Anesthesiology
50:199–204, 1979

Pre- and Postsynaptic Effects of Pancuronium at the Neuromuscular Junction of the Mouse

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The dose-effectiveness of pancuronium as it relates to membrane potentials, action potentials, electrical membrane constants, miniature endplate potentials, endplate potentials, and quantal release was studied in murine phrenic nerve–diaphragm preparations *in vitro*. Emphasis was placed on comparison of presynaptic with postsynaptic effects of pancuronium under similar experimental conditions. At low concentrations of pancuronium (5×10^{-8} g/ml or less), no presynaptic effect was found. At high concentration (5×10^{-7} g/ml), pancuronium depressed quantal release to 26 per cent of control in cut-fiber preparations and 40 per cent of control in high-magnesium preparations. Postsynaptic effects, as measured by the amplitude of miniature endplate potentials and relative depolarization induced by 20 μM carbachol, revealed depression to 16 and 22 per cent of control, respectively, at a pancuronium concentration of 5×10^{-7} g/ml. Pancuronium had no effect on directly elicited action potentials and electrical membrane constants. The authors conclude that presynaptic as well as postsynaptic effects of pancuronium in paralytic doses are essential in contributing to the total efficacy of neuromuscular depression. (Key words: Muscle, skeletal: endplate. Neuromuscular junction: action potential; synapses. Neuromuscular relaxants: pancuronium. Neuromuscular transmission.)

SINCE ITS INTRODUCTION by Buckett et al.,[1] pancuronium bromide has been used widely in clinical anesthesia because it lacks the ganglion-blocking and histamine-releasing properties of *d*-tubocurarine. Its mechanism of action is thought to resemble that of *d*-tubocurarine,[1–3] namely, it competitively occupies postjunctional receptors. Evidence for a postjunctional effect of pancuronium was obtained from a decrease in the sensitivity of muscle to an intra-arterial injection of acetylcholine.[1] In addition, neuromuscular blockade due to pancuronium could be reversed by anticholinesterase,[4] a finding that is also compatible with a postjunctional effect. Although there is no doubt that pancuronium possesses prominent postjunctional effects, more recent studies have indicated that it may have significant prejunctional actions. By use of a bioassay method, Gergis *et al.*[5] found that pancuronium increased the release of acetylcholine in low concentrations and blocked the release in high concentrations. Sohn *et al.*,[6] however, found that pancuronium depressed terminal motor nerve activities even in subparalytic concentrations. Galindo[7] reported that pancuronium depressed the acetylcholine release and also prolonged the refractory period of prejunctional structures. Blaber[8] found pancuronium depressed the rates of refilling of the store and depletion of the store, but did not affect the probability of release, a fact contradictory to Galindo's findings.

Because of the difficulty in assessing the different experimental conditions in the literature, the principal site of action of pancuronium remains undetermined (see Buckett[9] for a review). In none of the experiments mentioned above was a complete dose–response relationship for both presynaptic and postsynaptic action of pancuronium reported. Such a relation is important because it may provide an answer to the question of whether a presynaptic action of pancuronium is of functional importance in its total effect on neuromuscular transmission. In the present experiments we have re-examined the question of the sites of action of pancuronium. We have also investigated the effects of pancuronium on the muscle membrane outside the neuromuscular junction, since few data are available concerning this aspect of the action of pancuronium.

Methods

Phrenic nerve–diaphragm preparations from Swiss mice weighing 35 to 45 g were studied *in vitro*. The mice were anesthetized with ether during dissection. The diaphragm with attached phrenic nerve was bathed in a 25-ml lucite chamber containing a modified Krebs' solution (mM: NaCl 150, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) 3, glucose 11; *p*H 7.3–7.4) and bubbled with pure oxygen. The thoracic surface of the diaphragm was pinned upward on the bottom of the chamber, which was coated with a thin layer of transparent Sylgard® (Dow Corning Corporation). The Krebs' solution was circulated continuously via a dual-syringe system and peristaltic pump (911 Holter) at a rate of 10–15 ml/min. To obtain cellular detail, we used a 20× water-immersion objective[10] with a

