European Journal of Pharmacology, 98 (1984) 99–107
Elsevier

COMPARISON OF PINAVERIUM BROMIDE, MANGANESE CHLORIDE AND D600 EFFECTS ON ELECTRICAL AND MECHANICAL ACTIVITIES IN RAT UTERINE SMOOTH MUSCLE

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Received 10 February 1983, revised MS received 15 August 1983, accepted 1 November 1983

J. MIRONNEAU, C. LALANNE, C. MIRONNEAU, J.P. SAVINEAU and J.L. LAVIE, *Comparison of pinaverium bromide, manganese chloride and D600 effects on electrical and mechanical activities in rat uterine smooth muscle,* European J. Pharmacol. 98 (1984) 99–107.

The effects of pinaverium bromide, were compared with those of D600 and manganese chloride (Mn), on membrane potentials, ionic currents and isometric contractions in uterine smooth muscle strips from pregnant rats. Pinaverium bromide (10^{-7}-10^{-6} M) depressed twitch contractions and K-contractures within 15-20 min while D600 (2×10^{-6} M) and Mn (10^{-3} M) abolished both contractions. D600 and pinaverium bromide were more potent inhibitors in K-depolarized preparations than in polarized tissues. At a supramaximal dose (10^{-5} M), pinaverium bromide decreased the rate of rise, amplitude, and rate of repolarization of the action potential, and prolonged the potential duration. The inward Ca current was depressed and the reduction in Ca_i was responsible for the decrease in K current. Pinaverium bromide (10^{-5} M) depressed the myometrial contractions induced in Ca-free solution by acetylcholine (10^{-4} M) and by prolonged membrane depolarizations. Mn (2.5×10^{-3} M) only reduced the Ach-induced contraction and D600 (10^{-5} M) had no effect on intracellular Ca stores. The results indicate that pinaverium bromide has Ca channel blocking properties similar to those of currently used Ca antagonists; it may also exert an effect to depress contractions supported by intracellular Ca release.

Smooth muscle Electrophysiology Contraction Ca antagonists Intracellular Ca stores

1. Introduction

Uterine contraction can be activated by two different mechanisms: the first consisting of the Ca inward current and the second of the release of Ca from intracellular stores (Edman and Schild, 1962; Mironneau, 1973). These observations indicate that the electromechanical properties of rat myometrium are similar to those of intestinal smooth muscles (Hurwitz, 1975; Bolton, 1979; Kuriyama, 1981). Under these conditions, the rat myometrium can be used as a smooth muscle model to elucidate the mechanisms of action of substances which are believed to act at the cellular level.

Pinaverium bromide (N-(bromo-2-dimethoxy-4,5 (benzyl)N((dimethyl-6,6 norpinalyl-2)-2 ethoxy)-2 ethyl morpholinium bromide) exerts an inhibitory action on the contractions induced by barium chloride in intestinal smooth muscles from rats and guinea-pigs (Bretaudeau and Foussard-Blanpin, 1980).

The purpose of the present study was to test whether pinaverium bromide has Ca channel blocking properties in smooth muscle, and to compare its effects with those of D600 and manganese chloride. The actions of these substances were examined on isometric contractions (in polarized and depolarized preparations), on the action

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potential time course, and on ionic currents of myometrium isolated from rats at the end of pregnancy. Ca-free EGTA-containing solutions were used to determine whether pinaverium bromide and Ca antagonists may affect intracellular Ca release.

2. Materials and methods

2.1. Preparation

Isolated longitudinal strips from pregnant rat myometrium (70-100 μm in diameter; 3-4 mm in length) taken at the end of pregnancy (18-19 days) were used in these experiments. After a stabilizing period (approx. 10-30 min) in the reference solution, the preparation was ready for electrical and mechanical recordings.

2.2. Mechanical recordings

The experimental chamber consisted of a $3 \times 3 \times 20$ mm open-topped channel connected at one end to a four-way tap opening directly into the channel, allowing rapid change of the perfusing solutions. The solution entered the channel at a rate of 10 ml/min, so that the liquid was completely changed in about 1 s. The other end of the channel opened into a drain, so as to avoid perfusion by stagnant solutions. About 2 mm from the tap, one end of the strip was tied to the floor of the chamber by means of a nylon loop. The other end of the strip was fixed to the lever of a highly sensitive isometric force transducer (Aksjeselskapet Mikro-Electronikk Ltd, Norway) with very low drift, good linearity and high sensitivity. The muscle was stimulated either electrically by 10 ms pulses through platinum electrodes on each side of the channel, or by perfusion with a concentrated K solution (60 mM).

