} *M* physostigmine. Since the nicotinic agents tested are not susceptible to the enzymatic hydrolysis of cholinesterases, the potentiation of their nicotinic effects is probably due to the ACh released by these nicotinic agents from the nerve terminals.

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