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Neuromuscular blocking agents inhibit receptor-mediated increases in the potassium permeability of intestinal smooth muscle

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1 The neuromuscular blocking agents tubocurarine, atracurium and pancuronium have been tested for their ability to inhibit receptor-mediated increases in the K^+ permeability of intestinal smooth muscle.

2 All three agents, as well as the bee venom peptide apamin, reduced both the resting efflux of ^{86}Rb and the increase in efflux caused by the application of either bradykinin (1 μM) or an α_1-adrenoceptor agonist, amidephrine (20 μM), to depolarized strips of guinea-pig taenia caeci. This suggested that, like apamin, the neuromuscular blocking agents inhibit the Ca^{2+}-dependent K^+ permeability ($P_{K(Ca)}$) mechanism which in this tissue is activated by a variety of membrane receptors.

3 The concentrations (IC_{50}s) of atracurium, pancuronium and (+)-tubocurarine which reduced the effect of amidephrine on ^{86}Rb efflux by 50% were 12, 37 and 67 μM respectively.

4 Also in keeping with an ability to block $P_{K(Ca)}$, the neuromuscular blockers and apamin reduced the inhibition by amidephrine and bradykinin of physalaemin-mediated contractions of the taenia caeci. The IC_{50} values were 15, 31 and 120 μM for atracurium, tubocurarine and pancuronium respectively, and 2.3 nM for apamin.

5 Each of the neuromuscular blockers, and apamin, increased the spontaneous contractions of the rabbit duodenum and blocked the inhibitory effect of amidephrine thereon.

6 It is concluded that the $P_{K(Ca)}$ mechanism in the longitudinal smooth muscle of the intestine resembles that of hepatocytes and sympathetic ganglion cells in its susceptibility to inhibition by neuromuscular blocking agents, as well as by apamin.

Introduction

Apamin, an 18-amino acid peptide from bee venom, blocks Ca^{2+}-activated K^+-permeabilities ($P_{K(Ca)}$) in several tissues, including liver and intestinal smooth muscle (for reviews see Lazdunski, 1983; Jenkinson *et al.*, 1983; Habermann, 1984). Studies with apamin congeners have shown that the arginine residues at positions 14 and 15 are essential for biological activity. The consideration that the separation between the positive charges that these arginines carry is similar to that of the two charged nitrogens in neuromuscular blocking agents, such as tubocurarine, prompted tests of the ability of the latter compounds to block the $P_{K(Ca)}$ mechanism in liver cells. This was found to occur, and it was also observed that the neuromuscular blockers would displace labelled apamin from its binding sites on intact hepatocytes, suggesting a common site of action (Cook & Haylett, 1983; 1985). The aim of the present work was to find whether neuromuscular blocking agents would also inhibit the apamin-sensitive $P_{K(Ca)}$ mechanism in the longitudinal smooth muscle of the intestine. This tissue, as exemplified by the taeniae of the guinea-pig caecum, has long been known to respond to activation of α-adrenoceptors and purine receptors with an increase in K^+-permeability (P_K) (Jenkinson & Morton, 1967a,b; Bülbring & Tomita, 1969; Tomita & Watanabe, 1973; Den Hertog, 1982).

Two sets of experiments were performed. In the first, the change in permeability and its modification by neuromuscular blockers were studied directly by using ^{86}Rb or ^{42}K efflux from guinea-pig taenia caeci as a measure of P_K. In the second, the alterations in P_K were assessed indirectly from changes in the spontaneous mechanical activity of the rabbit duodenum and

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in the contractile response of the guinea-pig taenia caeci to physalaemin.

Methods

The experiments were performed *in vitro* at 37°C with the taenia of the guinea-pig caecum and with whole segments of rabbit duodenum. Except where noted, the standard bathing fluid contained (mM): NaCl 118, KCl 4.62, $MgSO_4$ 1.16, KH_2PO_4 1.18, $NaHCO_3$ 25, $CaCl_2$ 2.5, and glucose 11. It was maintained at pH 7.4 by gassing with 5% CO_2 plus 95% O_2. Propranolol 1 μM was included, to block β-adrenoceptors.