2.3. Electrical recordings

Electrical activity was recorded with the double-sucrose gap method (Mironneau, 1973). With this method an estimate of the gap potential was obtained before each experiment. The preparation in the test gap was perfused with a high K solution (135.6 mM for 5 min), and the electronic set-up was connected for current-clamp. Then, when the high K solution was changed to the reference solution, the preparation repolarized to a stable value. Because the observed gap potential was close to the resting potentials recorded by intracellular microelectrodes, this gap potential may be accepted as representing the average resting potential of the cells in the test compartment. Adequate transmembrane voltage control was limited by the presence of a significant series resistance, and uniformity of the voltage control was limited by the multicellular and inherent cable properties of the muscular strips (Bolton et al., 1981). Therefore, the following precautions and tests were applied: (1) the length of the test compartment (150-200 μm) was relatively short in comparison with the space constant of the preparation (2.5 mm in resting conditions, Kuriyama and Suzuki, 1976). The low strip diameter/test compartment ratio (0.5) minimized sucrose-ionic solution diffusion and allowed viable action potentials to be obtained; (2) direct evidence of voltage-clamp control was obtained by measuring the voltage independently in the test gap with a microelectrode (Mironneau et al., 1980).

The results were expressed as follows: V (mV), variation of the membrane potential, the resting potential being taken as zero (holding potential $E_r = 0$). Positive values of V represent a depolarization, negative values a hyperpolarization; I (μA), membrane current. Positive values of I correspond to an outward current, negative values to an inward current.

2.4. Solutions

Physiological solutions had the following composition: (a) reference solution (mM): NaCl 130; KCl 5.6; $CaCl_2$ 2.1; $MgCl_2$ 0.24; glucose 11. The solution was aerated with O_2 and buffered by Tris-HCl (8.3 mM) at pH 7.4. (b) In Ca-free solution, $CaCl_2$ was omitted and 0.5 mM EGTA was added. At this concentration EGTA was able to chelate superficial Ca ions from the cell membrane in less than 1 min since triggered action potentials were suppressed. However, the uterine

membrane remained polarized at -32 ± 6 mV (Mironneau et al., 1982). (c) High K solutions were prepared by substituting NaCl for KCl in equimolar amounts: (1) 135.6 mM K^+ to estimate the gap potential of the strip at the beginning of each electrical recording. (2) 60 mM K^+ to depolarize the membrane at a steady state value (-20 ± 3 mV, n = 50) in order to induce a maximal K contracture (Gabella, 1978). In some experiments, the possible interference between experimental stimulations and release of endogenous neurotransmitters was eliminated by use of tetrodotoxin (5×10^{-7} M), phentolamine (3×10^{-6} M) and propranolol (3×10^{-6} M). The responses obtained in the presence of these drugs were not substantially different from responses obtained in their absence. All solutions were maintained at 30 ± 1°C.

2.5. Drugs

Pinaverium bromide was a gift from L.T.M. Laboratory (Paris) and D600 from Knoll AG (Ludwigshafen, FRG). D600 and manganese chloride were used as inhibitors of the Ca inward current (Hagiwara and Nakajima, 1966; Fleckenstein, 1977) and tetraethylammonium chloride (TEA) as an inhibitor of the K current (Hille, 1967).

2.6. Calculations and statistical analysis

The experimental results were expressed as mean $\pm$ S.E.M. and significance was tested ($p \leqslant 0.01$) by means of Student's t-test. A microcomputer (Tektronix 4052) was used for calculations of electrical and mechanical parameters.

