Enhancement by Carbachol of Transmitter Release from Motor Nerve Terminals

(miniature endplate potential frequency/cholinomimetics/nerve terminal depolarization/
prejunctional cholinceptive sites/cholinergic neurosecretion)

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ABSTRACT In the endplates of rat phrenic nerve-diaphragm, application of the acetylcholine-like compound, carbachol, causes a marked increase in transmitter release, as measured electrophysiologically using miniature endplate potential frequency. Washing out of carbachol reverses the increase in frequency. The ability of carbachol to increase transmitter release is greatly enhanced by perfusion of the preparation with Ringer solution containing elevated K+. At concentrations of carbachol greater than 30 μM, the onset of the postjuncti- 
onal blocking action of carbachol is too rapid and ob- 
scures the increase in miniature potential frequency. The rate of increase in transmitter release is dependent on the concentration of carbachol applied and can be antagonized by d-tubocurarine (10-60 nM) and other blocking compounds. These findings, in contrast to previous reports, indicate that cholinergic nerve endings, like adrenergic nerve endings, respond to applied acetylcholine-like drugs with measurable increases in transmitter output.

The actions of acetylcholine (ACh) and similar compounds (cholinomimetics) on nerve membranes have been well in- 
vestigated. ACh causes membrane depolarization of sensory nerve fibers, non-myelinated vagal C-fibers, sympathetic nerve fibers, and preganglionic and motor nerve endings (1-4). At adrenergic nerve terminals, ACh brings about the release of neurotransmitter (2). By contrast, application of cholinomimetics to cholinergic nerve endings has been reported to be ineffective in causing the release of transmitter from available storage sites. Electrophysiological studies at the neuromuscular junction and sympathetic ganglia have failed to detect any increase in the rate of spontaneous release of transmitter packets (miniature potentials*) in response to applied ACh or the cholinomimetic drug, carbachol (3, 4). ACh, in fact, has been shown to cause a decrease in the output of transmitter evoked by motor nerve stimulation (4, 5). Similarly, radio- 
tracer studies at perfused sympathetic ganglia have shown that the application of carbachol apparently does not cause ACh release from transmitter storage sites, even though depolarization of the nerve terminals by K+ and nerve stimu- 
lation bring about the release of ACh into the perfusion stream (6). These findings imply that depolarization of cholin- 
ergic nerve terminals by cholinomimetics is ineffective in caus- 
ing the release of ACh and that fundamental differences

exist between adrenergic and cholinergic nerve terminals. Al- 
ternatively, the experimental conditions may have been un- 
suited for the demonstration of such an effect on cholinergic nerves of the cholinomimetic drugs. The present findings show that carbachol does increase mepp frequency at motor nerve endings and suggest that cholinomimetics are capable of promoting transmitter release from cholinergic nerve endings.

MATERIALS AND METHODS

Hemi-diaphragms from young male Sprague-Dawley rats (100-130 g) were removed under ether anesthesia and mounted in a 5-ml chamber. Bath temperature was kept at 33°C by heating the perfusion fluid, gassed with 95% O2-5% CO2, before entry into the chamber. A constant flow rate of 2 ml/ 

min was maintained using a Holter roller pump. The control solution contained: (mM) NaCl 120; KCl 5; CaCl2 2; MgCl2 1; NaH2PO4 1; NaHCO3 24; glucose 17. "K+ Ringer" was made by raising KCl to 13, CaCl2 to 3, and lowering NaCl to 110 mM. The effect of elevating the concentration of K+ was an increase in basal mepp frequency (7), presumably due to nerve terminal depolarization (8). Because the frequency increase consisted of both fast and slow components (9), dia- 

phragms were equilibrated for at least 1 hr to ensure steady-state conditions. In the majority of cases, 500 nM tetrodo- 
toxin was added to solutions to reduce muscle twitching and the possibility of antidromic backfiring along the motor nerve. Tetrodotoxin has been reported to have no effect on spontaneous transmitter release (10), and no differences in results were seen in the presence or absence of tetrodotoxin.

Glass microelectrodes filled with 3 M KCl (15-20 MΩ) were connected to a high impedance input preamplifier. To prevent baseline drift, the output was ac-coupled to the oscilloscope. A variable low-pass filter was used to improve the mepp signal-to-noise ratio. Record traces were photographed on moving 35-mm film. The output from the preamplifier was also dc-coupled to a chart recorder to monitor resting mem- 

brane potentials and postjunctional depolarizations by applied drugs. Mepp frequency was used to assess transmitter release and drug effects were indicated by alterations in mepp fre- 

quency. Single junctions were observed for a control period of 10 min, a period of drug exposure, and in most cases, a period of wash-out of drug-containing solutions. Focal endplate recording was indicated by mepp rise times less than 1 msec. Other criteria included stable resting potentials about -50 mV, mepp amplitudes at least 0.5 mV, and measurable frequencies about 20-100/sec.

