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Some antagonists of dantrolene sodium on the isolated diaphragm muscle of the rat

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The effects of a number of potential antagonists of dantrolene sodium have been studied on twitches of the isolated hemidiaphragm preparation of the rat stimulated directly at a frequency of 0·1 Hz, after complete neuromuscular block produced by tubocurarine or erabutoxin *a*. The substances selected as possible dantrolene antagonists were uranyl ions, thiocyanate ions, adrenaline, caffeine, quazodine, quinine, 4-aminopyridine and the calcium ionophore A23187, all of which facilitate excitation-contraction coupling in one way or another. Contracture was the main feature of the response to A23187, the increase in the tension of the dantrolene-depressed twitches being very slight. All the remaining compounds increased the amplitude of the twitches, but only 4-aminopyridine, quinine, quazodine and caffeine were capable of restoring to the control amplitude twitches that had been maximally depressed by dantrolene. Of these, 4-aminopyridine and quinine were the most potent on a molar basis.

The muscle relaxant activity of dantrolene sodium was first described by Snyder, Davis & others (1967). Subsequent studies showed that its action is exerted directly on the muscle fibres at a site beyond membrane excitation in the series of events leading to contraction; most evidence indicates that it acts by interfering with the release of 'activator' calcium ions from the sarcoplasmic reticulum, probably as a secondary consequence of a primary action at the triadic junctions through which the release of 'trigger' calcium ions is impaired (Ellis & Carpenter, 1972; Putney & Bianchi, 1974; Takauji, Takahashi & Nagai, 1975; Homma, Kurihara & Sakai, 1976). Dantrolene sodium is of therapeutic value in the control of chronic muscle spasticity associated with conditions such as multiple sclerosis, cerebral palsy, stroke, and spinal cord injury, and may be of use in relieving the spasticity associated with malignant hyperpyrexia. For a review of its actions and uses see Pinder, Brogden & others (1977).

Since dantrolene sodium is thought to act by impairing the access of calcium ions to the contractile proteins, a number of substances that are considered to facilitate excitation-contraction coupling by various actions involving calcium ions were tested as dantrolene antagonists on the isolated diaphragm of the rat. The potential antagonists tested were uranyl ions, thiocyanate ions, the calcium ionophore A23187, adrenaline, caffeine, quinine, quazodine, and 4-aminopyridine.

Uranyl ions, like zinc ions, act on the plasma membrane to prolong the repolarization phase of the action potential, and the duration of the action potential is increased in this way (Sandow & Isaacson, 1963, 1966; Sandow, 1965; Edman, Grieve & Nilsson, 1966). As a consequence the period during which Ca^{2+} is released from the sarcoplasmic reticulum (i.e., the plateau of the active state) is also prolonged, and twitch tension is thereby augmented.

Thiocyanate ions are the most potent anions of the lyotropic series ($SCN^- > I^- > NO_3^- > Br^-$) in augmenting muscle twitches (Sandow, 1965). These anions are preferentially absorbed by the membranes of the T-tubules and they are thought to act by facilitating the transfer of excitation from the T-tubules to the sarcoplasmic reticulum, so that mechanical threshold is lowered (Bianchi, 1968; Chapman, 1969; Luttgau, 1970; Andersson, 1972). Consequently, the release of Ca^{2+} from the sarcoplasmic reticulum occurs earlier, at a smaller than normal level of depolarization by the action potential; release then continues for the rest of the duration of the spike potential that is above the lowered mechanical threshold. This type of twitch augmentation is associated with a reduction in the latent period, an increase in the rate of development of tension, and a prolongation of the plateau of the active state. According to Moulds (1977), the actions of thiocyanate ions and of dantrolene are the precise opposite of each other on several parameters of muscle activity.

The calcium ionophore A23187 produces a pronounced increase in the twitch tension of single muscle fibres from the depressor muscle of the

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barnacle (Hainaut & Desmedt, 1974). The evidence obtained by these workers indicated that this effect was a consequence of facilitation, by A23187, of Ca^{2+} release from the sarcoplasmic reticulum in response to membrane depolarization.

Adrenaline is thought to increase the twitch tension of fast-contracting muscles, such as the diaphragm, by an action on β_2-adrenoceptors, leading to adenylate cyclase activation and the production of cyclic AMP (Bowman & Nott, 1969, 1974). It has been proposed that cyclic AMP facilitates Ca^{2+} uptake by the sarcoplasmic reticulum during the intervals between stimulation, so that more is then available for release by an action potential; enhanced contractility thereby results.

