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Effect of Pinaverium Bromide on Electrical and Mechanical Activity of Smooth Muscle Cells

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Summary. Pinaverium bromide exerts antagonistic effects on the contractions induced by $BaCl_2$ in intestinal smooth muscle. We have therefore investigated its effects on the electrical and mechanical activity and on the ^{45}Ca exchange of guinea-pig taenia coli and ileum. In the concentration range 10^{-7} to 10^{-5} M this drug does not exert an effect on the resting potential of either preparation. At 10^{-6} M no effects could be observed on the spontaneous electrical activity in *taenia coli*. However, in the ileum the frequency of the action potentials, as well as their amplitude and maximum rate of rise and of repolarization were significantly reduced, causing inhibitory effects on the concomitant mechanical responses. This effect is more pronounced at 10^{-5} M, and in some tissues the spontaneous activity is completely abolished. Similar effects have been observed in *taenia coli* at this concentration of pinaverium bromide.

The contractions induced by K-depolarization and by 10^{-5} M carbachol are inhibited in both preparations in a dose-dependent way. Also the contraction induced by carbachol in Ca free solution, as well as the restoration of that contraction by exposing the tissues to Ca containing solutions are reduced in the presence of 10^{-5} M pinaverium bromide. Pinaverium also inhibits the stimulation of the ^{45}Ca efflux induced by K-depolarization and by stimulation with 10^{-5} M carbachol.

In rabbit ear artery it was found that the contraction induced by noradrenaline is much less sensitive to pinaverium bromide than that induced by depolarization. It was also observed that this substance does not significantly affect the noradrenaline induced contraction in Ca free solution, neither does it prevent the restoration of that contraction by exposure of the tissue to Ca containing solutions.

These effects are similar to those observed with other Ca-entry blocking agents, and therefore suggest that pinaverium bromide mainly acts by blocking voltage-dependent Ca-channels.

Key words: Smooth muscle – Ca entry blockers – Contraction – ^{45}Ca fluxes

Introduction

Pinaverium bromide (N-(bromo-2-dimethoxy-4,5 benzyl)-N ([(dimethyl-6,6 norpinanyl-2)-2 ethoxy]-2 ethyl) morpholinium bromide), a substance synthezised by Baronnet et al. (1974), was found to exert in intestinal smooth muscle preparations an inhibitory effect on the contractions induced by $BaCl_2$ (Bretaudeau et al. 1980). This substance has been used as a spasmolytic agent in clinical conditions characterized by disturbances of the motility of the colon and of the biliary tract (Paris et al. 1977).

We have tried to elucidate in the present experiments the mechanism of action of this substance and to find out whether its action is limited to visceral smooth muscle cells by comparing its effects on guinea-pig *taenia coli* and ileum with those on rabbit ear artery. The latter preparation is characterized by a clear distinction between electromechanical and pharmacomechanical coupling, and allows ^{45}Ca fluxes to be followed which can be analyzed quantitatively (Droogmans et al. 1977).

In this paper we now demonstrate that pinaverium bromide exerts actions which resemble those of calcium entry blockers, and that it exerts a larger inhibitory effect on voltage-dependent than on other receptor-operated mechanisms.

Methods

Longitudinal smooth muscle strips of guinea-pig caecum and ileum, and helical strips of rabbit ear artery were dissected and mounted in a muscle chamber of 1 ml (Casteels and Droogmans 1981). For contraction experiments the tissues were superfused with oxygenated physiological solutions at a rate of 4 ml/min. The tissues were fixed at one end to the bottom of the chamber, and the other end was connected to an isometric force transducer. A resting tension of 5–10 mN was applied to the tissues, and they were allowed to equilibrate for 1 h. The tension development was recorded on a potentiometric pen recorder.

For recording electrical activity, the intestinal preparations were pinned to the bottom of a perfusion chamber and sufficiently stretched to facilitate prolonged insertion of a microelectrode. The transmembrane potential was measured by means of conventional micro-electrodes, filled with 3 M KCl and having a resistance varying between 80 and 100 MΩ. The electrical activity and its time derivative, measured by means of an analogue differentiator, were displayed on an oscilloscope screen and recorded with a pen writer.