Experiments with guinea-pig taenia caeci

^{86}Rb efflux Strips of taenia 1–2 cm in length were tied with cotton thread to holders made of stainless steel tubing through which 5% CO_2 plus 95% O_2 was passed. After mounting, the strips were incubated for 30 min in the standard bathing solution. They were then depolarized by transfer to a K^+-rich solution of the following composition (mM): $KMeSO_4$ 121, $MgSO_4$ 1.2, NaH_2PO_4 1, $KHCO_3$ 25, $CaSO_4$ 1.5, RbCl 1, glucose 11, propranolol 0.001. They were kept in this solution for 120 min, during the final 90 min of which ^{86}Rb was included at a specific activity of $\sim 1\ \mu Ci\ ml^{-1}$. At the end of this 'loading' period the strips were washed in ^{86}Rb-free K^+-rich solution for 22 min, long enough to allow ^{86}Rb to diffuse from the extracellular fluid. They were then transferred at 2 min intervals through a series of test-tubes containing 5 ml of the K^+-rich solution. The effects of agonists on ^{86}Rb efflux were examined by their inclusion in 3 consecutive tubes; when the influence of apamin or a neuromuscular blocker on the response to an agonist was to be tested, it was included both in the 'agonist' tubes and in the 3 immediately preceding tubes. The ^{86}Rb content of each tube was determined by Cêrenkov counting in a liquid scintillation spectrophotometer, as was the amount of ^{86}Rb left in the muscle strip, which at the end of the experiment was digested in 0.1 ml concentrated nitric acid at 100°C. After the count rates were corrected for differences in quenching, rate coefficients for ^{86}Rb efflux in each collection period were calculated (see e.g. Jenkinson & Morton, 1967a). The increase in efflux caused by an agonist was expressed as the difference between the peak rate coefficient during agonist action and the mean of the values for the two collection periods that immediately preceded the application of the agonist. The same procedure was followed when agonists were tested in the presence of putative channel blocking agents, and these 'test' responses were expressed as a percentage of the 'control' (i.e. agonist alone) values. In most experiments, 5 strips from the same animal were used: 2 received agonist only (controls) and the 3 others were exposed to the same concentration of the agonist but in the presence of the blocking agent under study.

^{42}K rather than ^{86}Rb was used on a few occasions.

Tension recording Strips of taenia 1–2 cm in length were mounted under an initial tension of 0.5–1 g in 10 ml organ baths. Their mechanical activity was recorded isometrically using a Swema SG 4–3 transducer feeding to a potentiometric chart recorder. After an equilibration period of 60–90 min, the tissue was exposed at 10 min intervals to a submaximal concentration (usually 10 nM) of physalaemin. The resulting contractions were well maintained. The inhibitory action thereon of either amidephrine (an α-adrenoceptor agonist, usually applied at 1 μM) or bradykinin (1 μM) was examined by adding the agent for 45 s in the presence of physalaemin, applied 45 s beforehand. This combination was repeated several times at 10 min intervals until both the physalaemin contraction and its inhibition became reproducible. The reduction of the inhibition by either apamin (added 10 min before physalaemin) or a neuromuscular blocker (added 15 min before) could then be studied (see Figure 3).

Experiments with rabbit duodenum

Lengths of duodenum, 2–3 cm, were mounted under a tension of 1 g in organ baths containing 40 ml of the standard bathing solution, to which atropine, mepyramine and propranolol (all at 1 μM) had been added. Spontaneous mechanical activity was recorded using an isotonic transducer feeding to a potentiometric chart recorder. After the tissue had equilibrated for 60–90 min, amidephrine was applied, at 5 min intervals, at a concentration (100–300 nM) sufficient to reduce the spontaneous contractions by 70–100%. When the amidephrine inhibition had become reproducible, the effects thereon of apamin and of neuromuscular blocking agents, applied 5 min beforehand, were tested.

The ability of the test agents to reduce the inhibitory response to amidephrine was assessed by expressing the amidephrine inhibition in the presence of the agent as a % of the mean of the previous 2 control responses to amidephrine at the same concentration.

Materials

Radiochemicals ^{86}RbCl and ^{42}KCl were obtained from New England Nuclear Chemicals and The Radiochemical Centre, Amersham, respectively.