Caffeine has previously been shown to antagonize dantrolene in frog sartorius muscle (Ellis & Carpenter, 1972). Caffeine and quinine produce a number of effects on muscle mechanics that may contribute to their ability to augment contractility (Sandow, 1964, 1965, 1970; Bianchi, 1968; Weber & Herz, 1968; Batra, 1974, 1975). They act on the plasma membrane to prolong the duration of the action potential, and they impair the re-uptake of Ca^{2+} into sarcoplasmic reticulum and mitochondria. These two effects are probably those mainly responsible for the twitch augmentation produced in the focally-innervated muscle fibres that comprise mammalian muscles. In higher concentrations, even in the absence of membrane depolarization, caffeine and quinine may cause the release of intracellularly bound Ca^{2+} and thereby induce contracture. However, this effect is largely confined to the slow tonic muscle fibres of amphibians and avians. Caffeine also possesses phosphodiesterase inhibiting activity and can thereby cause accumulation of cyclic AMP. This effect may contribute to its twitch-augmenting activity, but it is unlikely to play a major role (Weber, 1968; Bowman & Nott, 1974). Quazodine is chemically related to caffeine and produces similar mechanical effects (Nott & Winslow, 1973). Its mechanism of action is probably essentially the same.

4-Aminopyridine has been found both to facilitate neuromuscular transmission by an action on the nerve endings through which acetylcholine output is enhanced (Fastier & McDowall, 1958; Lemeignan & Lechat, 1967; Foldes, Agoston & others, 1976a; Foldes, Braak & others, 1976b; Bowman, Harvey & Marshall, 1976, 1977), and, apparently according to the species, to enhance contractility by a direct action on the muscle. Bowman & others (1976, 1977) found the direct muscle effect to be trivial compared to the neuromuscular effect in chick muscle, and Lemeignan & Lechat (1967) failed to observe it at all in rabbit muscle. On the other hand, the remaining workers quoted above reported the direct effect in rat muscle to be at least as important as that on transmission.

METHODS

Hemidiaphragms with their phrenic nerves were removed from freshly killed Wistar rats of either sex of between 250 and 350 g, and set up according to the method of Bülbring (1946). Each preparation was mounted on a Perspex holder in a 50 ml organ bath containing Krebs-Henseleit solution at 37° and continuously gassed with 5% CO_2 in oxygen. The composition of the Krebs-Henseleit solution was, in g litre^{-1}: NaCl, 6·95; KCl, 0·4; $CaCl_2$, 0·28; $MgSO_4.7H_2O$, 0·14; NaH_2PO_4, 0·14; $NaHCO_3$, 2·1; dextrose, 2·0. A resting tension of 0·015–0·025N was applied to the tissue. Isometric twitches were recorded by means of a Grass (model FT03) strain gauge coupled to a Grass polygraph (model 79). Twitches of the diaphragm were evoked at a frequency of 0·1 Hz either by stimulating the phrenic nerve through electrodes of the type described by Burn & Rand (1960), or by stimulating the muscle directly through platinum wires attached to the Perspex holder on which the muscle was mounted. For indirect stimulation, rectangular pulses of 100 μs duration and of about twice the strength required to evoke a maximal twitch were applied to the phrenic nerve from a Grass (model S4) stimulator. For direct stimulation, the pulse duration was increased to 1 ms and the strength was increased until the amplitude of the twitches matched that of the maximal twitches evoked by indirect stimulation. During direct stimulation, neuromuscular transmission was completely blocked by the addition to the bath fluid of either 6 μg ml^{-1} of tubocurarine or 15 μg ml^{-1} of the irreversible cholinoceptor antagonist erabutoxin *a* (Tamiya & Arai, 1966). At intervals, stimulation was briefly switched back to the nerve to check that neuromuscular transmission remained blocked.

The drugs used were dantrolene sodium (Norwich Pharmacal Co.), (−)-adrenaline hydrochloride, quinine dihydrochloride, sodium thiocyanate, and uranyl acetate (BDH), quazodine (Mead Johnson), tubocurarine chloride (Duncan Flockhart), 4-aminopyridine (Koch-Light), A23187 2-[(3β,9α,11β-trimethyl)-8-(2-pyrrolecarboxymethyl)-1,7-dioxaspiro-[6,6]undecyl-2β-methyl]-5-methylaminobenzoxazole-4-carboxylic acid (Eli-Lilly), and erabutoxin *a*

(supplied by Professor N. Tamiya of Tohoku University, Japan). Solutions of hydrated dantrolene sodium (3·99 mg ml^{-1} ≡ 10^{-2} M) were prepared in polyethylene glycol 400, and appropriate quantities were added to the organ bath from a microsyringe. A23187 was dissolved in dimethylsulphoxide to produce a stock solution of 10^{-1} M (10 mg in 0·19 ml). Appropriate μl volumes of this solution were added from a microsyringe to vigorously stirred 10 ml volumes of Krebs-Henseleit solution at 37° to produce faintly opalescent colloidal solutions. These were then poured directly into the organ bath which already contained 40 ml of Krebs-Henseleit solution. The remaining drugs were dissolved in distilled water at high concentration and then diluted with Krebs-Henseleit solution before addition to the organ bath. All solutions were freshly prepared for each experiment.

Statistical analysis of the differences between means was derived from Student's *t*-test. *P* values of less than 0·05 were regarded as being significant.