For flux experiments, the tissues were exposed for the time periods indicated in the Results to solutions containing ^{45}Ca. After this loading procedure the preparations were mounted isometrically in a perfusion chamber and superfused with non-radioactive solutions at a rate of 1–2 ml/min. The superfusate was collected and radioactivity was measured by liquid scintillation counting. At the end of the experiment the

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tissue was weighed and digested overnight in 30 % H_2O_2. The ash was dissolved in 1 ml of a 5 mM EDTA solution and the amount of ^{45}Ca present in this solution was measured. By adding in reverse order the radioactivity in each fraction to that remaining in the tissue, the desaturation curve was obtained. The fractional loss was calculated by dividing the number of counts released in each fraction by the collecting time and by the average number of counts present in the tissue at that time.

The normal physiological solution was a Hepes buffered Krebs' solution of the following composition (mM): Na^+ 132; K^+ 5.9; Mg^{2+} 1.2; Ca^{2+} 1.5; Cl^- 143.8; Hepes 11.6; glucose 11.5. The pH was kept at 7.2 and the temperature was 35° C. Solutions with increased $[K]_0$ were prepared by replacing part of the NaCl by an equimolar amount of KCl. Ca-free solutions always contained 2 mM EGTA, giving a free Ca concentration of less than 10^{-9} M.

Pinaverium bromide was a gift from LTM, Paris, France and D600 from Knoll AG, Ludwigshafen, FRG.

The experimental results are expressed as mean ± S.E.M., and significance was tested at the 0.01 level by means of Student's *t*-test.

Results

Effect of Pinaverium Bromide on the Spontaneous Electrical and Mechanical Activity of Longitudinal Smooth Muscle of Guinea-Pig Ileum and Taenia coli

At a concentration of 10^{-7} M pinaverium bromide did not affect the spontaneous electrical activity either of the ileum or of the *taenia coli*. The frequency of the action potentials remained the same as in control solution. Neither the amplitude of the action potentials nor their maximum rate of rise and of repolarization were affected by this concentration of pinaverium bromide (Fig. 1 a, b). Increasing the concentration of pinaverium bromide to 10^{-6} M significantly reduced the frequency of the action potentials in the ileum, and also markedly reduced their amplitude as well as their maximum rate of rise and of repolarization (Fig. 1 a, b). However the spontaneous electrical activity in guinea pig *taenia coli* was not significantly affected. Increasing the concentration up to 10^{-5} M further reduced the spontaneous activity of the ileum, and even completely inhibited some tissues. Similar effects of pinaverium bromide were observed at this concentration in *taenia coli*. In this tissue the electrical activity either was completely abolished, or the amplitude of the action potentials and their rate of rise and of repolarization were appreciably reduced, while their frequency was little affected (Fig. 1 c).

Pinaverium bromide even at a concentration of 10^{-5} M did not exert any significant effect on the resting potential of either preparation.

We have also investigated the spontaneous mechanical activity in parallel experiments. These data are consistent with our observations obtained in the electrophysiological experiments. At concentrations between 10^{-7} M and 10^{-6} M, pinaverium bromide did not affect significantly the pattern of spontaneous mechanical activity of the ileum or of *taenia coli* (Fig. 2). Increasing the concentration to $2 \cdot 10^{-6}$ M did not modify the spontaneous mechanical activity in *taenia coli*, but in the ileum the frequency and the amplitude of the spontaneous contractions were reduced. At 10^{-5} M the contractions of the ileum became weaker and in some of the tissues the spontaneous activity ceased. Also the spontaneous mechanical activity of *taenia coli* was sometimes completely inhibited at this concentration and when some spontaneous contractions persisted, their amplitude was markedly reduced (Fig. 2).

Effect of Pinaverium Bromide on the Contractions Induced by Agonists and by K-Rich Solutions

The force development of both intestinal preparations induced by increasing $[K]_0$ from 5.9 mM was inhibited in a dose-dependent way by pinaverium bromide (Fig. 3 a). A similar type of inhibition is observed for contractions induced by 10^{-5} M carbachol (Fig. 3 b). Thirty minutes after washing out 10^{-5} M pinaverium bromide the amplitude of the contraction induced by K-depolarization or by 10^{-5} M carbachol had not recovered to the control value, although the spontaneous electrical and mechanical activity had returned to normal.