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Received from the Division of Neurology, State University of New York at Stony Brook, and Neurology Service, Veterans Administration Hospital, Northport, New York. Accepted for publication May 19, 1978. Supported by Veterans Administration General Research Fund 8206-01.

total magnification of 400×. The objective has a working distance of 3 mm and permits electrodes to be positioned above the muscles with micromanipulators. Resting potentials, action potentials, endplate potentials and miniature endplate potentials were recorded in a conventional manner using glass microelectrodes filled with 3 M KCl with an impedance of 5 to 15 megaohms. A high-impedance electrometer (Industrial Science Association) with a gain of 10 was coupled to a Tektronix 565 and a 5403 storage oscilloscope. Through amplification, the signals were recorded on FM tape (Hewlett Packard 3939). An audio-monitor incorporating a digital meter was DC-coupled to the electrometer and displayed the resting potential continuously. The monitor also provided an audio signal whose frequency depended on the potential measured by the recording electrode.[11]

The signals were photographed (Grass C-4 camera) and measured for further analysis. Neural stimulation was delivered by a pair of platinum wires embedded in a 0.03-inch plastic tube and immersed in a chamber near the entry of the nerve to the muscle. The stimuli were 0.1-msec square waves supplied by a Grass S88 stimulator. The voltages used were two and a half times the threshold required for initiating contraction, and usually ranged between 7 and 15 volts. To record directly stimulated action potential and threshold for action potential, a current-passing electrode was placed intracellularly near the recording electrode at a distance of 0.05–0.15 mm. In order to minimize the effect of variation in the resting potential, the potentials of the cell were locally controlled to a level of 80 mV by passing a hyperpolarizing current of 100 msec duration through the membrane. Once it has reached a steady state of hyperpolarization, a 1-msec depolarizing pulse was introduced from another synchronized stimulator (Grass S88) in parallel with the first. The voltage of the depolarizing pulse was then increased in steps until an action potential was generated. The threshold for initiation of an action potential was determined by reading the resting potential at which an action potential was produced. The time constant for the recording circuit is usually 10 μsec. Maximal rates of rise and fall of action potentials were obtained by using an RC differentiating circuit (time constant 1.1×10^{-4} sec). The neuromuscular junction was located by the presence of a miniature endplate potential with a rise time of less than 1.2 msec. Pancuronium in doses ranging from 0.5×10^{-8} to 50×10^{-8} g/ml in modified Krebs' solution was perfused via a reservoir into the preparation chamber. The temperature of the bath solution was monitored (YSI, Tele-thermometer) and was maintained constant at 30 ± 1 C. (Melsunger, Thermomix 1420). At least 30 min were allowed for the muscles to equilibrate with the bath solution before readings were made. In some instances, the same cell was continuously observed during perfusion.

To determine quantal content, endplate potentials were recorded either in cut-fiber preparations or by using high magnesium ion (6–10 mM) concentrations to block transmission. For cut-fiber preparation the muscle fiber of the diaphragm was removed 2–3 mm

TABLE 1. Effects of Pancuronium on Electrical Membrane Constants of Mouse Diaphragms (Mean ± SE)

	Cells (Number of Animals)	Input Resistance (MΩ)	λ (mm)	τm (msec)	Rm (ohm/cm²)	Cm (μF/cm²)
Control	29 (4)	0.71 ± 0.05	0.47 ± 0.05	9.27 ± 1.86	1,014 ± 78.6	9.7 ± 1.9
Pancuronium, 1×10^{-6} g/ml	24 (4)	0.69 ± 0.05	0.43 ± 0.04	9.67 ± 0.95	956 ± 76.7	11.4 ± 1.3
Pancuronium, 1×10^{-5} g/ml	19 (3)	0.65 ± 0.05	0.49 ± 0.04	14.0 ± 2.8	1,083 ± 116.5	13.5 ± 2.2