RESULTS

Dantrolene sodium in concentrations of 1·5 × 10^{-8} M (5 ng ml^{-1}) and above depressed the indirectly evoked and the directly-evoked twitches of the diaphragm. Fig. 1 illustrates the mean cumulative log concentration-response curve compiled from the results of experiments on 8 directly-stimulated muscles (continuous line). A similar curve plotted from results on muscles stimulated through their nerves was indistinguishable (dashed line in Fig. 1). These results are similar to those of Ellis & Carpenter (1972). The mean concentration of dantrolene to produce 50% twitch depression in 8 preparations was 7·4 × 10^{-7} M (about 0·25 μg ml^{-1}). The maximum depression never exceeded 85% and was produced by a mean concentration of 6 × 10^{-5} M (20·5 μg ml^{-1}) dantrolene sodium, which approximates to the maximum solubility of the drug in water. Larger concentrations sometimes caused a very small return of the twitches towards the control level. This effect was probably due to the amount of solvent (polyethylene glycol 400) used to dissolve the higher concentrations of dantrolene, since the same amounts of solvent alone also often caused a slight increase in twitch tension. The failure of dantrolene to abolish the twitches completely may have been due to its insolubility at higher concentrations. However, a contractile remnant which is resistant to dantrolene is characteristic of the drug's action; it is also evident in *in vivo* studies and differs in degree according to the type of striated muscle (see for example, Nott & Bowman, 1974).

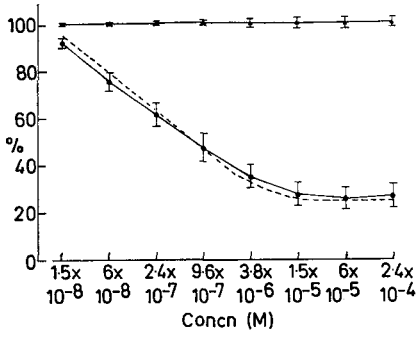


FIG. 1. Mean log concentration-response lines showing the effects on the directly-evoked twitch tension of dantrolene in polyethylene glycol 400 (●, n = 8), and of the equivalent concentrations of polyethylene glycol alone (▲, n = 5). The vertical bars represent the standard errors of the means. The dashed line represents the mean results of similar experiments on 3 diaphragms stimulated through their phrenic nerves. Ordinate—Per cent control twitch tension.

The effects of each of the potential antagonists of dantrolene on the directly-evoked twitches were examined in the following three ways. (1) In one series of experiments, concentrations of the drug were added cumulatively in the absence of dantrolene until the maximal effect for that drug was produced. In these, and all other experiments, each increment in concentration was added at the peak of the effect of the previous addition. (2) In a second series of experiments, the same cumulative concentrations were added in the continued presence of a concentration of dantrolene that produced about 50% depression of the maximal twitches. (3) In a third series of experiments, dantrolene was carefully added to the preparation until a concentration sufficient to produce the maximum or almost the maximum twitch depression was present. In its continued presence, the potential antagonist was then added cumulatively until the maximum increase in twitch tension was produced.

All of the compounds tested increased the maximal twitches of the directly-stimulated muscle, and on the basis of the maximum extent of this effect they were divided into the following three broad classes.

1. Calcium ionophore, A23187

In experiments on 3 diaphragms this compound, in concentrations of 5 × 10^{-5} M, produced a small increase in the twitch tension (10–34%) of muscles that had not been treated with dantrolene. Higher

concentrations of A23187 (1–2 × 10^{-4} M) did not produce a greater increase in twitch tension, but caused a slowly developing increase in the background tension of the muscle (i.e., a contracture) upon which the slightly augmented twitches were superimposed. These effects of A23187 were slowly reversible by washing the tissue. The same volume of dimethylsulphoxide as that used to dissolve the highest concentration of A23187 (i.e., 0·1 ml dimethylsulphoxide added to a 50 ml organ bath) was without effect on the contractions of the diaphragm.

In a further 3 experiments on diaphragms treated with dantrolene so that the twitches were close to being maximally depressed, A23187 produced a somewhat greater percentage increase in twitch tension (28–66%) than that produced in the absence of dantrolene. The contractural response to A23187 was depressed by dantrolene approximately in proportion to the depression of the twitches. This is evident in Fig. 2 which compares responses to A23187 in two preparations, one in the presence and one in the absence, of dantrolene. In the latter case, the gain on the recording was reduced so that the amplitude of the maximal twitches approximately matched that of the dantrolene-depressed twitches in the other experiment. Under these conditions, the two responses to A23187 appeared closely similar.

2. Adrenaline, uranyl acetate and sodium thiocyanate

In the absence of dantrolene, adrenaline and uranyl acetate produced mean maximal increases in twitch tension of 24% (s.e.m. 8·5) and 26% (s.e.m. 4) respectively. When the twitches were about 50% depressed by dantrolene, the maximum mean percentage increases in twitch tension produced were 31% (s.e.m. 7) for adrenaline and 45% (s.e.m. 6) for uranyl acetate, but they were far short of restoring the twitches to normal control amplitudes. The concentration-response curves for these two substances were shallow (Fig. 3).

