Azathioprine:

Effects on Neuromuscular Transmission

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The neuromuscular effects of azathioprine were examined in the in-vitro cat soleus muscle preparation. In concentrations ranging from 10 to 1,000 μg/kg, administered intra-arterially, the agent caused motor axons to fire repetitively and produced a dose-related increase in the force of contraction. The drug reversed neuromuscular blockade produced by d-tubocurarine and potentiated the neuromuscular blockade produced by succinylcholine. The effects of theophylline, a phosphodiesterase inhibitor, on neuromuscular transmission were identical to those produced by azathioprine. Using an in-vitro assay preparation, azathioprine was found to produce 50 per cent inhibition (IC₅₀) of phosphodiesterase at a concentration of 2 × 10⁻⁴ M. In the same preparation, theophylline had an IC₅₀ of 1 × 10⁻⁴ M. Neither agent in concentrations to 10⁻² M affected cholinesterase activity measured in vitro. It is concluded that the effects of azathioprine on neuromuscular transmission are due to inhibition of phosphodiesterase in the motor nerve terminal. (Key words: Antagonists, neuromuscular relaxants, azathioprine; Neuromuscular relaxants, azathioprine.)

Azathioprine (Imuran*), a derivative of mercaptopurine, is an immunosuppressive drug used clinically to prevent rejection of transplanted kidneys. While the drug is usually given orally, it may be administered intravenously during the transplant operation. In the course of one such operation, in which the neuromuscular blockade induced by pancuronium was monitored by recording the force of contraction of the neurally stimulated adductor pollicis muscle, one of us (L.W.) observed that the administration of azathioprine antagonized the action of pancuronium (fig. 1). This phenomenon was subsequently observed in three other patients in whom muscle relaxation was produced by administration of either d-tubocurarine or pancuronium.

Azathioprine has no obvious relationship to the classic anticholinesterase agents, such as edrophonium and neostigmine. However, purines are structurally related to xanthines, and one of the latter, theophylline, has received attention recently as an inhibitor of phosphodiesterase.† It occurred to us that azathioprine might also inhibit phosphodiesterase, and this might be the mechanism by which it exerts its anticholinesterase action.

Methods and Materials

Cat Soleus Preparations

Cats weighing 2 to 3 kg were anesthetized with alpha-chloralose, 70 mg/kg (iv). Tracheotomies were performed and the animals were artificially ventilated with a constant-volume respiratory pump. Arterial blood was withdrawn periodically from a catheter in a femoral artery for determination of arterial blood pH, PₐO₂, and PₐCO₂. These were kept within the

<table>
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<th>ABBREVIATIONS</th>
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<td>IC₅₀ = concentration that produces 50 per cent inhibition</td>
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<td>PDR = post-drug repetitive activity</td>
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<td>PTF = posttetanic potentiation</td>
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physiologic range for the cat by adjusting respiratory rate and volume. The two heads of the gastrocnemius muscle were removed and the soleus nerve and muscle isolated. The exposed tissues were covered with mineral oil pools thermoregulated at 37°C. The calcaneus was cut and the tendon of the soleus muscle was attached to a force transducer through a steel rod. Supramaximal rectangular pulses of 0.1-msec duration were applied to the soleus nerve by means of bipolar platinum-iridium electrodes at a frequency of 0.4 Hz. Every 5 min a 400-Hz stimulus train was interposed for 10 sec. This produced a tetanic contraction in the muscle and led to posttetanic potentiation (PTP) of contraction strength.

In some animals a dorsal laminectomy was performed on vertebrae L3-S1. Filaments containing single axons from decentralized ventral roots were placed on bipolar platinum-iridium electrodes. The soleus muscle and its nerve were exposed and stimulated as described above. The ventral root electrodes recorded antidromically conducted action potentials, which were stored on magnetic tape for later analysis and photography. For details of the method, see Standaert.2

Denervated muscles were prepared by anesthetizing animals with ketamine, 25 mg/kg, im, and aseptically cutting the sciatic nerve at the sciatic notch. Two weeks later the animal was anesthetized with alpha-chloralose, 70 mg/kg, iv, and the leg prepared and mounted as above. Electrodes for direct stimulation were formed by sewing 33-gauge stainless steel wire in concentric loops at the musculotendinous junction and approximately 1 cm cranial from it.