TABLE 2. Effects of Pancuronium on Directly Stimulated Action Potentials of Mouse Diaphragms (Mean ± SE)

	Cells (Number of Animals)	Resting Potential (mV)	Action Potential (mV)	Maximal Rate of Rise of Action Potential (V/S)	Maximal Rate of Fall of Action Potential (V/S)	Duration of Action Potential as Measured at 0 mV Level (msec)	Threshold (mV)*
Control	22 (3)	−80.3 ± 0.9	103.6 ± 3.1	360.6 ± 24.6	58.1 ± 3.3	0.57 ± 0.05	−50.8 ± 0.8
Pancuronium, 5×10^{-7} g/ml	17 (3)	−80.1 ± 0.7	105.4 ± 3.0	346.0 ± 23.0	60.3 ± 7.7	0.58 ± 0.01	−48.1 ± 1.1
Pancuronium, 1×10^{-5} g/ml	20 (3)	−80.5 ± 1.0	108.1 ± 2.6	332.8 ± 24.4	66.5 ± 9.4	0.58 ± 0.02	−47.4 ± 1.6

* See text.

on either side of the main intramuscular nerve branch according to the method of Hubbard and Wilson.[12] The potassium ion concentration in the cut-fiber preparation was decreased to 2.5 mM instead of 5.0 mM to avoid nerve block. The equilibrium potential of −12 mV was used to correct nonlinear summation of endplate potentials in cut-fiber preparations,[12] and −15 mV was used in the high-magnesium experiments.

Miniature endplate potentials and endplate potentials were analyzed with the aid of a computer (IBM 360/44), which applied Martin's[13] correction. The quantal content (m) of the endplate potentials was determined by the ratio of the mean amplitude of the endplate potential to that of the miniature endplate potential. At least 100 consecutive endplate potentials evoked at 1 Hz and 50–100 miniature endplate potentials were used for the estimate of m. At higher concentrations of pancuronium the miniature endplate potential was decreased so that it was not visible. In such circumstances, m was estimated indirectly based on Poisson assumptions that

$$m = \frac{1}{\overline{CV}^2}$$

where $\overline{CV}$ was the coefficient of variation of the amplitudes of endplate potentials.[14]

The membrane constants were calculated by applying the cable theory of Hodgkin and Rushton[15] and the square-pulse analysis of Fatt and Katz.[16] The potential, V, produced by a steady current, I, through the membrane is given by the formula:

$$V = \tfrac{1}{2}\, I(rm \cdot ri)^{1/2}[\exp - x/(rm/ri^{1/2}]$$

where x is the electrode separation measured by the micrometer attached to the eyepiece, rm the transverse resistance, and ri the internal longitudinal resistance per unit length of the fiber. The potential changes V were measured at three interelectrode distances (0.05 to 0.5 mm). The log V/I was plotted against the distance in millimeters between electrodes. The data were analyzed further only when there was a linear relationship by visual inspection between these three plots. A linear regression line was then derived by the least-square method based on the observed data. The input resistance, $Rin = \tfrac{1}{2}\,(rm \cdot ri)^{1/2}$, is the antilog of the intercept of the linear regression line of the log V/I axis when X = 0. From the inverse of the slope of the linear regression line the space constant $\lambda = (rm/ri)^{1/2}$ was obtained. From these two values rm and ri were calculated. The transverse resistance of a unit area is $Rm = 2\,\tau\,p\,rm$, where fiber radius (p) was measured directly in isometrically fixed hematoxylin and eosin-stained specimens after

