TOXICOLOGY AND APPLIED PHARMACOLOGY 71, 391–397 (1983)

Relative Refractory Period as a Measure of Peripheral Nerve Neurotoxicity

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Received March 10, 1983; accepted July 14, 1983

Relative Refractory Period as a Measure of Peripheral Nerve Neurotoxicity. ANDERSON, R. J. (1983). *Toxicol. Appl. Pharmacol.* **71**, 391–397. A method is presented for converting the relative refractory period to a linear form which can be used for quantifying changes in peripheral nerve conduction and membrane excitability. Since the relative refractory period is very consistent in nerves taken from animals of a given age, strain, and size, small alterations due to exposure by neurotoxic agents can readily be detected. In the present study rat sciatic nerves exposed to phenol developed a peripheral neuropathy which was detected by an increased relative refractory period. Diisopropylfluorophosphate (DFP), another neuropathic agent, produced a shift in the relative refractory period which was also consistent with the time course of its known neurotoxicity. On the other hand, acrylamide did not change the relative refractory period although the rats exhibited deficits when measured behaviorally (roto-rod and inclined screen tests). This observation is consistent with the view that the acrylamide toxicity initially affects nerve endings and spares the nerve axon. Erythrosin B, an agent which enhances nerve excitability, produced a shift in the relative refractory period which indicated that the nerve was less refractory. These examples point to the value of the relative refractory period as an index of the extent and type of neurotoxicity induced by agents which affect peripheral nerve axons, and suggest that this method may be useful in determining the mechanism of action of neurotoxic agents.

A variety of electrophysiological measures have been used to detect functional deficits in peripheral nerves, both clinically (Rossi *et al.*, 1981; Behse and Buchthal, 1978) and experimentally (Bosch *et al.*, 1979; Miyoshi and Goto, 1973; DeJesus *et al.*, 1978). Of these, conduction velocity is perhaps the most widely used because it can be determined accurately in both sensory (DeJesus *et al.*, 1978) and motor nerves (Rossi *et al.*, 1981). Although there is a correlation between conduction velocity and fiber diameter (Hursh, 1939; Hung, 1954; Behse and Buchthal, 1978), conduction velocity typically does not change after exposure to neurotoxic agents, except in cases of demyelination (Waxman, 1978; Foster *et al.*, 1980). Peripheral nerve axonopathy preferentially decreases action potential amplitude and duration (Fullerton and Barnes, 1966; Hopkins and Gilliatt, 1971; DeJesus *et al.*, 1978). However, these latter measurements are quite dependent on electrode placement, leading to great variability in the measurements from one recording session to the next.

As an alternative, several investigators have proposed using the nerve refractory period as an index of neurotoxicity (Roberts and Trollope, 1979; Smith, 1980). The refractory period is a measure of membrane excitability which describes the limits of both conduction velocity and nerve recovery after depolarization. The range of excitability during the relative refractory period gives a profile of the nerve in a partially functional state, with the

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0041-008X/83 $3.00

most refractory axons not responding and only the most excitable axons contributing to the diminished response. Furthermore, in contrast with amplitude measures, relative refractory period is a time-dependent measure, which is less affected by electrode placement, thus decreasing the variability between recording sessions and facilitating comparisons.

The initial studies which used relative refractory period measurements as a means of assessing neurotoxicity were successful but presented some limitations. The three-dimensional graphic representations of the relative refractory period presented by Smith (Smith, 1980; Smith and Hall, 1980), although impressive visually, are hard to manipulate statistically. An alternate mathematical manipulation proposed by Roberts and Trollope (1979) determines a "minimum refractory period" point, roughly approximating the length of the absolute refractory period. Although this method facilitates statistical comparisons, it is a measure of only the most refractory fibers in the nerve bundle and does not provide information about possible changes in the relative refractory period. The present study incorporates the best features of both of these techniques into a method which transforms the relative refractory period into a linear function.