We have also investigated the effect of pinaverium bromide on the contraction of *taenia coli* and of ileum induced by 10^{-4} M carbachol after exposure to Ca-free solution for a fixed period of time. This contraction is elicited by Ca released from an intracellular Ca-store and it can be evoked only once in Ca free solution (Casteels and Raeymaekers 1979). Moreover it can only reappear after refilling of the store by exposure of the tissue to Ca containing solution. In order to exclude the effect of Ca-influx during spontaneous electrical activity on the refilling of the agonist sensitive Ca-store, these experiments were performed at 18° C. Tissues, which had been stimulated with carbachol in Ca-free medium, were exposed either for 10 min to normal physiological solution containing 1.5 mM Ca and 5.9 mM K, or for 5 min to this physiological solution and for 5 additional min to a physiological solution containing 1.5 mM Ca and 59 mM K. Subsequently these tissues were superfused for 2 min with a Ca-free solution to wash out extracellular calcium, and they were then stimulated with 10^{-4} M carbachol in Ca-free medium. The amplitude of the transient contraction induced by carbachol was used to estimate the degree of refilling of the Ca-store. Figure 4 summarizes the results obtained on *taenia coli* with both loading procedures in the absence and in the presence of 10^{-5} M pinaverium bromide during the procedure of loading with calcium. The contractile response induced by carbachol is appreciably increased by loading during K-depolarization. However, it is significantly reduced for both loading procedures if pinaverium bromide is present. These findings suggest that pinaverium bromide lessens the filling of the store. However it cannot be excluded that it also affects the release of calcium from the store or that it interferes directly with the contractile proteins. In order to rule out the latter mechanisms, pinaverium was applied not during the loading procedure but during the exposure to Ca-free solution 1 min before the stimulation with carbachol. This procedure did not inhibit the carbachol induced contraction, but enhanced it significantly (132 ± 5 % of control value; mean of 4 tissues). Similar results were obtained with longitudinal smooth muscle of ileum.

Effect of Pinaverium Bromide on the Contractile Responses of Rabbit Ear Artery to K-Depolarization and Noradrenaline

Because the activation of the vascular smooth muscle cells of the rabbit ear artery by concentrations of noradrenaline up to 10^{-6} M is not accompanied by a modification of the resting