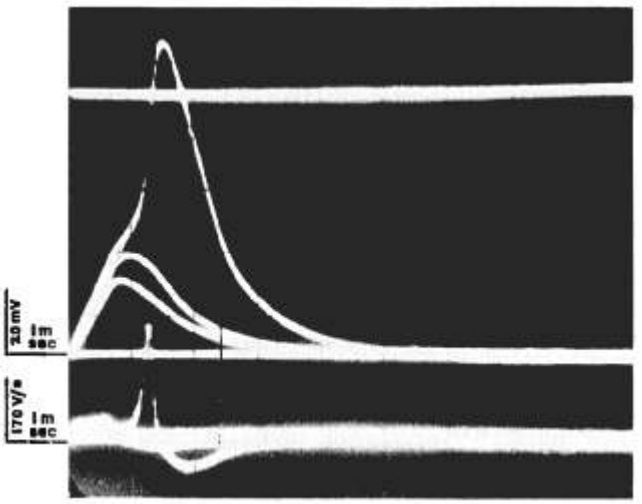

FIG. 1. Directly stimulated action potential of mouse diaphragm muscle fiber in pancuronium 10^{-5} g/ml. First horizontal line, 0 mV (outside the cell): second horizontal line, resting potential at −85 mV (20 mV/division); action potential 104 mV; threshold, −49 mV; third horizontal line, rates of rise (306 volts/sec) and fall (68 volts/sec). Time scale, 1 millisecond/division.

the experiment. We measured a total of 84 cells in three 40–45-g mice and obtained a value for p of 27.1 ± 6.4 μm (mean ± SD). The capacity of the membrane Cm was calculated from the measured Rm after determination of the time constant τm according to Cm = τm/Rm. The τm was determined on the basis of the spreading rate of electrotonus in that

$$\tau m = 2\,\frac{\Delta t}{\Delta x}\,\lambda$$

where Δx (mm) is the distance between two recording electrodes and Δt (msec), the time difference between the increase or decrease in amplitude of the electronic potential to half of its steady-state value.

To determine the significance of the results, the control as well as pancuronium-treatment data from different mice were pooled. A two-tailed Student t test was performed, and $P < 0.05$ level was considered significant.

Results

Pancuronium had no significant effect on resting membrane potentials. For a total of 36 cells, the mean resting potential at a concentration of 5×10^{-8} g/ml was −75.8 ± 1.6 mV (±SE), whereas the control value was −77.3 ± 1.2 mV (56 cells). At higher concentrations of pancuronium, 1×10^{-6} g/ml and 1×10^{-5} g/ml, the resting potentials were −74.8 ± 3.5 mV (20 cells) and −73.7 ± 1.2 mV (17 cells), respectively, which were not significantly different from the control value. No effect on electrical mem-

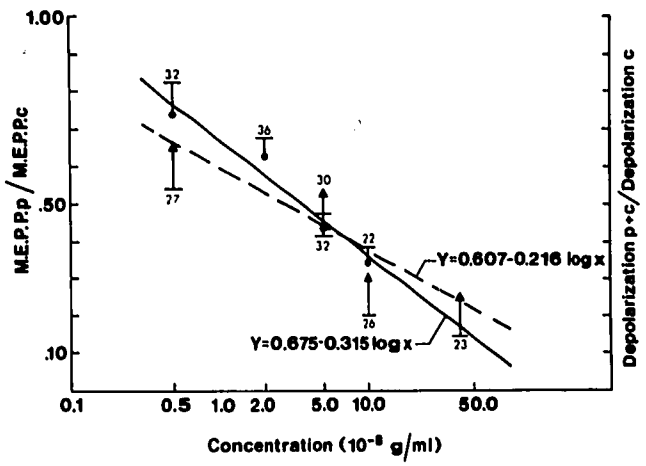

FIG. 2. Postjunctional effect of pancuronium. *Ordinate (left)*, ratio of amplitude of miniature endplate potentials (MEPP) with pancuronium to control; *ordinate (right)*, ratio of depolarization produced by 20 μM carbachol plus pancuronium to depolarization with carbachol alone. *Abscissa*, concentration of pancuronium in ml; this can be converted to μM by a factor of 0.0136. Number in parentheses indicates the total number of muscle fibers. *Bar*, SE of mean. *Solid line*, effect on MEPP; broken line, effect on carbachol-induced depolarization.

brane constants was found (table 1). Action potentials maintained a normal configuration and normal characteristics in muscle fibers treated with pancuronium in doses as high as 1×10^{-5} g/ml (fig. 1, table 2).

