NEUROMUSCULAR TOXICITY OF ANTICHOLINESTERASE AGENTS

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Since the discovery of physostigmine in 1864, later elucidation of the mechanism for its pharmacological action in 1929 and the development of organophosphorus compounds during the World-War II, a great number of chemical agents including carbamates and organic phosphorus compounds have been synthesized as potent inhibitors of acetylcholinesterase (AChE) for uses in therapeutics, insecticides and nerve gases. These anti-cholinesterase agents (Anti-ChE), by inhibiting AChE located at the cholinergic synapse, retard the rapid hydrolysis of the neurotransmitter "acetylcholine" (ACh) released from the central as well as the peripheral cholinergic neurons, and thereby potentiate or ironically deteriorate the cholinergic transmission in brain (particularly the respiratory center), autonomic ganglia, various parasympathetic effector organs (including eye, heart, gastro-intestinal and respiratory tracts and xocrine organs) and the motor nerve-skeletal muscle systems. These effects provoke cholinergic crisis and cause *respiratory failure*, the major outcome of the acute lethal toxicity due to Anti-ChE.

Among various sites of Anti-ChE action, the effect on the motor nerve-skeletal muscle system is unique in that the neurotransmission of this system is not mediated by muscarinic receptor but by muscle-type nicotinic Ch receptor, a ligand-gated cation channel. Activation of this channel by ACh results in an increase of permeability to both Na^+ and K^+ which induces endplate potential (EPP). This depolarization, when reached a threshold, triggers muscle action potential along the whole muscle fiber by activation and opening of Na^+ . The muscle contraction then occurs because of Ca^{2+} release from sarcoplasmic reticulum. In most mammalian skeletal muscle, the amplitude of EPP is large enough (3-5 times of threshold) to trigger muscle action potentials while the duration is very short (within 2 msec) because of rapid hydrolysis of ACh released from nerve termi-

nals. Therefore, on repetitive stimulation with train pulse, the successive EPPs do not superimpose in normal physiological condition. The basal membrane potential is well maintained (~ 80 mV) and muscle action potential can be successfully elicited by successive EPPs in response to repetitive pulse.

When AChE in the synaptic cleft is inhibited, the released ACh cannot be hydrolyzed within msec range of time duration. The amplitude of single EPPs is therefore increased and the duration prolonged to tens of msec, which often provokes two or three repetitive firings of action potential. The twitch amplitude on single stimulation is thus increased by Anti-ChE. Whereas, on repetitive stimulation with train pulse at frequencies higher than 50 Hz, the successive EPPs now superimpose, build up and cause marked sustained depolarization of endplate region to more positive than -50 mV, resulting in a complete inactivation of Na^+ channel. As a result, the repetitive stimulation, in different with single stimulation, can no longer elicit action potentials continuously (Fig. 1) and the tetanic muscle contraction fails (Fig. 2). A desensitization of ACh receptor appears unlikely and does not contribute to the failure of tetanic contraction.

In addition to the above postsynaptic phenomenon, we have further found that the nerve terminal is brought to a chaotic situation in the presence of Anti-ChE. When the nerve is stimulated with a train of pulse at 100 Hz for only 50 msec, an explosive yet sustained and massive release of ACh is triggered which depolarized the endplate rapidly from -80 mV to -40 mV for up to 1000 msec. During this period the muscle is made unable to respond with any action potential because Na^+ channel nearby the endplate region is inactivated. Moreover, continuous stimulation of the nerve during this pathological ACh release cannot evoke additional EPP, implying that the nerve terminal is also inactivated because of depolarization. Since the explosive release maintained at its plateau for a prolonged time with no sign of desensitization, it

Fig. 1. Action potentials and regenerative endplate depolarization in mouse phrenic nerve- diaphragm preparations. The nerve was stimulated repetitively at 75 Hz in the absence (upper) or presence of $0.3 \mu\text{M}$ neostigmine (lower). The calibrations were 20 mV and 40 msec. The dotted line indicates zero mV membrane potentials. Note the cumulative depolarization and spontaneous upstroke of membrane potential (arrow) and the subsequent failure of EPPs.

Fig. 2. Antagonism by β -eudesmol alone or in combination with obidoxim against the tetanic fade induced by DFP. The phrenic nerve was stimulated at 75 Hz for 3 sec. Panels from left to the right show, respectively, the responses in control, after treatment with $10 \mu\text{M}$ DFP for 80 min followed by wash, after further treatment with vehicle (upper) or $20 \mu\text{M}$ β -eudesmol for 40 min (lower) and after further addition of $0.05 \mu\text{M}$ obidoxim.

appears likely that a Ca^{2+} channel rather than Na^{+} channel is recruited for the nerve terminal depolarization. The above electrophysiological findings on the pre- and post-synaptic site can account for the acute failure of tetanic contraction and respiratory paralysis in Anti-ChE intoxication. It is found that in companion with these sustained endplate depolarization, the endplate area of muscle is made locally contracted during the period of explosive depolarization, implying that there could have occurred intensive influx and/or intracellular release of Ca^{2+} . Indeed, an increase of myoplasmic Ca^{2+} at endplate region is reported after repetitive stimulation in the presence of Anti-ChE by Kimura and his associates. The explosive release of ACh at nerve terminals also implicates that there could be also a massive influx of Ca^{2+} at the nerve terminals. It seems possible that these pathological increase of intracellular Ca^{2+} in the motor nerve and muscle may contribute to untoward effect "myoneuropathy" often seen after intoxication from organophosphate Anti-ChE.