Although less potent, sodium thiocyanate was more powerful than adrenaline or uranyl acetate in increasing twitch tension, and its concentration-response curve was steeper (Fig. 3). In the absence

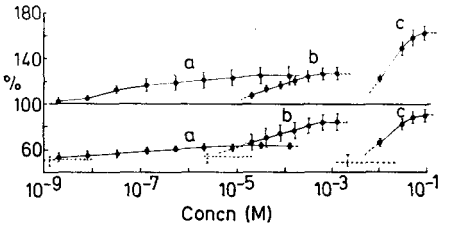


FIG. 3. Effects of adrenaline (a), uranyl acetate (b), and sodium thiocyanate (c) on the tensions of maximal directly-evoked twitches (curves above the 100% line) and in a separate series of experiments, on those twitches depressed to about 50% by dantrolene (curves below the 100% line). The horizontal dashed lines show the mean dantrolene-depressed levels for each drug and the vertical dashed lines show the *ranges* of dantrolene depression. All other vertical lines are the standard errors of the means. Each point is the mean of measurements on 6 diaphragms. Only one potential antagonist was added to each diaphragm. Ordinate—Per cent maximal twitch tension.

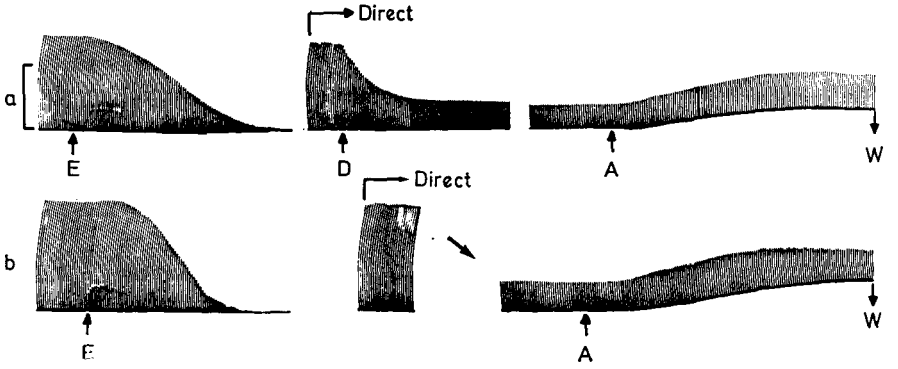


FIG. 2. Maximal twitches of hemidiaphragms were initially evoked by stimulation of their phrenic nerves. At E, erabutoxin *a*, to produce a bath concentration of 15 μg ml^{-1}, was added and left in contact with the tissue for the remainder of the experiments. Neuromuscular transmission was completely blocked and direct stimulation was then applied as indicated until the end of the experiments. At D in a, dantrolene, to produce a bath concentration of 1·5 × 10^{-5} M, was added and left in contact with the tissue. At A in both experiments A23187 was added to produce a bath concentration, in each case, of 2 × 10^{-4} M. In a, the gap in the record after dantrolene corresponds to 10 min. In b, the oblique arrow indicates a reduction in gain to make the twitches approximately equal in amplitude to the dantrolene-depressed twitches in the other experiment. At W, the tissue was washed (recovery not shown). The vertical calibration corresponds to 0·02 newtons.

of dantrolene, sodium thiocyanate produced a mean maximal increase in twitch amplitude of 64% (s.e.m. 8); in the presence of sufficient dantrolene to produce about 50% twitch depression, sodium thiocyanate maximally increased the twitches by 111% (s.e.m. 10·5), so that it was just capable of restoring the twitches to the control amplitude from this level of depression. The concentration of sodium thiocyanate necessary to produce the maximum increase in twitch tension, also produced a slight increase in the background tension of the muscle. Fig. 3 graphically expresses the results obtained with adrenaline, uranyl acetate and sodium thiocyanate in the absence and in the presence of sufficient dantrolene to produce about 50% twitch depression.

When adrenaline or uranyl acetate was added in the presence of sufficient dantrolene to produce the maximum twitch depression (i.e., about 80% depression), they were incapable of restoring the twitches even to the 50% depressed level. Under similar circumstances, sodium thiocyanate was maximally capable of restoring the twitches to about the 50% depressed level. Thus, these three substances were classified together because they exerted only limited antagonistic actions, being effective in overcoming dantrolene depression only when it was relatively small.

3. Caffeine, quazodine, quinine and 4-aminopyridine

Each of these substances, in sufficient concentration, was capable of at least doubling the amplitude of the directly-evoked twitches in the absence of dantrolene and of increasing, to above normal control size, the amplitude of twitches depressed to about 50% by dantrolene. The results with all four compounds in this group are expressed graphically in Fig 4. The concentration-response curves were relatively steep.