Drugs Histamine acid phosphate, pancuronium bromide and (+)-tubocurarine chloride were

obtained from Burroughs Wellcome. The following were also used: atropine sulphate (Aldrich), bradykinin triacetate (Sigma), carbachol (BDH), methoxyverapamil (D-600; Knoll AG), mepyramine maleate (May & Baker), (−)-noradrenaline bitartrate (Koch-Light), physalaemin (Sigma), (±)-propranolol hydrochloride (ICI). Atracurium besylate and (±)-amidephrine mesylate were gifts from Burroughs Wellcome and Mead Johnson respectively. Apamin was kindly provided by Dr P.N. Strong, Department of Pharmacology, UCL.

Results

The increase in K^+ permeability which follows the activation of α-adrenoceptors in intestinal smooth muscle is readily studied by measuring the concomitant rise in the efflux of ^{42}K or ^{86}Rb. To avoid the complication of changes in ion flux secondary to alterations in membrane potential, the agonist can be applied in a solution containing a high enough concentration of K^+ to depolarize the muscle cells completely (Jenkinson & Morton, 1967a). Such an experiment is illustrated in Figure 1a which shows the rise in ^{86}Rb efflux that follows the application of the α_1-adrenoceptor agonist amidephrine to the taenia of the guinea-pig caecum. The mean increase in the rate coefficient was 38 ± 3% (s.e.mean, $n = 9$).

This response was inhibited by apamin, (+)-tubocurarine, atracurium and pancuronium. The block by apamin confirms the conclusions of Banks *et al.* (1979), Maas & Den Hertog (1979), Maas *et al.* (1980) and Den Hertog (1981). The effectiveness of the neuromuscular blockers is a new observation, and is illustrated in panels b and c of Figure 1 and in Figure 2 (see also Figure 5 and Table 1). In the experiment depicted in Figure 2, bradykinin was used as the agonist instead of amidephrine, in order to exclude the unlikely possibility that the reduction in the effect of amidephrine on ^{86}Rb efflux was a consequence of α-adrenoceptor blockade.

An incidental finding was that apamin and the neuromuscular blocking agents also consistently reduced the resting efflux of ^{86}Rb from the depolarized taenia, as can be seen in Figure 1 (b and c) and Figure 2.

Effects of neuromuscular blocking agents on inhibitory responses in smooth muscle

Two further series of experiments were done to study the action of the neuromuscular blockers on intestinal smooth muscle under more physiological conditions.