Reagents Employed

Acetylthiocholine iodide, dithiobisnitrobenzoic acid acetylcholinesterase purified from Electrophorus electricus, and phosphodiesterase purified from bovine heart were obtained from Boehringer Mannheim Biochemicals. Azathioprine was obtained from Burroughs-Wellcome and theophylline from Sigma Chemical Company. For the soleus muscle preparations, azathioprine, theophylline, d-tuboemarure, gallamine, pancuronium, or succinylcholine was dissolved in 0.85 per cent sodium chloride solution and administered intraarterially (ia) in a volume of 0.1 ml/kg.

Enzyme Assays

Phosphodiesterase was dialyzed overnight against 15 liters of pH 7.4 Tris-HCl (50 mM). Phosphodiesterase activity was assayed according to the method of Huang and Kemp.3 Acetylcholinesterase was dissolved in pH 8.0 phosphate buffer (0.01 M), and acetylthiocholine iodide was used as the substrate. Acetylcholinesterase activity was assayed according to the method of Ellman et al.4 The degree of inhibition of either enzyme is expressed in terms of the IC50 value, that is, the concentration of inhibitor that gave 50 per cent inhibition, determined from the plot of log concentration of inhibitor vs. percentage enzyme inhibition.

Results

Neuromuscular Effects

Azathioprine in doses ranging from 10 μg to 1 mg/kg, ia, increased the contraction strength of the soleus muscle to between 150 and 300 per cent of control. The effect was immediate in onset and lasted 10 to 120 seconds, depending on the dose of azathioprine (fig. 2). The increase in the force of contraction was accompanied by trains of repetitive potentials in motor axons (fig. 3). This post-drug repetitive activity (PDR) was present as long as the contraction strength remained above control values. These doses of the drug also increased
the durations of the posttetanic repetitive activity (PTR) in the motor axons, and the posttetanic potentiation (PTP) of muscle strength.

Azathioprine, in doses greater than 100 μg/kg, ia, reversed the neuromuscular blockade produced by prior administration of d-tubocurarine. The maximum effect was observed with a dose of 330 μg/kg. The reversal began immediately and the maximal effect occurred within 1 minute. Reversal was virtually complete, i.e., the strength of contraction returned to within 10 per cent of control values, but in no instance did administration of azathioprine increase the strength of contraction to above control values. A typical record of the reversal of a blockade induced by d-tubocurarine can be seen in figure 4. Azathioprine also reversed the depression of PTR and PTP produced by d-tubocurarine. Blockade of neuromuscular transmission produced by gallamine or pamecuronium was also overcome by azathioprine. The time course of the effect and the doses needed were exactly the same as for the reversal of blockade produced by d-tubocurarine.

In contrast to its effect on the blockade produced by nondepolarizing agents, azathioprine potentiated the neuromuscular blockade.
produced by succinylcholine (fig. 4). Similarly, it potentiated the depression of PTR and PTP induced by succinylcholine.

Azathioprine had no effect on denervated muscle. Injection of as much as 2 mg/kg had no effect on the strength of contraction of the directly stimulated muscle. The agent also did not affect the time course or amplitude of the contractions produced in denervated muscle by rapid intra-arterial injection of 0.5–5 μg/kg acetylcholine.

Theophylline also increased the force of contraction of neurally stimulated muscle (fig. 5). Threshold was at 100 μg/kg and maximum effect at 600 μg/kg. The onset was immediate or within 10 seconds, and the effect lasted 60 to 120 seconds. Associated with the increase in the force of contraction was the appearance of repetitive activity (PDR) in motor axons. The pattern of PDR produced by theophylline was identical with that produced by azathioprine. These doses of theophylline also increased the durations of PTR and PTP.

Theophylline in concentrations greater than 200 μg/kg reversed neuromuscular blockade induced by d-tubocurarine (fig. 5). Maximal effect was observed with 400 μg/kg. The onset was immediate, with the maximum occurring within 40 seconds. The reversal was virtually complete, but in no case did theophylline increase the strength of contraction to above control values. Theophylline also reversed the depression of PTR and PTP induced by d-tubocurarine. Like azathioprine, theophylline...
Fig. 6. A comparison of the degrees of inhibition of phosphodiesterase activity by azathioprine and theophylline. The ordinate represents percentage inhibition of phosphodiesterase. Phosphodiesterase activity in the absence of inhibitors is 12.4 ± 0.83 nmol cyclic AMP hydrolyzed/mg protein/min. The abscissa represents the concentrations (mol/L) of the inhibitors. Each point represents the mean ± SEM of six determinations.

potentiated the neuromuscular blockade produced by succinylcholine (fig. 5). It also potentiated the depression of PTR and PTP induced by succinylcholine.