Abbreviations: ACh, acetylcholine; mepp, miniature endplate potential; dTC, d-tubocurarine.

*The so-called miniature endplate potentials (mepps) for neuro- 
muscular junction or miniature excitatory postsynaptic potentials for ganglia.
Fig. 1. Effect of carbachol on mepp frequency and amplitude. Sample records from a preparation in K⁺ Ringer, showing reduction in mepp amplitude and concomitant increase in mepp frequency on exposure to 10 μM carbachol. Figures (from left to right) (1) control, mean frequency = 31/sec, mean amplitude approx. 1 mV. (2) 5.5 min after application of carbachol, frequency = 137/sec. (3) 1 min after washing out drug, frequency too high and amplitudes too low to measure mepps. (4) 5.3 min after beginning wash, frequency = 57/sec. (5) 12 min after wash, frequency = 32/sec.

RESULTS

The primary effects of cholinomimetic drugs at the chemo-sensitive motor endplate are membrane depolarization and blockade of the postjunctional response to the transmitter substance. Because of the postjunctiional blockade, the ability to assess nerve terminal activity by using frequency or amplitude of mepps is, for all measurable purposes, abolished. In initial studies with carbachol, however, apparent increases in mepp frequency were observed during the onset of receptor blockade. Examination of such records gives results which are equivocal, probably because of the low resting frequency of mepps (1–2/sec) and the rapid onset of postjunctiional blockade. Preparations perfused with K⁺ Ringer solution raise mepp frequency and provide larger statistical samples of mepps before and, particularly, during the bath application of carbachol. Results of studies using K⁺ Ringer solution show unequivocal increases in the frequency of mepps during the application of carbachol, and sample records from a single junction demonstrating this effect are presented in Fig. 1. The left panel shows the control period of recording and the middle panel shows the effect of carbachol. There is a clear increase in mepp frequency, along with a decrease in mepp amplitude, the latter presumably due to postjunctiional blockade. Although cholinomimetics cause an increase in recording noise level (11), the increase in mepp frequency reported here is not an illusion due to increased noise, because the increase in frequency is readily apparent at a time when the basal noise level is unchanged (panel 2 in Fig. 1). In Fig. 2 (top), the effect of carbachol on mepp frequency is plotted with time for another similar junction. Note that the magnitude of the increase cannot be accurately estimated due to the disappearance of mepps at the peak effect of carbachol. The time between the initial increase in mepp frequency and the disappearance of mepps is typically less than 2 min for 30 μM carbachol. On wash-out of the drug, mepps reappear at a frequency higher than control but return to the initial level within 5 min. Upon reapplication of carbachol, mepp frequency is again increased. Fig. 2 (bottom) shows the resting membrane potential of the same endplate and the membrane depolarization in response to carbachol. The onsets of the mepp frequency increase and endplate depolarization are too close to determine whether one event preceded the other (Fig. 2).

Although it is not possible to measure the peak effect of the frequency increase during the application of carbachol, the dose–response effects of carbachol can be estimated by comparing the rate of mepp frequency increase with the concentration of carbachol applied. Assuming that the frequency increase can be fitted with a straight line as indicated in Fig. 2
the inhibition of the frequency increase by 10 nM dTC and of complete blockade at 30-60 nM dTC. Endplate depolarization by the test dose of carbachol is reduced but not abolished at the dTC doses which block the frequency increase. Because of its prejunctional inhibitory action (13), the effect of DMAE [α,α’-bis (dimethylammonium acetaldehyde diethylacetal)-p,p’-diacetylphosphonyl dibromide], an analogue of hemicholinium-3, was also tested. However, DMAE in doses having no effect on mepp size did not cause a selective block of the mepp frequency increase produced by carbachol. DMAE at 3 μM blocks both effects of carbachol but at a lower concentration (0.3 μM) is without effect on either response to carbachol (13 trials). Similar studies using diphenylethanoin, which suppresses nerve terminal excitability (14), show a parallel reduction in mepp frequency increase and endplate depolarization in concentrations up to 10 μM. Collectively, these findings suggest that the cholinocceptor responsible for the increase in mepp frequency by carbachol is also sensitive to blockade by the same agents as those known to block the postjunctional receptors.