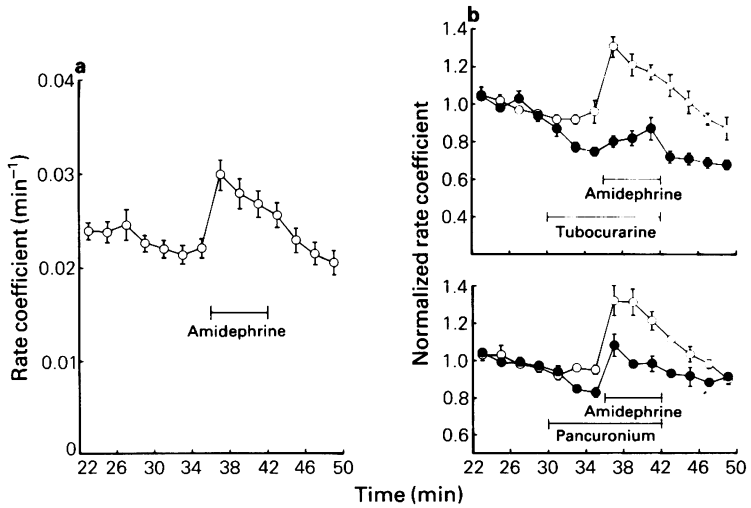

Figure 1 (a) The effect of amidephrine (20 μM) on the efflux of ^{86}Rb from strips of guinea-pig taenia caeci bathed in K^+-rich solution at 37°C. Ordinate scale, rate coefficient for ^{86}Rb efflux; abscissa scale, time after transfer of strips from radioactive to inactive solution. The values plotted are the means, and vertical lines show s.e. mean, of the rate coefficients observed in 9 experiments. (b) Inhibition by tubocurarine (100 μM, present during the longer bar) of the effect of amidephrine (20 μM) on ^{86}Rb efflux. To facilitate comparison, the rate coefficients were 'normalized' by calculating, for each strip, the average of the rate coefficients observed for the 4 pre-drug collection periods from 22 to 30 min. All the coefficients observed for a given strip were then divided by this average. The points plotted are the means (with vertical lines showing s.e.mean) of these normalized rate coefficients. The open and closed circles show the 'control' (strips exposed to amidephrine alone; $n = 10$) and 'test' (amidephrine applied in the presence of tubocurarine; $n = 7$) values, respectively. (c) As (b), but showing inhibition by pancuronium (30 μM). Control, $n = 4$; test, $n = 3$.

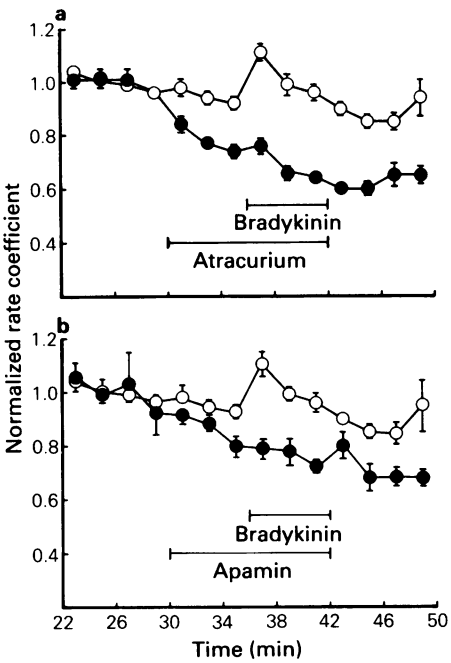

Figure 2 The effect of (a) atracurium (30 μM) and (b) apamin (30 nM) on the resting efflux of ^{86}Rb from the taenia caeci, and on the increase in efflux caused by bradykinin (1 μM). In each case $n = 6$ in the absence and 3 in the presence of the inhibitor. Other details as in Figure 1. (○) 'Control' strips, exposed to bradykinin alone: (●) 'test' strips, bradykinin applied in the presence of atracurium (a) or apamin (b).

It has long been known that activation of the α-adrenoceptors can inhibit both the spontaneous mechanical activity of the taenia caeci, and the contractile response to agonists such as carbachol. This inhibition can be explained as a consequence of an increase in P_K (Jenkinson & Morton, 1967b) and is itself blocked by apamin, presumably because of interference with the $P_{K(Ca)}$ mechanism (Banks *et al.*, 1979; Den Hertog, 1982).

Preliminary experiments to test whether the neuromuscular blocking agents mimicked this action of apamin were complicated by the finding that the blockers could additionally reduce the contractile response of the taenia caeci to both carbachol and histamine. However, this did not occur with the agonist physalaemin, a tachykinin related to substance P (for studies of the mechanism of action of tachykinins on intestinal smooth muscle, see Benham & Bolton, 1983 and Holzer & Petsche, 1983). Accordingly physalaemin was used in the next series of experiments. Typical records are shown in Figure 3. It was found that each of the neuromuscular blocking agents tested was able to reduce the inhibitory effect of α-adrenoceptor activation on the physalaemin contractions. The blocking action of apamin was additionally confirmed (see also Figure 5 and Table 1). Apamin, tubocurarine and atracurium also blocked inhibition of physalaemin-induced contractions by bradykinin (1 μM) rather than amidephrine, indicating that this effect was not a consequence of α-adrenoceptor blockade (see also Table 1).