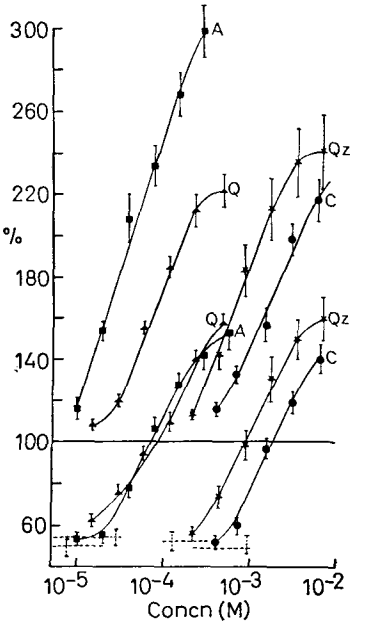


FIG. 4. Effects of 4-aminopyridine (■A), quinine (▲Q), quazodine (★Qz) and caffeine (●C) on the tensions of maximal directly-evoked twitches (curves above the 100% line) and on those of twitches depressed to about 50% by dantrolene (curves starting at about 50% level). All other details as for Fig. 3.

In the presence of sufficient dantrolene to produce the maximal degree of twitch depression, all four compounds were capable of restoring the twitches to at least the control amplitude. The effect of 4-aminopyridine is illustrated in Fig. 5.

In the experiment of Fig. 5, 4-aminopyridine was added gradually in cumulating quantities, so that full restoration of the twitches to control amplitude developed slowly. When the concentration that was necessary to restore the twitches (i.e., about 8 × 10^{-5} M) was added abruptly, antagonism developed

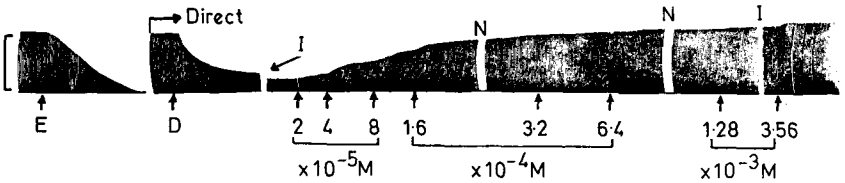


FIG. 5. Effects of 4-aminopyridine on dantrolene-depressed, directly-evoked twitches. The preparation was initially stimulated through its phrenic nerve. At E, erabutoxin *a* was added to produce a bath concentration of 15 μg ml^{-1} which was left in contact with the tissue for the remainder of the experiment. Neuromuscular transmission was completely blocked and remained so throughout; the gaps marked N indicate periods of nerve stimulation. The muscle was stimulated directly as indicated, at all other times. The gaps in the record marked I correspond to 10 min. At D, dantrolene was added to give a final bath concentration of 1·5 × 10^{-5} M. The concentrations beneath the remaining arrows indicate the accumulated concentrations of 4-aminopyridine present in the bath following each addition. The tissue was not washed throughout the period of the experiment illustrated. The vertical calibration on the left is equivalent to 0·02 newtons.

more rapidly and was complete within 5 min. Similar pictures were obtained with caffeine, quazodine and quinine.

Of the 4 compounds in this group, 4-aminopyridine was the most potent and the most powerful in increasing the contractility of the directly evoked twitches in the absence of dantrolene. Quinine was the next most potent in this respect, and was about equipotent and equieffective with 4-aminopyridine in increasing the dantrolene-depressed twitches (Fig. 4). Quazodine and caffeine were about equieffective with quinine but less potent, quazodine being slightly more potent than caffeine.

As illustrated in the graphs of Figs 3 and 4, in the presence of dantrolene, none of the antagonists in any concentration was capable of increasing the twitch tension to the amplitude that it produced in the absence of dantrolene.

The results so far described were obtained on directly-stimulated preparations, care being taken throughout each experiment to ensure that neuromuscular transmission remained fully blocked either by tubocurarine or by erabutoxin *a*. In addition to its direct action on contractility, 4-aminopyridine facilitates neuromuscular transmission, and it was therefore of interest to compare its effects on twitches evoked by nerve stimulation, with those on directly-evoked twitches. Fig. 6 illustrates log concentration-response curves comparing the increase in maximal twitch amplitude produced by 4-aminopyridine under the two conditions of stimulation. The compound was active at lower concentrations in the indirectly stimulated preparations, and the maximal effect produced (about a four-fold increase in amplitude) was greater than that in the directly stimulated preparations (about a three-fold increase in amplitude). Since the concentrations to produce the two effects overlapped, the higher concentrations applied to the indirectly-stimulated preparations might be expected to have been acting both at the neuromuscular junction and directly on the muscle, but there is some doubt that this was so, as explained below.

In the experiments from which the data in Fig. 6 were compiled, the 4-aminopyridine was added in small cumulative concentrations. With the smaller concentrations, the onset of the increase in twitch tension and the time to reach peak effect were prolonged. For example, in a typical experiment, the onset of the response to the first addition of 5×10^{-7} M of 4-aminopyridine did not occur until about 5 min after its addition, and the maximum effect produced by this concentration took about 27 min to develop. Larger concentrations added abruptly produced effects that developed much more rapidly. The slow onset of the effects of low concentrations may explain the fact that some workers have thought it necessary to use much larger doses.