To test the validity of this method of analysis, several known neurotoxic substances were chosen, each being a prototype of a different neurotoxic mechanism. Phenol induces a progressive nerve degeneration after a single, direct exposure (Schaumburg *et al.*, 1970). One would expect that the loss of axons due to this agent would be reflected as a change in electrophysiologic parameters. Diisopropylfluorophosphate (DFP) is known to produce a peripheral neuropathy in mammalian nerves after systemic administration (Lowndes *et al.*, 1974). Since the onset of histologic damage induced by DFP is delayed, there might be electrophysiologic changes prior to the ultimate neuropathy. Acrylamide induces a neuropathy which appears to begin at the nerve terminals and then progresses proximally (Jennekens *et al.*, 1979). Electrophysiologic changes induced by this agent would be expected only after the neurotoxicity reached the axons being recorded. Some neurotoxic agents, instead of producing nerve damage, produce an increase in nerve excitability. One such compound is erythrosin B. Data are presented to demonstrate the value of the relative refractory period method by comparing the responses of normal nerves with the responses of nerves exposed to these neurotoxic agents.

METHODS

Dosing schedules. Male Sprague–Dawley rats (175 to 250 g, Zivic-Miller Co., Allison, Penn.) were used for each of these experiments. Animals were housed in a colony room maintained at 22 ± 2°C with a 12-hr light/dark cycle; they were provided feed (Purina Rat Chow) and water *ad libitum*. All animals were dosed in groups of five. For the acrylamide experiments, rats were given daily ip injections of acrylamide (30 mg/kg) dissolved in saline for 15 days. This dosing schedule produces the initial signs of behavioral toxicity (Loeb and Anderson, 1981). The control group was given saline injections in an equal volume (1 ml/day) for 15 days. All animals were evaluated electrophysiologically 24 hr after the final dose.

For the DFP experiments, rats were given daily ip injections of DFP (1 mg/kg) dissolved in saline for 5 days. This dosing schedule was used to induce the initial pathological effects, prior to delayed neuropathy. The control group was given saline injections in an equal volume (1 ml/day). All animals were evaluated electrophysiologically 24 hr after the final dose.

For the phenol experiments, each animal was anesthetized with 50 mg/kg pentobarbital ip and the left hindlimb dissected to expose the sciatic nerve, with care being taken not to disrupt the hindlimb innervation and blood supply. A small plastic liner was positioned under the nerve to isolate it from surrounding tissues. A 2.5% solution of phenol was applied to a 5-mm section of the nerve for 15 min. The nerve was then flushed with 20 ml of saline. The wound was sutured and the wound edges irrigated with betadine solution. This exposure procedure is known to induce significant histologic damage in the nerves (Schaumburg *et al.*, 1970). Control animals were given the same surgical treatment except that the nerves of these animals were exposed for 15 min to saline instead of phenol. Three weeks later the incision was reopened for electrophysiological evaluation.

Erythrosin B is known to have excitatory effects after acute exposure (Augustine and Levitan, 1980). For these experiments, the rat sciatic nerve was dissected and placed in a nerve chamber with three compartments. The com-

partments were made water tight by sealing the nerve with Vaseline. The end compartments were filled with mineral oil and contained stimulating and recording electrodes, respectively. The central compartment contained saline during control measurements. The compartment was then replaced with erythrosin B (1 mM), and the refractory period measurements were taken again. Following this procedure, the test compartment was replaced with saline and another set of measurements was made. Only those experiments which showed at least a partial recovery toward control during the wash-out period were used in the data analysis.

Electrophysiologic recording. Each rat was anesthetized with 1.5 g/kg of urethane administered ip. The sciatic nerve was dissected free of surrounding tissues and cut distally at the point of insertion into the gastrocnemius muscle. The nerve was positioned on pairs of bipolar stimulating and recording electrodes. The exposed nerve was immersed in oxygenated perflurocarbon solution which provided both electrical insulation and a source of oxygen to the nerve.