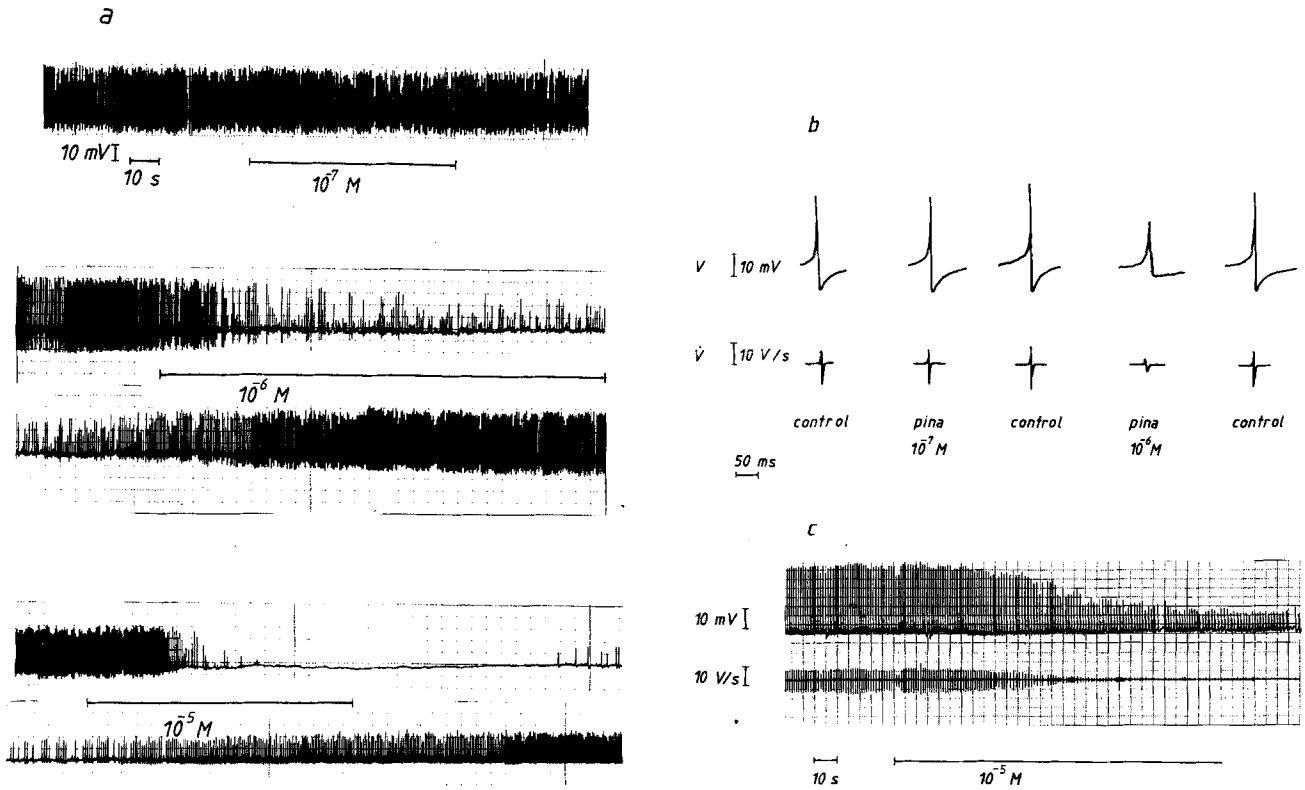

Fig. 1a—c. Effect of pinaverium bromide on the spontaneous electrical activity of guinea-pig ileum. Record (**a**) shows a continuous recording of action potentials. The addition of different concentrations of the substance is indicated by horizontal bars beneath the recording. Record (**b**) shows single action potentials and their time derivative ($\dot{V}$). Record (**c**) shows the effect of 10^{-5} M pinaverium bromide on the spontaneous electrical activity of guinea-pig *taenia coli* and on its time derivative. The records shown are taken from single experiments but are typical of those obtained in the 5 experiments of this type which were carried out

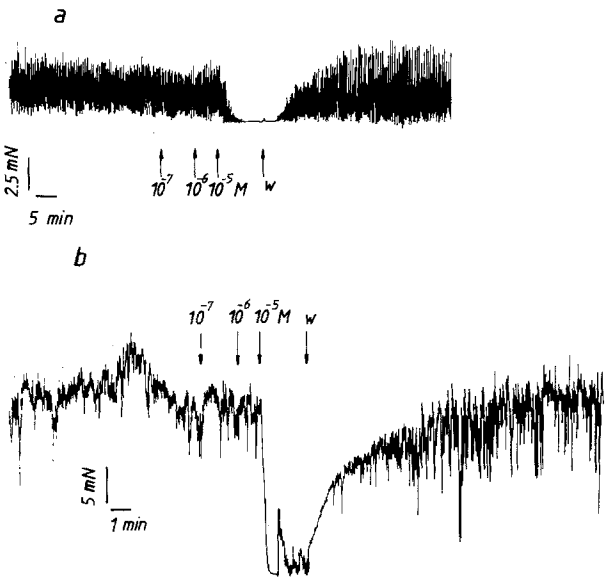

Fig. 2. Effect of different concentrations of pinaverium bromide, added in a cumulative way as indicated by the *arrows*, on the spontaneous mechanical activity of the ileum (**a**) and of guinea-pig *taenia coli* (**b**). The washing out of the substance is indicated by *W*