Pancuronium produced significant depression of the amplitude of miniature endplate potentials at concentrations of 5×10^{-9} g/ml or more (fig. 3). At high concentrations of pancuronium (more than 1×10^{-8} g/ml) the amplitudes of miniature endplate potentials were so depressed that they were indistinguishable

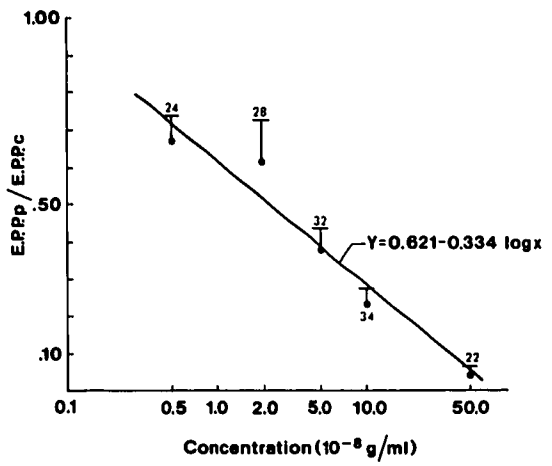

FIG. 3. Effect of pancuronium on the amplitude of EPP. *Ordinate*, ratio of endplate potentials (EPP) amplitude with pancuronium to control. *Abscissa*, concentration of pancuronium. Number in parentheses indicates total number of muscle fibers. *Bar*, SE of mean.

from baseline noise. Nevertheless, the linear regression line obtained by the least-squares method showed that the amplitude of miniature endplate potentials was 16 per cent of control at a concentration of 5×10^{-7} g/ml. The frequency of miniature endplate potentials was not significantly altered. At low concentrations of pancuronium (2×10^{-8} g/ml), the frequency of miniature endplate potentials was 0.89 ± 0.02/sec (25 cells), while the control value was 0.92 ± 0.04/sec (30 cells); at 5×10^{-8} g/ml the frequency of miniature endplate potentials was 0.83 ± 0.10/msec (28 cells) and the control value was 1.0 ± 0.08/sec (30 cells).

Postsynaptic sensitivity can also be evaluated by the depolarization produced by carbachol, since this agent acts exclusively on postsynaptic receptors. The depolarization produced by carbachol, 20 μM, decreased proportionally with the concentration of pancuronium (fig. 2). The dose-effective linear regression curve is quite similar to that measured by the decrease in the amplitude of miniature endplate potentials produced by pancuronium.

Increasing concentrations of pancuronium progressively decreased the amplitude of endplate potentials. This decrease was statistically significant at concentrations of 5×10^{-8} g/ml or more. Figure 3 represents the sum of the results of both cut-fiber and high-magnesium preparations, since we found that there was no qualitative difference between the two preparations. Furthermore, the effect on the amplitude of endplate potentials was compared with the control endplate potential in each muscle. The least-squares linear regression line showed a 95 per cent depression of the amplitude of endplate potentials at a pancuronium concentrations of 5×10^{-7} g/ml.

Endplate potentials in three intact diaphragms perfused with physiologic concentrations of magnesium and calcium were also recorded. The amplitude of endplate potentials was 1.45 ± 0.4 mV (24 cells) at a pancuronium concentration of 5×10^{-7} g/ml. If one assumes the amplitude of endplate potentials at an undepressed neuromuscular junction is 40 mV, this represents a 96 per cent depression of transmission efficacy at this concentration. This corresponds closely to data shown in figure 3.