We have next examined the Ca^{2+} channel subtypes which could be involved in the normal (physiological) release of ACh and the Anti-ChE-provoked explosive (pathological) release. By using specific inhibitors for subtypes of Ca^{2+} channel, e.g., verapamil and nifedipine for L-type, ω -conotoxin GVIA for N-type, ω -agatoxin IVA for P-type, ω -conotoxin MVIIC for Q-type, the Ca^{2+} channel mediating the physiological release of ACh from mammalian motor neuron is identified to be P-type, whereas the pathological explosive release is mediated mainly by L-type channels as revealed by the marked shortening of the pathological release by verapamil and nifedipine. Some N-type may be also involved. In the presence of P-type channel antagonists, increased number of pulse is needed for triggering explosive release, while the amplitude and duration of the explosive release is unaffected once it is triggered. Since the explosive release is extremely sensitive to inhibition by tubocurarine, it is inferred that the explosive release of ACh is mediated by L-type Ca^{2+} channel, which in turn is activated by depolarization caused by accumulated ACh in the synaptic cleft by acting on the nicotinic receptor on the nerve terminals. Hexamethonium and choline are weak curare-like compounds and appear to antagonize the Anti-ChE-induced sustained endplate depolarization as well as the tetanic failure better than tubocurarine. These compounds should be considered as potential candidate for intoxication from Anti-ChE.

The effect of obidoxim (an reactivator of phosphorylated AChE) on the explosive release of ACh and reversal of neuromuscular transmission affected by Anti-ChE is therefore studied by comparing its effect against the neuromuscular failure induced by neostigmine, a reversible carbamate Anti-ChE and that by diisopropyl fluorophosphate (DFP), an irreversibly acting organophosphorus Anti-ChE. Obidoxim antagonized the explosive release induced by both Anti-ChEs and restored the tetanic contraction in isolated mouse diaphragm preparations. The EC_{50} , however, is significantly larger for neostigmine than for DFP; being $300 \mu\text{M}$ against neostigmine and $0.6 \mu\text{M}$ against DFP when DFP was washed out. The obidoxim EC_{50} increased dose-dependently on increase of DFP-concentration ($1\sim30 \mu\text{M}$) if not washed out, and increased to $30 \mu\text{M}$ when $30 \mu\text{M}$ of DFP was used. This result indicates that obidoxim restore the neuromuscular transmission by dual mechanism; one by reactivation of AChE phosphorylated by organophosphorus Anti-ChE and the other by a direct effect, possibly by an curare-like anti-nicotinic effect or by effect on the nicotinic receptor channel. At hundred micromolar range, obidoxim has significant direct depressant effect on the EPP amplitude, particularly in the presence of Anti-ChE.

Diazepam and β -eudesmol (a terpenol from Chinese herbs) also antagonize the explosive release of ACh and restore the tetanic contraction. Unlike the different potency of antagonism by obidoxim against neostigmine and DFP, β -eudesmol displayed the same potency against both types of Anti-ChE. Interestingly, the antagonistic effect of β -eudesmol is synergistic with that of obidoxim (Fig. 2), particularly for the muscle intoxicated with DFP. The study of the antidotal effect of β -eudesmol against the lethal toxicity of neostigmine and DFP in mice reveals that DFP lethality is greatly reduced by β -eudesmol when used in combination with atropine and obidoxim. The LD_{50} of subcutaneous DFP was increased from 4.2 mg/kg to 6.4 mg/kg after pretreatment with β -eudesmol, 7.8 mg/kg after atropine, 10.6 mg/kg after atropine plus β -eudesmol, 281 mg/kg after atropine plus obidoxim, and to a value larger than 800 mg/kg after a combination of atropine, obidoxim and β -eudesmol. No such synergism is found against the lethality of neostigmine. β -Eudesmol may become one effective adjunct to the therapy of intoxication from carbamate and organophosphorus Anti-ChE. Whether or not β -eudesmol acts synergistically with benzodiazepines for treatment of Anti-ChE poisoning

remains to be elucidated.

Another observation worthy-mentioning is the interaction of Anti-ChE with various L-type Ca^{2+} antagonists, proadifen, chlorpromazine, imipramine and phencyclidine. These compounds, probably acting on nicotinic receptor allosterically, are generally inactive against the neuromuscular transmission. Whereas, in the presence of Anti-ChE, these drugs now produce marked neuromuscular blocking action by rapid desensitization of ACh receptors especially when the nerve is repetitively stimulated. The combination use of these compounds with any Anti-ChE in clinic could result in unexpected muscle paralysis.

In addition to the acute neurotoxicological effects of Anti-ChE, possible effects on motor nerve and muscle after chronic treatment with subtoxic dose of neostigmine in rats are studied. After 7 days of treatment with neostigmine, the hemidiaphragm of rats contained only half normal amount of nicotinic ACh receptors at the endplate zone. Evidently, there occurs down-regulation of the nicotinic receptor when the metabolic enzyme is decreased and the transmitter accumulated, presumably in order to compensate the pharmacologically enhanced synaptic transmission. Interestingly, the release of neurotransmitter is also halved after chronic treatment, indicating down-regulation occur not only at the post-synaptic receptor level but also at the presynaptic transmitter level. It was later confirmed by Albuquerque and his associates that the subsynaptic structure has also changed; synaptic folds being flattened followed by vacuolation and muscle necrosis. These myopathic changes are likely induced by the increase of myoplasmic Ca^{2+} as outlined above for the acute neuromuscular effects of Anti-ChE. It is reported that this myopathy can be ameliorated by various agents including nicotinic ACh receptor antagonists and L-type Ca^{2+} channel antagonists.

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