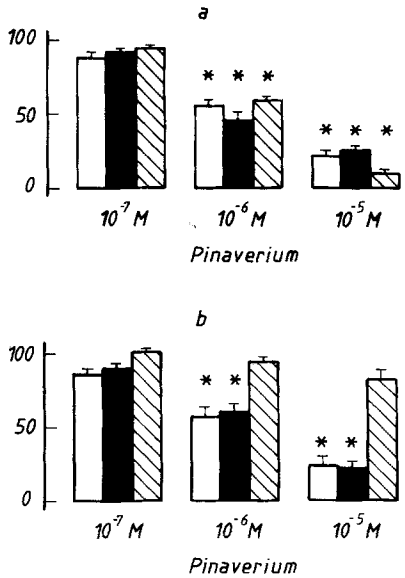

Fig. 3. Effect of different concentrations of pinaverium bromide on the contractions induced by depolarization with 59 mM K in guinea-pig ileum and *taenia coli* and in rabbit ear artery (**a**), and on the contractions evoked by 10^{-5} M carbachol in guinea-pig ileum and *taenia coli* and by 10^{-5} M noradrenaline in rabbit ear artery (**b**). The data obtained in the ileum are represented by *open columns*, those in the *taenia coli* by *solid columns* and those in the ear artery by *hatched columns*. The *height* of the column represents the mean value obtained on 4 tissues, and the *vertical lines* indicate S.E. of mean. The values are given as a % of the contraction in the absence of pinaverium bromide. An *asterisk* denotes a significant difference with the control value ($P < 0.01$)

potential (Droogmans et al. 1977), the use of this preparation might by very helpful in analyzing the effects of pinaverium bromide on voltage sensitive and receptor operated channels.

10^{-6} M pinaverium bromide slightly inhibits the contraction induced by K-depolarization, while 10^{-5} M almost

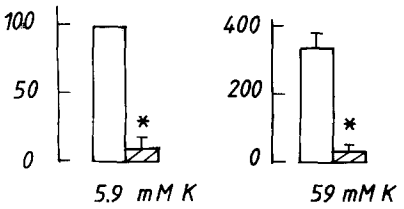

Fig. 4. Effect of pinaverium bromide on the filling of the carbachol sensitive Ca store of guinea-pig *taenia coli* in a solution containing 5.9 mM K and in one containing 59 mM K. The *height* of the columns gives the mean amplitude of the contraction induced in 4 tissues by 10^{-4} M carbachol after an exposure of 2 min to Ca-free solution, and is given as a % of the contraction after a 10 min loading period in normal physiological solution. The *vertical lines* indicate the S.E. of mean. The *open columns* in each panel correspond to control tissues, and the hatched columns to tissues which have been loaded in the presence of 10^{-5} M pinaverium bromide. Note the different scale for tissues loaded in 5.9 mM K and for those loaded in 59 mM K. The *asterisk* denotes a significant difference between control and pinaverium bromide treated tissues (paired *t*-test; $P < 0.01$)

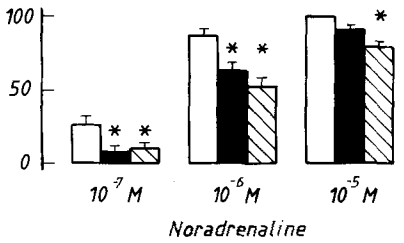

Fig. 5. Effect of D600 and pinaverium bromide on the amplitude of the contractions evoked by different noradrenaline concentrations in rabbit ear artery. Each column represents the mean value obtained on 4 tissues, and is expressed as a % of the contraction induced by 10^{-5} M noradrenaline in control solution. The *vertical lines* indicate the S.E. of mean. The *open columns* represent the data obtained in control solutions and the *solid columns* those obtained in solutions with 10^{-6} M D600 added and the *hatched columns* those obtained in solutions containing 10^{-5} M pinaverium bromide. An *asterisk* denotes a significant difference with the corresponding control value (paired *t*-test; $P < 0.01$)

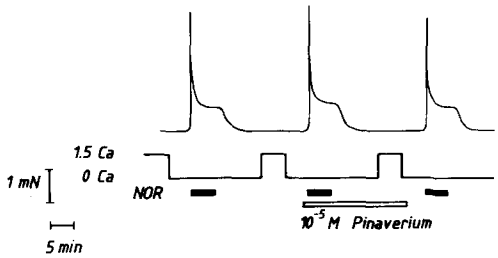

Fig. 6. Effect of 10^{-5} M pinaverium bromide on the contraction induced by 10^{-5} M noradrenaline in rabbit ear artery after exposing the tissues for 5 min to Ca-free solution. Before each stimulation with noradrenaline in Ca-free solution, the tissues were incubated for 5 min in a solution containing 1.5 mM Ca in order to refill the noradrenaline sensitive Ca-store. The 1st contraction represents the control. Pinaverium bromide was added to the Ca-free solution 1 min before the 2nd stimulation with noradrenaline. Pinaverium bromide was still present during the refilling of the store by exposing the tissue to the Ca-containing solution. This procedure was followed by a 3rd stimulation with noradrenaline. Pinaverium bromide was washed out after 1 min superfusion with Ca-free solution, and 4 min before stimulation with the agonist