A further series of experiments was performed to compare the anticurare action of 4-aminopyridine with that of its antidantrolene action. To study the anticurare action, hemidiaphragms were stimulated through their phrenic nerves, and sufficient tubocurarine (1·5–2·5 μg ml^{-1}) was added to produce a 90–95% block of the twitches. In the continued presence of tubocurarine, 4-aminopyridine was then added either in a cumulative manner, or as a single larger dose. Fig. 7 illustrates the antagonistic action of a single dose. Complete restoration of the twitches to control amplitude was produced by

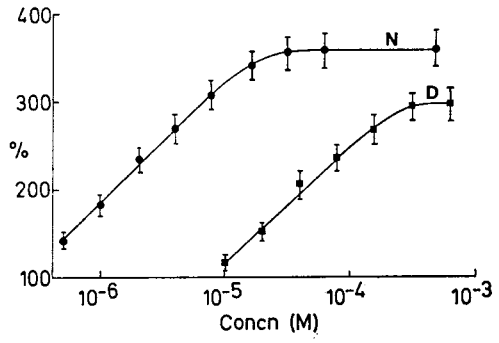


FIG. 6. Mean log concentration-response lines comparing the increases produced by 4-aminopyridine in the tension of maximal twitches evoked by nerve stimulation (●N), and by direct stimulation (■D) in the presence of tubocurarine or erabutoxin *a*. Each point is the mean of measurements made on 6 hemidiaphragms. The vertical lines are the standard errors of the means. Ordinate—Per cent maximal twitch tension.

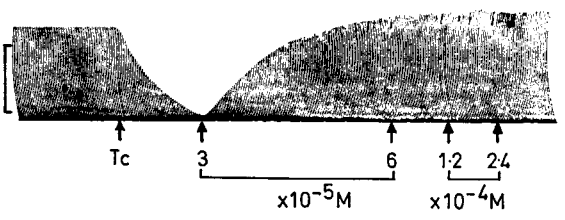


FIG. 7. Anti-curare action of 4-aminopyridine. Maximal twitches were evoked throughout by stimulation of the phrenic nerve. Tubocurarine (2 μg ml^{-1}) was added at Tc. Without washing the tissue, 4-aminopyridine was then added cumulatively. The concentrations given below the remaining arrows denote the accumulated bath concentration of 4-aminopyridine following each addition. The vertical calibration corresponds to 0·02 newtons.

2–3 × 10^{-5} M of 4-aminopyridine. The onset of the anticurare effect required larger concentrations (about 10^{-5} M) than those necessary to produce the smallest detectable increase in the twitches of the indirectly-stimulated muscle (about 5 × 10^{-7} M), but the anticurare concentrations were smaller than those necessary to augment the amplitude of directly evoked twitches whether depressed by dantrolene or not. 4-Aminopyridine was 5–8 times more potent in restoring indirectly-evoked tubocurarine-depressed twitches to the control level, than in restoring directly-evoked dantrolene-depressed twitches to the control level. However, when indirectly-evoked twitches were about 50% depressed by dantrolene, the concentrations of 4-aminopyridine necessary to increase their amplitude in 4 experiments were similar to those found effective in the absence of dantrolene (i.e., 10^{-6} M and above), and full restoration of twitch amplitude was produced by concentrations around 10^{-5} M. This observation suggests that under these conditions, the main effect of 4-aminopyridine was exerted at the neuromuscular junction, despite the fact that dantrolene acts directly on the contractile mechanism; if this was so, the interaction was not a true antagonism.

A surprising finding was that once having added a concentration of 4-aminopyridine that restored indirectly-evoked tubocurarine-depressed twitches to normal or to just above normal, further addition of 4-aminopyridine did not usually produce bigger contractions. This effect is illustrated in Fig. 7. The lack of additional effect is despite the fact that the higher concentrations are amongst those that augment the contractility of directly-evoked twitches (Fig. 6). If, in an experiment such as that illustrated in Fig. 7, extra tubocurarine were then added and the muscle stimulated directly, then a further increase in twitch tension could usually be produced with 4-aminopyridine. The implications of this observation are not clear; they suggest that a physiologically-evoked action potential triggered from the endplate potential does not permit the access of 4-aminopyridine to the contractile mechanism, whereas the strong electrical impulse necessary to evoke contractions directly does do so.

Throughout the experiments with 4-aminopyridine, its effects were found to persist even though the tissue was washed repeatedly. Thus, having first administered 4-aminopyridine to a preparation, and despite subsequent frequent washing, larger than normal concentrations of tubocurarine or dantrolene were then required to depress the twitches. Furthermore, depending on the amount of 4-aminopyridine previously administered, the depression produced was then not reversible, or only weakly reversible, for periods ranging from 30 min to more than 3 h, at which time most experiments were abandoned. Fastier & McDowall (1958) and Lemeignan & Lechat (1967) also noted the persistent action of 4-aminopyridine.