To determine the refractory period, supramaximal twin rectangular pulses were applied at approximately 30-sec intervals. The interstimulus interval between the pair of pulses was adjusted to record data at approximately 20 intervals between 0.5 and 15 msec. From each pair of compound action potentials recorded in this manner, the peak amplitude, area under the waveform, and the interstimulus interval were calculated. (To obtain greater accuracy in determining the interstimulus interval, the elapsed time between the two stimulus artifacts was calculated from the digitized record by computer. This method permitted an error of less than 0.025 msec.) As the interval between the stimuli decreased, the size of the second action potential diminished, which indicated the relative refractory period of the nerve. By further decreasing the interstimulus interval, a point was reached where the second stimulus evoked no action potential. This point marked the absolute refractory period. For each pair of action potentials, the area of the second action potential was calculated as a percentage of the first. Similarly, the amplitude of the second potential was calculated as a percentage of the first.

Data analysis. The nerve relative refractory period approximates an exponential function (as shown in Fig. 1A). Interstimulus intervals which produce decrements in the action potential of 20 to 80% are of the greatest interest, since the nerve is most sensitive to small changes in interstimulus interval. (At longer intervals there is little or no decrement in the potential; at shorter intervals the nerve becomes completely refractory and there is no second action potential to measure.) Therefore, only intervals which gave a 20 to 80% decrease in the second action potential were used in the data analysis. We have found that an accurate reconstruction of the relative refractory period can be plotted if at least 10 pairs of data (percentage decrement versus interstimulus interval) within this range are used. The method of least-squares fit was used to determine the best fitting log-linear regression line for these data points according to the following equation:

$$Y = A \exp(BX)$$

where Y = 100 − the percentage decrement in the second action potential, X = the interstimulus interval, A = the Y-intercept, B = the slope, and exp = exponent, base e.

Using pairs of X and Y values, we calculated the best fitting log-linear regression line, which had a slope of B and a theoretical Y-intercept of A. These calculated values of A and B were then used in the equation to calculate the corresponding X values (the interstimulus intervals) when Y = 25, 35, 50, 65, and 75%. The result of this manipulation therefore gave the interstimulus intervals necessary to decrease the potential by standardized amounts between 25 and 75% of control. Since we had five animals in the treatment group, the calculated interval values from each experiment were averaged to obtain a mean and standard error. The plot of these averaged intervals (versus 25, 35, 50, 65, and 75%) therefore was a straight line function describing the relative refractory period of the treatment group. (We have found that control animals of a given age, weight, sex, and strain produce a nerve relative refractory period line having consistent slope with very small variation.)

RESULTS

Three weeks after the single exposure to phenol, three of five rats in this study showed signs of peripheral neuropathy. These animals exhibited loss of foot placement on the operated side. There was a significant slowing of the compound action potential recorded from these nerves and a marked reduction in the amplitude. As shown in Fig. 1, there was also a very prolonged relative refractory period. An example of one of the intoxicated nerves is shown in Fig. 1A and the mean change in refractory period is shown in Fig. 1B. Sham-operated controls and two of the phenol-treated rats exhibited no loss of motor control. The nerves from these animals had normal electrophysiologic characteristics.

It was clear from the loss of hindlimb movement that phenol has a neurotoxic effect, which the electrophysiologic measurements confirmed. However, the relative refractory period would be more useful as a measure of neurotoxicity if it could detect the early onset