Table 1. The effects of 10^{-5} M pinaverium bromide and 10^{-6} M D600 on the ^{45}Ca uptake by rabbit ear artery. Control tissues are compared to tissues depolarized by 59 mM K and to tissues which were depleted of Ca by stimulating them with 10^{-5} M noradrenaline in Ca-free solution. All tissues were exposed for 5 min to a solution containing 0.2 mM ^{45}Ca and then washed at 20° C in Ca-free solution. The amount of ^{45}Ca taken up by the tissues was estimated from the amplitude of the slowly exchanging Ca-fraction, as described previously by Casteels and Droogmans (1981). The values are given in $\mu mol \cdot kg^{-1}$ per 5 min. The Ca-antagonists were added 5 min prior to the loading procedure

Condition of tissue	No drug	Pinaverium bromide (10^{-5} M)	D600 (10^{-6} M)
Control	12.6 ± 1.3 (6)	13.8 ± 1.0 (6)	12.7 ± 0.6 (6)
K-depol.	25.3 ± 1.0 (6)	13.3 ± 0.3 (6)	14.8 ± 0.6 (6)
Ca depleted	22.4 ± 1.5 (6)	20.3 ± 1.4 (6)	20.2 ± 1.1 (6)

completely abolishes this contraction (Fig. 3a). The contractions of the ear artery induced by noradrenaline are much less sensitive to the action of pinaverium bromide than the contractions induced by membrane depolarization (Fig. 3b). This is in contrast to the findings in the intestinal preparations. We have also compared the effects of 10^{-6} M D600, a well-known blocking agent of the voltage-dependent Ca-channels, and 10^{-5} M pinaverium bromide on the contractions induced by noradrenaline in a solution containing 0.2 mM Ca (Fig. 5). It is obvious that both substances exert a similar effect on these contractions.

It has been reported (Casteels and Droogmans 1981) that calcium antagonists, such as D600 and nicardipine, neither affect the release of Ca from the noradrenaline sensitive Ca-store, nor the refilling of that store after its depletion. We have therefore also investigated the effect of 10^{-5} M pinaverium bromide on these parameters of the store. The experimental procedure and a typical record are shown in Fig. 6. From the data obtained in 4 tissues it was calculated that the mean amplitude of the contraction induced by 10^{-5} M noradrenaline in Ca-free solution is not significantly affected by the presence of 10^{-5} M pinaverium bromide (101 ± 7% of control). Also the contractions which are obtained after reloading the Ca-store by exposing the tissues to Ca containing solutions in the presence of 10^{-5} M pinaverium bromide (99 ± 4% of control) are not affected.

Effect of Pinaverium Bromide on the ^{45}Ca Exchange in Rabbit Ear Artery

The preceding experimental results suggest that pinaverium bromide interferes with the influx of Ca through voltage sensitive Ca-channels, and that it affects other receptor-operated mechanisms to a much lesser extent. We have therefore compared the effects of 10^{-5} M pinaverium bromide and of 10^{-6} M D600 on the ^{45}Ca-uptake of the ear artery. The experimental results are summarized in Table 1. The uptake under resting conditions is not affected by either of these substances, but the additional uptake of ^{45}Ca elicited by K-depolarization is largely blocked by both substances. These findings correlate with the effect on the contractile response under the same experimental conditions. They are also consistent with the inhibitory action of both drugs on the increase of the ^{45}Ca efflux induced by K-rich solutions (Fig. 7a). This increase is caused by the increased Ca-influx,