Figure 3 also illustrates the consistent observation that the physalaemin-induced contraction became

Table 1 Concentrations of apamin and of the neuromuscular blocking agents needed to cause a 50% reduction (responses 1–4) or 50% of the maximal increase (response 5) in the 5 responses listed

	Response				
	1 *Inhibition by amidephrine (1 μM) of the contractile response of the taenia caeci to physalaemin (10 nM)*	*2* *Inhibition by bradykinin (1 μM) of the contractile response of the taenia caeci to physalaemin (10 nM)*	*3* *Increase in ^{86}Rb efflux from taenia caeci exposed to amidephrine (20 μM)*	*4* *Relaxation of the rabbit duodenum by amidephrine (100–300 nM)*	*5* *Spontaneous contractions of the rabbit duodenum*
Apamin	2.33 ± 0.42 nM	—	—	0.92 ± 0.19 nM	0.93 ± 0.23 nM
Atracurium	15 ± 3 μM	26 ± 7 μM	12 ± 5 μM	24 ± 8 μM	5.4 ± 3.2 μM
Tubocurarine	31 ± 6 μM	58 ± 9 μM	67 ± 27 μM	54 ± 11 μM	9.0 ± 2.6 μM
Pancuronium	120 ± 34 μM	—	37 ± 17 μM	71 ± 14 μM	3.5 ± 0.7 μM

The values tabulated have been determined from the results presented in Figure 5 by fitting the best hyperbolae (by a non-linear least squares method, after Colquhoun *et al.* (1974), which also provided approximate standard errors). A Hill coefficient of 1.0 was assumed and responses 1–4 but not 5 were constrained to have maxima of 100%.

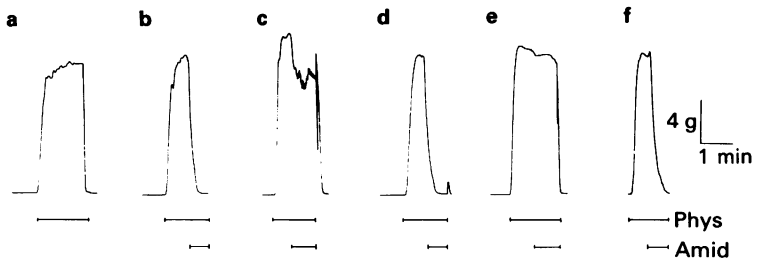

Figure 3 The influence of atracurium and apamin on the inhibition by amidephrine (Amid) of physalaemin (Phys)-induced contractures of guinea-pig taenia caeci. All records are from the same muscle strip. (a) The response to physalaemin (10 nM). (b) Inhibition of the physalaemin response by amidephrine (1 μM) added 45 s later. (c) As (b), but in the presence of atracurium (30 μM) added 15 min beforehand. (d) As (b), but 90 min after washout of atracurium. (e) As (b), but in presence of apamin (10 nM) added 10 min beforehand. (f) As (b), but 60 min after washout of apamin.

slightly greater in the presence of both apamin and atracurium. With apamin at 10 nM, the increase in amplitude was 21 ± 5% (s.e.mean, $n = 11$); with atracurium at 30 μM it was 14 ± 6% ($n = 9$). The effect introduces an unavoidable complication because as the contraction becomes greater, it can be expected to be less susceptible to inhibition by amidephrine. This may well have contributed to the observed reduction in the response to amidephrine seen in Figure 3c and e. However, we do not think that the effect can have been a major source of error since the amidephrine-induced inhibition became only marginally smaller when the physalaemin concentration was increased by the amount needed to match the enhanced contraction in the presence of the neuromuscular blockers.

Experiments with rabbit duodenum

An elegant experiment by A. Bowman (see Bowman, 1982) has shown that apamin abolishes the α-adrenoceptor-mediated inhibition (Bowman & Hall, 1970) of the spontaneous contractions of the rabbit ileum. We have confirmed this using rabbit duodenum, and have found that the neuromuscular blockers are also effective in this regard (Figure 4). An additional observation was that apamin and the neuromuscular blockers caused the amplitude of the spontaneous contractions to increase, with no corresponding change in frequency (Figure 4b,d). The size of the increase in amplitude, and the degree to which it was maintained, varied greatly from preparation to