DISCUSSION

In the Results section, the substances tested as potential antagonists of dantrolene have been divided into three groups according to the extent of the antagonism and the type of response produced. As is obvious from the Introduction, however, this is not meant to imply that substances in the same group necessarily possess the same mechanism of action.

Although the calcium ionophore A23187 has been found to produce a pronounced increase in the twitch tension of single barnacle muscle fibres (Hainaut & Desmedt, 1974), contracture was the more prominent feature of the response of the intact rat hemidiaphragm to this substance; the increase in twitch tension was not pronounced, and the compound did not therefore produce more than a trivial antagonism of dantrolene. Another antibiotic, designated X537A, which also acts as a calcium ionophore, produces qualitatively similar effects on the rat diaphragm (Levy, Cohen & Inesi, 1973) to those produced in the present experiments by A23187. The effects of X537A were ascribed largely to enhanced Ca^{2+} influx across the muscle fibre membrane, and it appears likely that this is the main action of both ionophores in intact mammalian muscle, even though in disrupted cells both have been shown to induce release of stored Ca^{2+} from isolated sarcoplasmic reticulum vesicles and mitochondria (Cashwell & Pressman, 1972; Scarpa, Baldassare & Inesi, 1972; Levy & others, 1973).

Adrenaline, which probably acts through the adenylate cyclase-cyclic AMP system, uranyl ions, which appear to act by prolonging the duration of the action potential, and thiocyanate ions which are thought to facilitate the transfer of excitation from the T-tubules to the sarcoplasmic reticulum (for references, see Introduction) were incapable of restoring dantrolene-depressed twitches to normal amplitude, except when the degree of dantrolene-induced depression was slight. It had been thought

possible that thiocyanate ions would be highly effective as dantrolene antagonists, since the literature suggests that both act at the same site but in opposite ways, and the experiments of Moulds (1977) have recently re-emphasized this inverse relationship.

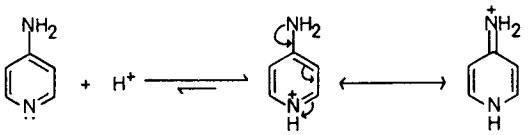
The most powerfully effective drugs included caffeine and quinine which, after excitation, inhibit the re-uptake of Ca^{2+} ions by internal storage sites, thereby delaying relaxation and allowing time for greater tension to be developed during a twitch. Quazodine, which is chemically related to caffeine, probably acts in a similar way. 4-Aminopyridine was also effective in completely restoring the dantrolene-depressed twitches. Its mechanism of action is not yet understood; some speculations are made later in this Discussion.

All of the twitch-potentiating agents facilitate excitation-contraction coupling in one way or another, and the fact that they increased the amplitude of dantrolene-depressed twitches, in some cases to more than control amplitude, is compatible with the evidence that dantrolene acts by impairing the access of calcium ions to the contractile proteins. However, none of the agents tested can be regarded as more than a physiological antagonist of dantrolene, because even concentrations that produced the maximum increase in twitch tension did not remove the effect of dantrolene. That is to say, in absolute terms, the maximum augmented twitch tension produced in the presence of dantrolene remained a great deal smaller than that in its absence.

Although quazodine, caffeine and quinine were capable of restoring dantrolene-depressed twitches to normal in the isolated muscles, they are unlikely to have any clinical value as dantrolene antagonists, should the need for such a drug arise, since the effective concentrations are almost certainly greatly in excess of those that could be tolerated by a patient. In anaesthetized cats, very large intravenous doses of quazodine (20 mg kg^{-1}) produced only a small (30%) increase in the dantrolene-depressed twitches (Nott & Bowman, 1974). Such doses exert pronounced cardiovascular effects, and caffeine behaves similarly. Clinically tolerable doses of quinine are used for their membrane stabilizing property in the therapy of muscle cramps; such doses produce concentrations that are considerably smaller than those that augment contractility.

4-Aminopyridine facilitates neuromuscular transmission by an action on the nerve endings through which acetylcholine release is increased, and it also increases contractility, at least in rat muscle, by a direct action (see Introduction for references). According to Fastier & McDowall (1958) directly- and indirectly-evoked twitches of the rat diaphragm were augmented to the same degree by the same concentrations of 4-aminopyridine. However, these authors apparently did not block neuromuscular transmission during the application of direct stimuli. Under such conditions the so-called 'direct' stimuli excite intramuscular nerve endings, and the evoked contractions are therefore at least partly the result of nerve stimulation. The present experiments clearly showed that both indirectly- and directly-evoked twitches were increased in tension, but that the concentration of 4-aminopyridine required to act directly on the muscle was considerably larger than that necessary to facilitate neuromuscular transmission. Although the muscle action potential was not recorded, the only known way in which a drug can increase the amplitude of maximal twitches by an action confined to the transmission process, is to facilitate transmission to the extent that repetitive firing of the muscle fibres is produced. It is therefore assumed that the increase in maximal twitch tension produced, at least by the smaller effective concentrations of 4-aminopyridine, was a consequence of increased transmitter output leading to repetitive muscle action potentials. The situation differs during partial neuromuscular block produced by tubocurarine, since here increased transmitter output can recruit the previously blocked fibres, and the twitches are restored for this reason. Repetitive firing does not occur.