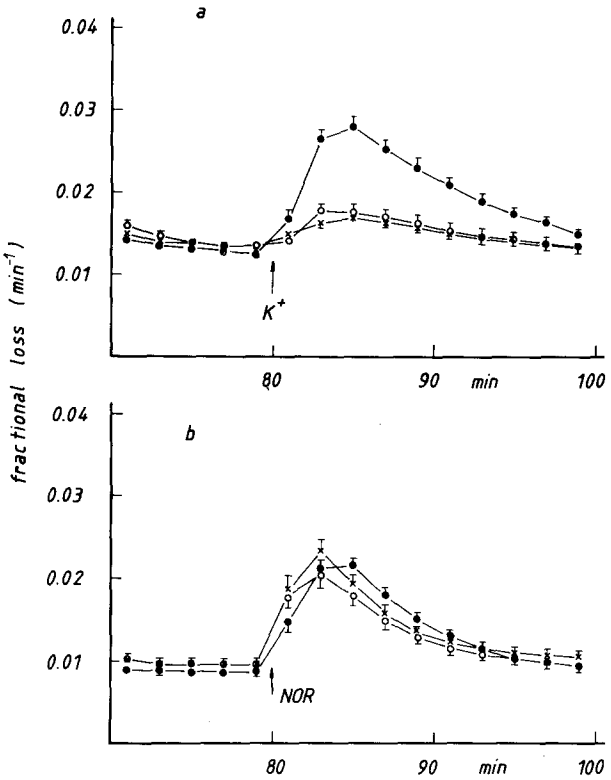

Fig. 7. Effect of 10^{-5} M pinaverium bromide and 10^{-6} M D600 on the increase of the fractional loss of ^{45}Ca from rabbit ear artery induced by K-depolarization in a Ca containing solution (**a**) and by 10^{-5} M noradrenaline in Ca-free solution (**b**). Each curve represents the mean of 6 tissues, and the initial 60 min of efflux are not shown. (●) Control; (×) pinaverium; (○) D600

which leads to an exchange of ^{40}Ca with cellular ^{45}Ca and thereby augments the ^{45}Ca-efflux (Droogmans et al. 1977).

The amounts of ^{45}Ca taken up by tissues, which had previously been depleted of calcium by stimulation with noradrenaline in Ca-free solution, are also represented in Table 1. These values include the amount of Ca taken up in the noradrenaline sensitive Ca-store during its refilling, and it is obvious that this refilling is not affected by either of the antagonists. The effect of both substances on the release of ^{45}Ca induced by noradrenaline in Ca free solution is shown in Fig. 7b. From this figure it can be deduced that this release is not reduced by either 10^{-6} M D600 or 10^{-5} M pinaverium bromide.

Effect of Pinaverium Bromide on the Stimulation of the ^{45}Ca Efflux Induced by K-Depolarization and Carbachol in Guinea-Pig Taenia coli

The existence in guinea-pig *taenia coli* of a large pool of extracellularly bound ^{45}Ca complicates considerably the demonstration of changes in degree of cellular labeling (Van Breemen and Casteels 1972). We have therefore, as suggested by these authors, used an efflux medium containing 20 mM Ca-EGTA and 0.5 mM Ca. The effects of K-depolarization on the ^{45}Ca efflux from *taenia coli* in the presence and in the absence of 10^{-5} M pinaverium bromide are represented in Fig. 8. It is obvious that this substance largely inhibits the stimulation of the ^{45}Ca efflux rate induced by depolarization. Pinaverium bromide also largely blocks the stimulatory effect of carbachol on the ^{45}Ca efflux rate (Fig. 8b). Both stimulations of the ^{45}Ca-efflux rate are caused by an increased entry of external Ca which can be blocked by D600 (Van Breemen et al. 1975). These findings therefore suggest that pinaverium bromide inhibits the Ca-influx induced by K-depolarization and carbachol in exactly the same way as D600.