The effect of pancuronium on quantal release evoked at 1 Hz was determined in four intact diaphragms without addition of a high magnesium ion concentration. For a total of 41 cells, the quantal content was 109.2 ± 10.4 at 5×10^{-7} g/ml pancuronium. However, interpretation of such values is difficult since we do not have control values with which to compare them. For this reason, we investi-

gated the action of pancuronium in cut-fiber preparations, with which it is possible to record endplate potentials without neuromuscular depressants. With such a method, significant decreases in quantal content produced by pancuronium were found at concentrations of 1×10^{-7} g/ml and 5×10^{-7} g/ml (table 3). Similar decreases in transmitter release were found in intact muscle fibers depressed by high magnesium ion concentrations. Pancuronium significantly depressed the quantal content at the concentration of 1×10^{-7} g/ml, as well as at 5×10^{-7} g/ml (fig. 4).

TABLE 3. Effects of Pancuronium on Quantal Content by Variance Method in Cut Mouse Diaphragms

	Cells (Number of Animals)	Quantal Content (Mean ± SE)
Control	41 (7)	309 ± 55
Pancuronium		
5×10^{-8} g/ml	11 (3)	222 ± 30
1×10^{-7} g/ml	12 (2)	180 ± 34*
5×10^{-7} g/ml	10 (2)	79 ± 10*

* $P < 0.05$.

Discussion

By use of the technique of determining muscle twitch responses, Buckett *et al.*[1] found that pancuronium exerted its action at the neuromuscular junction. Furthermore, from the interactions between pancuronium and various agents, such as edrophonium, succinylcholine and *d*-tubocurarine, they concluded that pancuronium is a nondepolarizing blocking drug. Actual measurement of the effect of pancuronium on the membrane potential was reported by Galindo.[17] In more sophisticated studies, we found that pancuronium had no effect on muscle membrane potentials, electrical membrane constants, and directly stimulated action potentials in concentrations far exceeding those in clinical use. Our single-fiber experiments merely support the conclusion of Buckett *et al.*[1] that pancuronium is a nondepolarizing drug acting exclusively at the neuromuscular junction.

It is well known that the amplitude of miniature endplate potentials is changed by the sensitivity of the postsynaptic receptor. Agents such as *d*-tubocurarine, which competitively combine with acetylcholine receptors, decrease the amplitude of miniature endplate potentials. The frequency of miniature endplate potentials, on the other hand, is influenced by agents affecting the presynaptic site of the neuromuscular junction. For example, nerve-terminal depolarization by an electrical current[18] or a high concentration of potassium or calcium[19] increases the frequency of miniature endplate potentials. In agreement with Galindo,[17] we have shown that low concentrations of pancuronium decrease the amplitude of miniature endplate potentials, with no effect on their frequency. This seems to suggest that in low concentrations pancuronium acts postsynaptically rather than presynaptically. Furthermore, our results show no significant effect on quantal release of low concentrations of pancuronium.

We did not study the effect of pancuronium in concentrations lower than 5×10^{-9} g/ml. Dretchen *et al.*,[20] however, reported an increase in the frequency of miniature endplate potentials as well as an increase in twitch tension in the frog sartorius muscle in concentrations between 10^{-15} g/ml and 10^{-11} g/ml pancuronium. At concentrations between 10^{-9} g/ml and 10^{-8} g/ml, pancuronium decreased the frequency as well as amplitude of miniature endplate potentials. The biphasic action of pancuronium at different concentrations reported by Gergis *et al.*[5] was not observed in our studies or in Galindo's studies of rat phrenic nerve–diaphragm preparations. We attribute this discrepancy solely to species differences.