**DISCUSSION**

The actions of ACh on sympathetic preganglionic nerve terminals have been investigated electrophysiologically by Nishi and Ginsborg (3). Although ACh clearly depolarizes these nerve terminals, the effect on miniature potential frequency is more difficult to document. High concentrations of ACh bring about only a modest change (3 out of 16 cells tested) in the release of transmitter (Nishi, ref. 3). A similar lack of effect of ACh on spontaneous transmitter release has been reported for the neuromuscular junction (4). By contrast, the present results provide unequivocal evidence that transmitter release at the neuromuscular junction can be evoked by cholinomimetic compounds. Katz and Miledi (11) have mentioned that ACh occasionally increases the frequency of mepps and relate the variability of the response to ACh to the initial level of the resting potentials of the nerve terminals. If this is so, then the effect of elevated K+ may be not only to increase the sampling rate of mepps, but also to increase the efficacy of carbachol in enhancing mepp frequency by lowering the nerve terminal resting potential.

The possibility does exist, however, that the increase in mepp frequency produced by carbachol can be explained by K+ released from the carbachol-depolarized endplate (15). Direct evidence against this possibility can be provided if, for example, the prejunctional response to carbachol is more sensitive to dTC than that of the endplate (16). Our results with dTC, while somewhat equivocal, indicate a partial separation of the two responses to carbachol. The present evidence not-
withstanding, there are strong arguments against the involvement of $K^+$ released from muscle in the presynaptic effects of cholinomimetics (17). Whereas close intra-arterial injection of anticholinesterase agents causes antidromic discharges in the motor nerve, injection of $K^+$ does not. Furthermore, at sympathetic ganglia, intense tetanic stimulation of postganglionic cells, which would be expected to release $K^+$, does not cause presynaptic depolarization (Nishi, ref. 3). Finally, it would appear highly unlikely that such depolarizations of nerve endings by cholinomimetics would occur by a mechanism different from that at other nerves, e.g., vagal C-fibers, where a role for $K^+$ release from other sites is untenable.

Since mepp frequency is a function of presynaptic polarization (7, 8), the increase in mepp frequency by carbachol is consistent with nerve terminal depolarization. The reported decrease by cholinomimetic drugs in transmitter release evoked by nerve stimulation (4, 5) may similarly be explained by depolarization of the nerve terminal, which would reduce the size of the incoming action potential (18). The effects of nerve terminal depolarization on transmitter release are not clear, however, for depolarization by $K^+$ increases both mepp frequency and evoked quantal release (19). These data indicate that the effects on transmitter release of nerve terminal depolarization by cholinomimetic compounds are different from those produced by $K^+$. It may be that low levels of depolarization increase calcium conductance (9) and facilitate evoked release, whereas higher levels of depolarization cause a depression of release by a reduction in the size of the action potential (18). Depending on the amount of depolarization, then, either an increase or a decrease in release might be seen. This possibility suggests that cholinomimetics under appropriate conditions might also increase transmitter release. The failure of investigators (4, 5) to observe increases in release may have been due to the use of $Mg^{++}$, which reduces the coupling between depolarization and transmitter secretion (pp. 122–123, ref. 19). With otherwise drug-free preparations, increases in evoked release have been reported for succinylcholine and decamethonium (20).

An important discrepancy remaining to be considered concerns the radiotracer studies at sympathetic ganglia, in which it was shown that carbachol fails to promote ACh release from transmitter storage sites (6). The failure to provide direct evidence of drug-induced transmitter release by the radiotracer method may be due to several factors. It should be noted that there is evidence for receptor desensitization (21) at nerve terminals, since application of high doses of cholinomimetics (2–4 mM) results in a transient depolarization (3, 4). It is, therefore, conceivable that the high doses of carbachol used in the radiotracer studies with perfused ganglia (6) cause a rapid desensitization and repolarization of the nerve terminals, so that transmitter release occurs for only a brief period. Because of the relatively long sampling time needed for the assay, the increase in ACh release by carbachol may be obscured. With the 100-fold lower doses of carbachol used in the present and other studies (4, 5), there would be a smaller, but more prolonged, depolarization so that, at the motor nerve terminal at least, the increase in transmitter release would be more persistent.

If the assumption is made that mepps reflect the release of ACh from storage sites, then the increase in mepp frequency caused by carbachol clearly indicates the ability of cholinomimetic agents to cause ACh release. Thus, exogenously applied cholinomimetic drugs are capable of promoting transmitter release at both cholinergic and adrenergic (2) junctions. Whether or not endogenous ACh is capable of effecting transmitter release as proposed by Burn and Rand (2) and Koelle (22) is highly problematical and remains to be established.

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