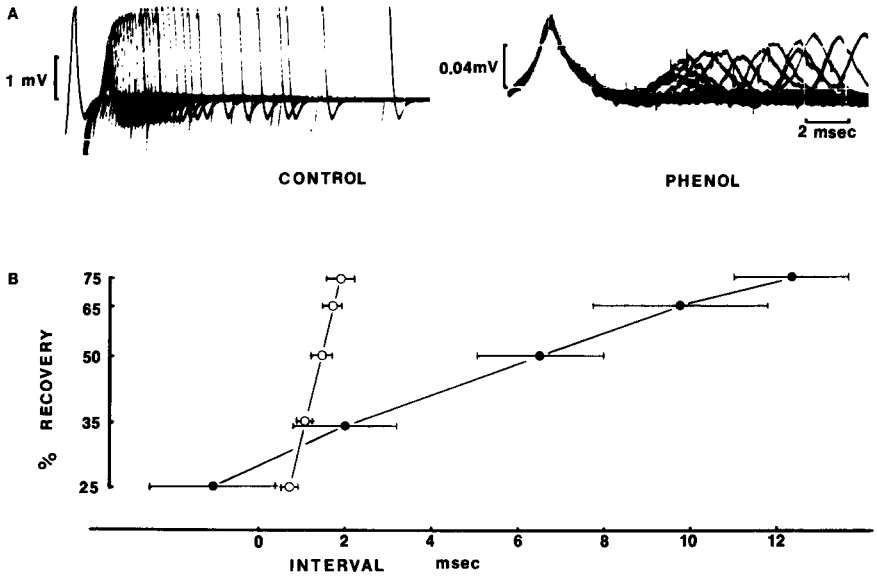

FIG. 1. Effect of phenol on relative refractory period. (A) show the sciatic nerve relative refractory period of a sham-operated control (left) and a rat whose sciatic nerve was exposed to phenol 3 weeks prior to recording (right). Each record is a multiple exposure photograph of the compound action potentials generated by twin pulse stimulation at interstimulus intervals from 0.5 to 10 msec. Time scale is the same for both traces. (B) is the average relative refractory period (mean ± SE) of the control and phenol-treated groups (n − 5), plotted as a function of the percentage of action potential recovery at each interstimulus interval.

of nerve dysfunction. Behaviorally, the rats dosed for 5 days with DFP showed no loss of motor control, determined by the roto-rod test. Each rat was able to remain on the rotating rod for at least 2 min. However, the refractory period was prolonged as shown in Fig. 2, suggesting neurotoxicity. The DFP-treated rats in this study lost considerable weight during the treatment period, and the contribution of malnutrition to the results cannot be ignored.

Figure 3 shows that there was no significant difference between the relative refractory period of the control and acrylamide-treated nerves. All of the features of the relative refractory period and conduction were within the normal range.

For the experiments with erythrosin B, the sciatic nerve was mounted in a nerve chamber to record the refractory period *in vitro*. After making control measurements, the test compartment was perfused with erythrosin B and the relative refractory period was again measured. The results are shown in Fig. 4. The shift in the relative refractory period was unchanged. However, at short interstimulus intervals the nerve was less refractory. This finding suggests that a greater number of axons

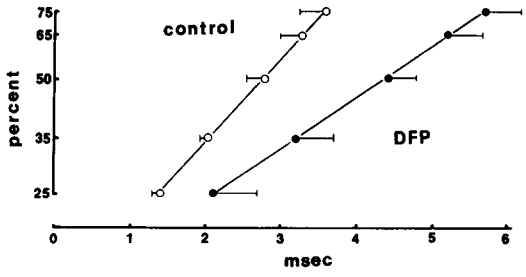

FIG. 2. Effect of DFP on the relative refractory period of the rat sciatic nerve. Each line represents the mean (±SE) of the relative refractory period of controls (open circles) or rats given DFP parenterally for 5 days (closed circles). Data are plotted as a function of the percentage of action potential recovery at each interstimulus interval. DFP-treated rats lost a significant amount of weight during the dosing interval and may in part account for the effect.

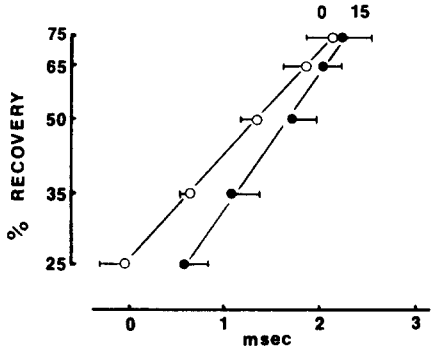

FIG. 3. Effect of acrylamide on the rat sciatic nerve relative refractory period. Each line represents the mean (±SE) of the relative refractory period of controls (open circles) or rats given acrylamide parenterally for 15 days (closed circles). Data are plotted as a function of the percentage of action potential recovery at each interstimulus interval.

in the nerve are capable of conducing nerve impulses with short interstimulus intervals. At longer intervals, nerve excitability was unaffected.

DISCUSSION

These results show that the relative refractory period can be converted to a linear representation which makes changes in nerve refractoriness readily apparent. Furthermore, for each of several agents known to be neurotoxic. the relative refractory period of the nerve changes in a manner consistent with the neurotoxicity.