At higher concentrations of pancuronium, significant decreases in quantal release were observed. It should be pointed out that the quantal content in figure 4 was determined in a solution containing a high magnesium concentration, which resulted in low m values in the range of 0.61 to 21.66. At physiologic levels of quantal release (usually 200–400 quanta/pulse), one might expect that presynaptic actions of pancuronium might contribute less to the depression

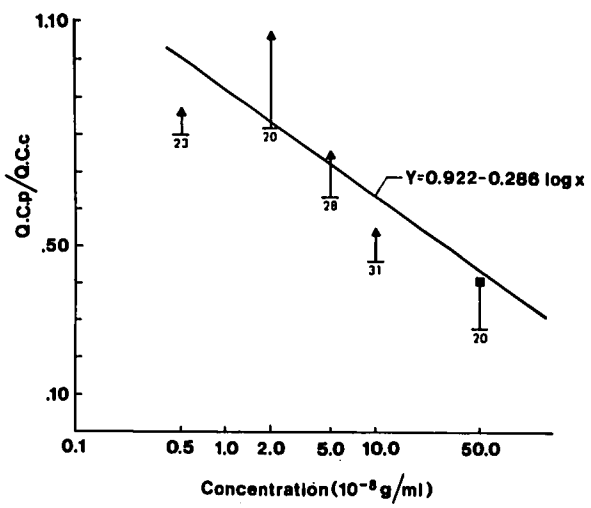

FIG. 4. Effect of pancuronium on quantal content evoked at 1 Hz. *Ordinate*, ratio of quantal content with pancuronium to control. *Abscissa*, concentration of pancuronium. Number in parentheses indicates total number of muscle fibers. *Filled triangle*, quantal content determined by direct method; *filled square*, quantal content determined by variance method (see text).

of neuromuscular transmission because of the marked nonlinear summation of the quantal component. For this reason, it is important to examine the effects of pancuronium in cut-fiber preparations in which neuromuscular transmission is not depressed. Our results (table 3) clearly demonstrate that pancuronium in high concentrations significantly decreased the undepressed quantal release. At 5×10^{-7} g/ml the quantal content was 26 per cent of control in cut-fiber experiments, whereas the quantal content in a solution containing a high magnesium concentration was 40 per cent of control. The discrepancy is probably due to different methologies used.

One of the purposes of our studies was to compare the presynaptic and postsynaptic effects of pancuronium at clinically effective concentrations. To do this, it is necessary to extrapolate the result of an *in-vitro* system to human studies. With the injection of pancuronium intravenously, extracellular action potentials, as recorded with a surface electrode, progressively decreased in amplitude, and eventually no response was seen.[21] It is clear that at clinically effective concentrations of pancuronium, endplate potentials, if present, must be small enough so that action potentials can not be generated. Galindo[7] reported that minimal endplate potentials that could initiate an action potential in the rat diaphragm were 2 mV in pancuronium-treated and 4 mV in *d*-tubocurarine-treated muscles. Boyd and Martin,[22] however, reported a minimal endplate potential of 10 mV in *d*-tubocurarine-treated cat muscles. Assuming the normal endplate potential is 40 mV, endplate potentials must be depressed by 75 (10 mV) to 95 per cent (2 mV) of the normal value at which no muscle action potential would be produced. In our *in-vitro* studies pancuronium, 5×10^{-7} and 1×10^{-7} g/ml, decreased the amplitude of the control endplate potential 95 and 77 per cent, respectively. It is, therefore, reasonable to conclude that at clinically effective concentrations pancuronium exerts its action presynaptically as well as postsynaptically.

For the postsynaptic effect of pancuronium, the measurement of the amplitude of miniature endplate potentials (fig. 3) showed a depression to 16 per cent of control at 5×10^{-7} g/ml. Depolarization produced by 20 μM carbachol revealed a decrease to 22 per cent of control in the presence of pancuronium 5×10^{-7} g/ml. On the other hand, the corresponding presynaptic effects of pancuronium 5×10^{-7} g/ml (fig. 4) were 40 per cent of control and 26 per cent of control (table 3). From such a comparison it is clear that pre- as well as postsynaptic effects of pancuronium are essential for total neuromuscular depression.

Pancuronium bromide was provided by Organon Inc., West Orange, New Jersey.

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