The pyridine N of 4-aminopyridine has a pKa value of 9·25 and the molecule is therefore largely in the protonated form at physiological pH values. The charge on the cationic form is delocalized, the extremes being represented by the two forms on the right-hand side of the equation.



Calcium ions play essential roles both in acetylcholine release from motor nerve endings, and in the contractile mechanism of muscle. It is therefore tempting to suppose that 4-aminopyridine in some way makes more Ca^{2+} available to the processes that couple excitation to transmitter release and to contraction; possibly in the protonated form it displaces Ca^{2+} from its binding sites. 4-Amino-

pyridine has been shown to prolong the duration of nerve action potentials by inhibiting potassium conductance (Pelhate & Pichon, 1974; Meves & Pichon, 1975). Tetraethylammonium ions and uranyl ions (Hille, 1967; Benoit & Mambrini, 1970) produce similar effects, and uranyl ions, at least, exert a similar change in the action potential of muscle fibres (see Introduction for references). Prolongations of the nerve and muscle action potentials might themselves explain an increase in evoked transmitter release, and an increase in contractility, respectively. However, an effect on the action potential could not account for the pronounced increase in miniature endplate potential frequency produced by 4-aminopyridine (Bowman & others, 1976). Possibly the changes in potassium conductance produced by 4-aminopyridine are secondary to a primary action through which the concentration of free intracellular Ca^{2+} is increased. A rise in axoplasmic free Ca^{2+} is known to increase the frequency of miniature endplate potentials (Hubbard, Jones & Landau, 1968).

The action of 4-aminopyridine on muscle differed from that of the calcium ionophore A23187, in that it did not produce contracture. This may therefore mean that 4-aminopyridine does not act on the plasma membrane to increase calcium influx. Indeed the slow onset of the effects of small doses of 4-aminopyridine and the great persistence of its effects suggest an intracellular site of action. Although it is a highly ionized compound, 4-aminopyridine clearly penetrates cell membranes, since it exerts pronounced central effects (Fastier & McDowall, 1958). Possibly it enters cells by aqueous diffusion or by making use of a facilitated transport mechanism. Once inside the cell, it may act to displace Ca^{2+} from intracellular binding sites.

Under the name Pimadin, 4-aminopyridine has been extensively employed in recent years in Bulgaria as an anticurare and also an analeptic agent (see, for example, Paskov, Stoyanov & Micov, 1973), so that clinical data regarding its unwanted effects and toxicity are available. The concentrations of 4-aminopyridine necessary to antagonize dantrolene by a direct action in the isolated diaphragm muscle were 5–8 times greater than those necessary to antagonize tubocurarine at the neuromuscular junction. However, the concentrations necessary to restore dantrolene-depressed muscular power were considerably smaller when the muscle was activated through its nerve, a situation which is more analogous to that in patients. Under these conditions, the increase in twitch tension is presumably largely or even entirely due to facilitation of the unimpaired transmission leading to repetitive firing of the muscle fibres, rather than to any true relief of the depressed contractility. It is doubtful whether an action on transmission would in fact improve voluntary muscular power during dantrolene depression, since repetitive firing and increased contractions, at least with other facilitatory drugs such as the anticholinesterases, do not occur during voluntary movements.

The present experiments also cast some doubt on the practical significance of the direct action of 4-aminopyridine on contractility, since its occurrence appeared to depend upon the application of strong stimuli directly to the muscle. Two pieces of evidence from other sources add some support to this view. First, 4-aminopyridine has been found not to affect the amplitude of twitches during partial neuromuscular block produced by depolarizing drugs (Lemeignan & Lechat, 1967; Foldes & others, 1976a). The action of 4-aminopyridine on transmission would not be expected to antagonize this type of block, but any direct action exerted on the muscle fibres might have been expected to increase the contractility of those fibres that were still responding. Second, Lemeignan & Lechat (1967) found 4-aminopyridine to be without effect on directly-evoked twitches of the chronically-denervated tibialis anterior muscle of the rabbit, although at the same time powerfully increasing the amplitude of the indirectly-stimulated contralateral muscle. After chronic denervation, electrical excitability is increased, and weaker stimuli (possibly too weak to drive in the 4-aminopyridine ions) are then adequate to excite the muscle.

There seems little doubt that 4-aminopyridine is a clinically-useful anticurare agent, and there may be sufficient human toxicity data already available to justify a trial in patients who have received excessive dantrolene medication. However, in view of the possibility that the direct action of 4-aminopyridine on contractility may be an artifact of the stimulation conditions, it is considered that further animal experiments to clarify this point are first desirable.

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