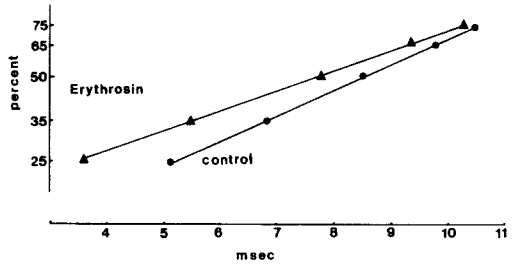

FIG. 4. Effect of erythrosin B on the sciatic nerve relative refractory period. Untreated sciatic nerves were placed in an isolated nerve chamber and the control relative refractory period (circles) recorded. The nerves were then exposed to erythrosin B and the relative refractory period was again recorded (triangles). Data are plotted as a function of the percentage of action potential recovery at each interstimulus interval.

The value of this method is that it is a quantitative description of changes in nerve excitability. Interpretation of these data will provide new information on how a toxic agent alters nerve function and complements other measurements such as conduction velocity, action potential amplitude, and duration.

After exposure to phenol which is known to induce histologic damage (Schaumburg *et al.*, 1970), there should be an accompanying functional deficit. It was not surprising, therefore, that the sciatic nerves from phenol-treated animals which exhibited such motor deficits also had a significant reduction in axonal conduction and a prolonged relative refractory period.

The slope and position of the relative refractory period line describe the range of interstimulus intervals through which the nerve is partially refractory. A parallel shift to the right, such as that induced by DFP, indicates that the absolute range of refractoriness has not changed. The relative refractoriness of the fibers within the nerve is the same. However, the nerve has a longer absolute refractory period (2 msec, compared with 1 msec in the control) and also does not completely recover until much longer intervals (6 versus 3.5 msec).

A change in the slope of the refractory period line is a representation of changes in the relative refractoriness of the fibers within the nerve bundle. A shallow slope, such as that resulting from phenol treatment, indicates that the nerve recovers more slowly after depolarization. A steep slope indicates that the nerve is less refractory. Erythrosin B did not alter the length of the relative refractory period. The nerve always recovered fully in 10 msec. However, the shift in slope at short interstimulus intervals suggests that erythrosin B selectively permits high frequency nerve traffic to be conducted without altering low fre-

quency conduction. Such a change in neuronal excitability is consistent with other acute effects of erythrosin B (Augustine and Levitan, 1980).

In addition to demonstrating positive results with these agents, it was also important to demonstrate that the relative refractory period remained normal after exposure to neurotoxic agents which are initially toxic to parts of the nerve other than the axon. The lack of an effect of acrylamide in the present experiments confirm the work of others who have shown that the earliest site of toxicity with acrylamide is the sensory (Lowndes *et al.*, 1978) and motor nerve terminals (Jennekens *et al.*, 1979) on the distal portion of the nerve and the spinal cord monosynapse connection (Goldstein and Lowndes, 1979) on the proximal end. At the stage of acrylamide intoxication used in our experiments the axon is normal histologically and also functionally, by either conduction velocity or refractory period measures.

These examples of prototype neurotoxic agents demonstrate that the relative refractory period changes as a function of nerve damage. There are several advantages of using this technique as a means of assessing neurotoxicity.

First, this linear representation of the relative refractory period makes changes easy to see when plotted graphically. Second, it is a quantitative treatment of the refractory period data and can be used to make statistical comparisons between test and control groups. Third, individual differences in a population of treated nerves are indicated by the size of the standard error bars of the relative refractory period line. Finally, the line gives a profile of the entire refractory period. Since shifts in the slope after drug treatment may be nonparallel, no single point within the relative refractory period can be taken as representative and as a result, important neurotoxic changes may be missed. The linear relative refractory period plot fully describes the characteristics of nerve refractoriness and facilitates comparisons. This type of analysis should thus provide added insight to the functional changes induced by neurotoxic agents.

ACKNOWLEDGMENTS

The author wishes to acknowledge the excellent technical assistance of Christopher Dunham. This work was supported in part by NIEHS.

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