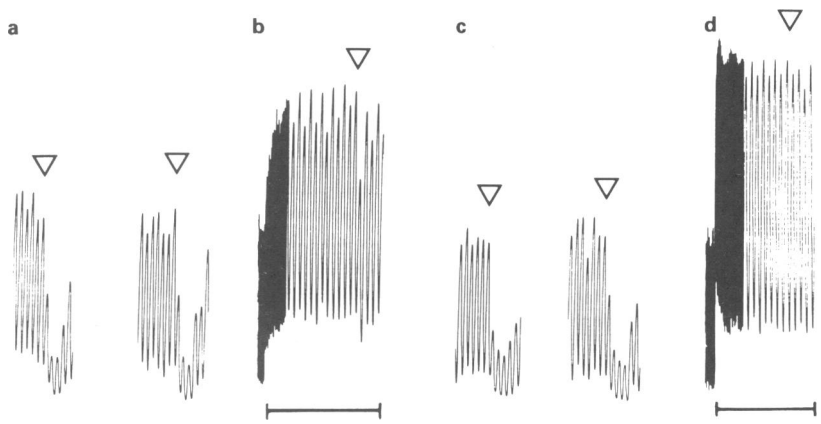

Figure 4 Effect of apamin and atracurium on the inhibition by amidephrine of the amplitude of spontaneous contractions (approximately 40% shortening) of rabbit duodenum. (a) Successive 'control' responses to amidephrine (300 nM for 30s, at ▽). (b) As (a) but in presence of apamin (10 nM) added 5 min earlier, as indicated by the bar; the chart speed was reduced for the first 4 min. (c) As (a), showing the recovery following washout of apamin. (d) As (b) but with atracurium (100 μM), rather than apamin. Apamin and atracurium caused unusually large increases in the amplitude of spontaneous contractions and the baseline in this experiment.

preparation. Concentration-response curves for these actions have been included in Figure 5 (see also Table 1).

Discussion

The main new finding is that neuromuscular blocking agents inhibit the increase in P_K that results from the action of α-adrenoceptor agonists and bradykinin on intestinal smooth muscle. Table 1 gives the concentrations needed. That they vary considerably is perhaps not surprising in view of the differences in the directness of the responses measured, and of the fact that the concentrations are approaching the range at which non-specific effects can be expected. The concentrations are, however, significantly greater than those found to be effective in guinea-pig hepatocytes, in which half-maximal inhibition of the action of agonists in causing a net loss of K^+ (an indirect measure of the underlying increase in P_K) was observed with atracurium, tubocurarine and pancuronium at 3, 3.5 and 3 μM, respectively (Cook & Haylett, 1983; 1985). Whether the 4 to 20 fold difference in the concentrations needed in smooth muscle and liver cells reflects a difference in the binding sites or in their environment remains to be determined.

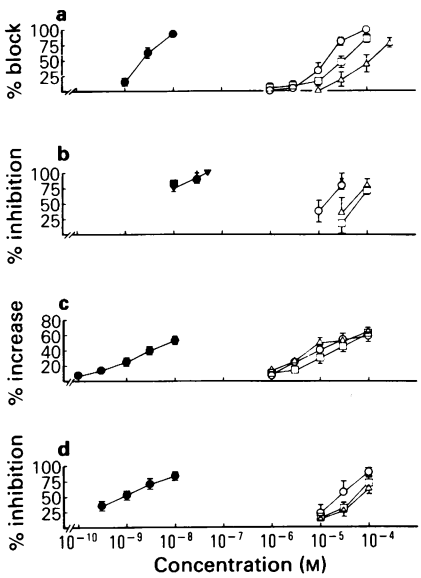

Figure 5 Concentration-dependence of the 4 actions of apamin (●) and the neuromuscular blocking agents (○, atracurium; □, tubocurarine; △, pancuronium). (a) Blockade of the inhibition by amidephrine (1 μM) of physalaemin (10 nM)-induced contractions of guinea-pig taenia caeci. Each point represents the mean, and vertical lines s.e.mean, of 3–7 observations. (b) Inhibition of the increase in ^{86}Rb efflux caused by amidephrine (20 μM); $n = 3$. Other values plotted are as follows: (×) and (+), inhibition by atracurium and apamin, respectively of the increase in ^{86}Rb efflux caused by bradykinin (1 μM, $n = 3$); (▼) and (■), inhibition by apamin of the increase in the efflux of either ^{42}K (▼, $n = 3$) or ^{86}Rb (■, $n = 2$) caused by noradrenaline (1 μM). (c) Potentiation of the spontaneous contractions of the rabbit duodenum ($n = 5–7$). The increases have been expressed as a % of the contraction before the drug was applied. (d) Inhibition of amidephrine-induced relaxation of the spontaneously active rabbit duodenum ($n = 4–6$).