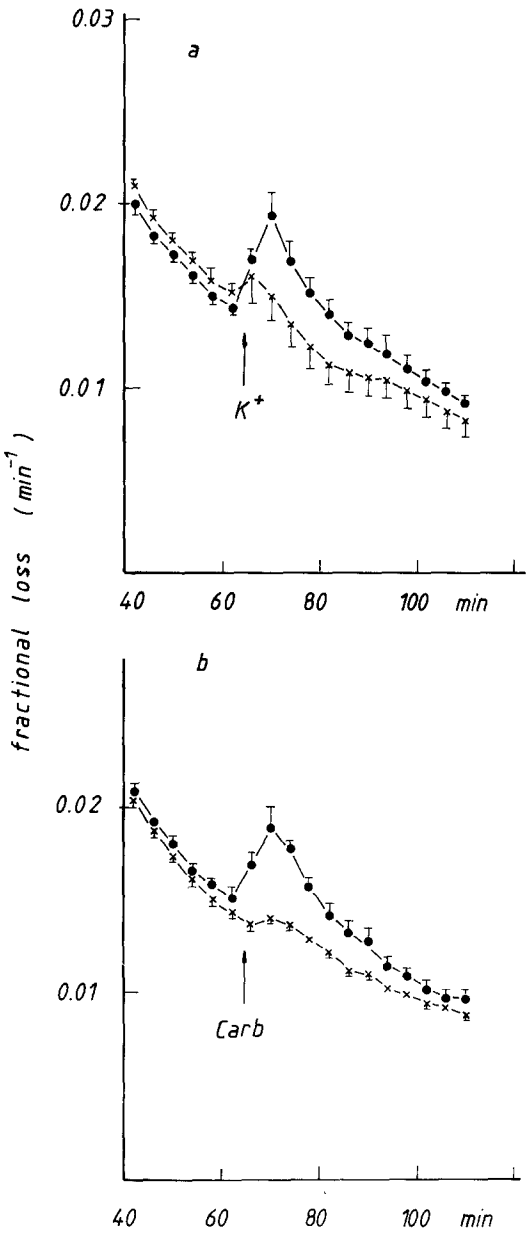

Fig. 8. Effect of pinaverium bromide on the stimulation of the ^{45}Ca efflux in guinea-pig *taenia coli* induced by K-depolarization (**a**) and by 10^{-5} M carbachol (**b**) in a solution containing 0.5 mM Ca and 20 mM EGTA. The (●) represent the control curve, and the (×) correspond to the efflux in a solution containing 10^{-5} M pinaverium bromide. Each curve represents the mean of 6 tissues, and the initial 40 min of efflux are not shown. The fractional loss (min^{-1}) is plotted as a function of the time of efflux (min)

Discussion

The effects of pinaverium bromide on the electrical activity of intestinal smooth muscle preparations are similar to those obtained with the Ca-antagonists D600 and verapamil in

taenia coli and other smooth muscle tissues (Golenhofen and Lammel 1972; Golenhofen and Hermstein 1975; Reiner and Marshall 1975). It is therefore likely that this agent exerts its main action on the voltage sensitive Ca channels that are activated by depolarization.

Also the effects of pinaverium bromide on the contractions induced by K-depolarization and by carbachol are similar to those exerted by D600 and verapamil on these contractions (Haeusler 1972; Riemer et al. 1974). Finally, pinaverium bromide prevents the recovery of the force development induced by carbachol on readmission of Ca to Ca-free solution, as has also been described by Ohashi et al. (1975) for D600. The finding that the presence of pinaverium bromide enhances in guinea-pig *taenia coli* and ileum the contractions induced by carbachol in Ca-free medium, makes it very unlikely that this substance interferes with Ca release or exerts an anti-muscarinic effect. The hypothesis that this action of pinaverium bromide would be due to a slowing down of the Ca loss from visceral smooth muscle during exposure to Ca-free solution, has to be investigated further.

The similarities between the effects of pinaverium bromide and the Ca antagonist D600 therefore suggest a common mode of action of both substances. The experiments with rabbit ear artery provide a clearer picture of the mechanism of action of pinaverium bromide. From the results obtained on this preparation, which are similar to those obtained with D600 (Casteels and Droogmans 1981), it is evident that pinaverium bromide selectively blocks voltage sensitive channels, and has only a slight effect on other receptor-operated mechanisms. It does not prevent the refilling of the noradrenaline sensitive Ca-store after it has been depleted by stimulation with the agonist in Ca-free medium.

Therefore our results suggest that pinaverium bromide, in the same way as the organic Ca antagonists D600, verapamil, nifedipine and nicardipine, blocks voltage sensitive Ca-channels, and affects receptor-operated channels and the intracellular Ca stores to a much lesser extent.

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