Theophylline had no effect on denervated muscle: as much as 5 mg/kg had no effect on the strength of contraction of stimulated muscle or on the contraction produced by injection of acetylcholine.

**ENZYME EFFECTS**

Azathioprine inhibited partially purified bovine heart muscle cyclic nucleotide phosphodiesterase activity with an IC50 of $2 \times 10^{-5}$ M. In the same preparation, theophylline had an IC50 of $1 \times 10^{-4}$ M. The inhibition curves for both compounds are shown in figure 6.

Neither azathioprine in concentrations as high as $10^{-2}$ M nor theophylline in concentrations as high as $5 \times 10^{-2}$ M caused any significant inhibition of acetylcholinesterase.

**Discussion**

This study shows that azathioprine has a facilitatory effect on neuromuscular transmission. The drug increases the force of contraction of neurally stimulated muscle and reverses the neuromuscular blockade produced by nondepolarizing agents. The latter effect occurs in man and in the cat.

It would appear that the facilitatory action can be ascribed to an effect on the motor nerve terminal, because administration of azathioprine caused the motor axon to fire repetitively in response to a single nerve stimulus (PDR). The PDR was conducted antidromically in the nerve and transmitted orthodromically to the muscle. In the latter, it produced brief tetanic contractions of the muscle. Many other agents produce this effect, and in each case the phenomenon has been traced to an action of the drug on motor nerve endings. In addition, azathioprine enhanced both PTR and PTP. Again, PTR and the subsequent PTP are thought to be prejunctional in origin. Finally, the agent did not alter the strength or the response to acetylcholine of denervated muscle; this suggests that direct depolarization of a postsynaptic site or modification of muscle contractile force did not play a significant role in the effects we observed.

The question arises: how does azathioprine affect the motor nerve terminal? A clue can be found in the similarity of the structures of azathioprine and theophylline (fig. 7). Recently, we demonstrated that a cyclic nucleotide system may be involved in the function of the motor nerve terminal. We suggested that the nerve action potential that invades the terminal stimulates the activity of adenylate cyclase and thereby increases the production
of cyclic AMP. The cyclic AMP, through a series of intermediate steps involving protein kinases, causes an influx of calcium ions and results in the release of transmitter. The cyclic AMP, which is the key to the hypothesized release mechanism, is destroyed rapidly by the enzyme, phosphodiesterase. Theophylline inhibits this enzyme, causing cyclic AMP to accumulate and accentuating its effects. If our hypothesis concerning cyclic AMP function in the motor nerve terminal is correct, then the present experiments prolonging the increase in concentrations of cyclic AMP prolonged the time of depolarization of the motor nerve terminal and, thereby, caused generation of repetitive activity (PDR). Transmitter release was increased also. The latter could cause reversal of neuromuscular blockade produced by d-tubocurarine and potentiation of neuromuscular blockade produced by succinylcholine.

A similar inhibition of phosphodiesterase is likely to be the mechanism by which azathioprine exerts its effects on neuromuscular transmission. First, azathioprine does not inhibit acetylcholinesterase. Second, all of the effects of azathioprine on neuromuscular transmission are identical to those produced by the known phosphodiesterase inhibitor, theophylline. Third, by direct chemical assay we have shown that azathioprine is a more potent inhibitor of phosphodiesterase than theophylline (IC_{50} 2 \times 10^{-5} M, 1 \times 10^{-4} M). Fourth, the relative potencies of theophylline and azathioprine in producing neuromuscular effects are the same as their relative activities in inhibiting phosphodiesterase. In both cases, only half to one tenth as much azathioprine is needed to produce an effect equivalent to that of theophylline. Finally, inhibition of phosphodiesterase would adequately explain the effects of azathioprine. The prolongation of the presence of cyclic AMP would prolong the permeability of the nerve ending to calcium. This would prolong the depolarization of the membrane of the nerve ending and increase the release of transmitter. The former would cause repetitive activity and a brief tetanic contraction of the muscle, and the latter would antagonize the action of d-tubocurarine and potentiate that of succinylcholine.

In conclusion, azathioprine has been shown to facilitate neuromuscular transmission, reverse the blockade of neuromuscular transmission produced by d-tubocurarine, potentiate the blockade of neuromuscular transmission produced by succinylcholine, and inhibit phosphodiesterase. We suggest that the effects of azathioprine on neuromuscular transmission are due to an inhibition of phosphodiesterase in the motor nerve terminal.

References