Two subsidiary findings deserve comment. The first is that the neuromuscular blocking agents, as well as apamin, reduce the resting efflux of ^{86}Rb from the depolarized taenia caeci. The most likely explanation is that the concentration of Ca^{2+} in the cytosol of the depolarized muscle cells is high enough, even in the absence of agonists, to activate the $P_{K(Ca)}$ mechanism, which is then susceptible to inhibition by the agents tested. A raised $[Ca^{2+}]_i$ is in keeping with the observation that the depolarized muscle develops tension, provided that the bathing fluid contains Ca^{2+} (Durbin & Jenkinson, 1961). This development of tension provided a means of testing another possible explanation for the reduction in ^{86}Rb efflux caused by the neuromuscular blockers and by apamin, namely that it could have been a consequence of a fall in Ca^{2+} influx due to a Ca^{2+}- antagonist action of these agents. However, comparison of the influence of methoxyverapamil (D-600) with that of atracurium, tubocurarine, pancuronium and apamin on Ca^{2+}-induced contractures of the depolarized taenia caeci showed that atracurium alone had a modest inhibitory effect at the concentrations used in the present studies (30 μM atracurium producing the same degree of inhibition as 20 nM D-600: unpublished observations of P.R. Gater & D.H. Jenkinson).

The second subsidiary observation was that the neuromuscular blockers and apamin increased the amplitude of the spontaneous activity of the rabbit duodenum as well as the contractile response of the taenia caeci to physalaemin. This, too, could be a consequence of blockade of a $P_{K(Ca)}$ mechanism which under normal circumstances, it can be supposed, becomes activated by the increase in $[Ca^{2+}]_i$ that occurs both during spontaneous activity and in response to agonists such as physalaemin. The increase in $P_{K(Ca)}$ would tend to reduce the electrical activity that underlies contraction: inhibition of the $P_{K(Ca)}$ mechanism by e.g. apamin or atracurium might, therefore, increase the electrical activity and the ensuing contraction. It is, however, puzzling that the stimulation of the rabbit duodenum was often transient (particularly with the neuromuscular blockers), and that lower concentrations of these blockers were needed for this

response than for the others we have studied (compare columns 5 and 1–4 in Table 1). Both points suggest that additional factors may have been at work, and this clearly requires further study. It is worth noting that Fedan *et al.* (1984) have observed a potentiation by apamin of contractile responses to noradrenaline, histamine and acetylcholine in guinea-pig vas deferens smooth muscle.

To summarize, our experiments showed that neuromuscular blocking agents were able to inhibit the apamin-sensitive $P_{K(Ca)}$ mechanisms in intestinal smooth muscle cells. The relatively high concentrations of the blockers needed make it unlikely that the effect on smooth muscle would be important in their clinical use. It is possible, however, that an ability to block $P_{K(Ca)}$ may contribute to the stimulant activity of tubocurarine injected directly into the brain (e.g. Feldberg & Lotti, 1970). In this respect the neuromuscular blockers could resemble apamin, whose stimulatory actions on the central nervous system may well be attributable to inhibition of neuronal $P_{K(Ca)}$. Such a hypothesis is strengthened by the recent observation that both tubocurarine (Pennefather *et al.*, 1985a) and apamin block one of the $P_{K(Ca)}$ mechanisms in sympathetic ganglion cells (Pennefather *et al.*, 1985b). It is interesting in relation to this that in each of the tissues in which the point has been examined (liver, intestinal smooth muscle, sympathetic ganglion cells), sensitivity to apamin is associated with susceptibility to the neuromuscular blockers. When, however, a $P_{K(Ca)}$ mechanism is not apamin-sensitive, as in red blood cells (Burgess *et al.*, 1981), the neuromuscular blockers are similarly ineffective (Cook & Haylett, 1985). This adds to the evidence for the existence of at least two distinct $P_{K(Ca)}$ mechanisms which can be differentiated on pharmacological grounds (see also North & Williams, 1985, and the review by Schwarz & Passow, 1983).

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