3. Results

3.1. Effects of pinaverium bromide, manganese chloride and D600 on isometric contractions in rat myometrium

Single pulses of electrical stimulation (10 ms; 2-5 V) applied to myometrial strips induced the development of isometric twitch contractions which lasted between 20 and 30 s. Cumulative concentration-response curves for the inhibitory effects of the drugs on the peak contraction were obtained with 5 different preparations. The amplitude of the contraction (in %) was plotted as a function of the external drug concentration (from 10^{-8}-10^{-3} M). Fig. 1 shows that the decrease in contraction was more pronounced with D600 and manganese chloride (Mn) than with pinaverium bromide. Half-maximal inhibition was obtained at concentrations of 3.7×10^{-7} M (D600), 6.4×10^{-7} M (pinaverium bromide) and 4×10^{-4} M (Mn). In all experiments, the measurements were only made when the preparations reached a steady state. For example, the depression of contractions in pinaverium bromide-containing solution began within 1-2 min, and reached a steady state value by 15-20 min. The effects of the 3 inhibitors on the contractions were reversible within 10-25 min. However, a faster recovery was observed following inhibition with manganese chloride (4-5 min). Whatever the inhibitors used, the blockade of contractions could be overcome by raising the extracellular Ca (6 mM) suggesting a competition of the inhibitors with Ca ions.

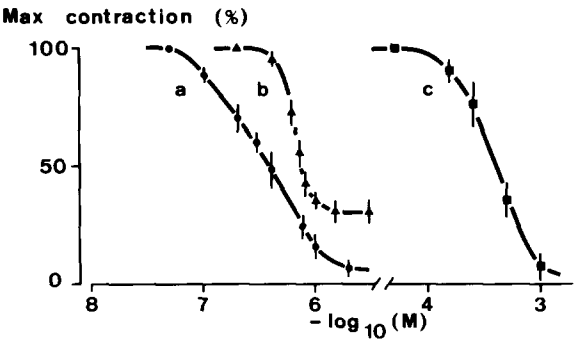

Fig. 1. Dose-response curves for the effects of D600 (●, a), pinaverium bromide (▲, b), and manganese chloride (■, c) on triggered isometric contractions in polarized rat myometrium. *Abscissa*: $-\log$ of substance concentration (M). *Ordinate*: percent of maximal contraction. Data points are means $\pm$ S.E.M. of 5 individual experiments.

Applications of a high K solution induced a sustained contracture the amplitude of which was dependent on the external Ca concentration (Edman and Schild, 1962). Each substance was then added and its effects on the contractile response

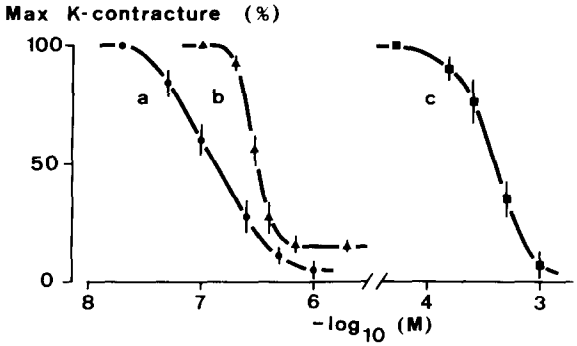

Fig. 2. Dose-response curves for the effects of D600 (●, a) pinaverium bromide (▲, b), and manganese chloride (■, c) on contractures induced by K-depolarization (60 mM). *Abscissa*: −log of substance concentration (M). *Ordinate*: percent of maximal K-contracture. Data points are means ±S.E.M. of 5 individual experiments.

measured when the muscle tension decreased to a new plateau. Fig. 2 shows the depressing action of the 3 substances on the maximal K contractures as a function of the external concentration. The half-maximal inhibition of responses was obtained at 1.4×10^{-7} M (D600), 3×10^{-7} M (pinaverium bromide) and 4×10^{-4} M (Mn). It is to be noted that D600 and pinaverium bromide were more effective on K contractures than on twitch contractions while manganese chloride depressed both contractions similarly. For example, pinaverium bromide (4×10^{-7} M) strongly depressed the K contracture (73 ± 6%) while it slightly affected the twitch contraction (5 ± 3%). Since the inhibitory action of pinaverium bromide on contractions was generally obtained within 15-20 min when low concentrations were used, we have studied the effects of a supra-maximal dose (10^{-5} M) in the following experiments in order to reduce as far as possible the time of perfusion with the drug (3-5 min).

3.2. Effects of pinaverium bromide, manganese chloride and D600 on contractions induced in Ca-free solution

The presence of an internal Ca store in rat uterus that can be released by acetylcholine has been reported previously by Edman and Schild (1962). They demonstrated that uterus washed in Ca-free solution could contract in response to high

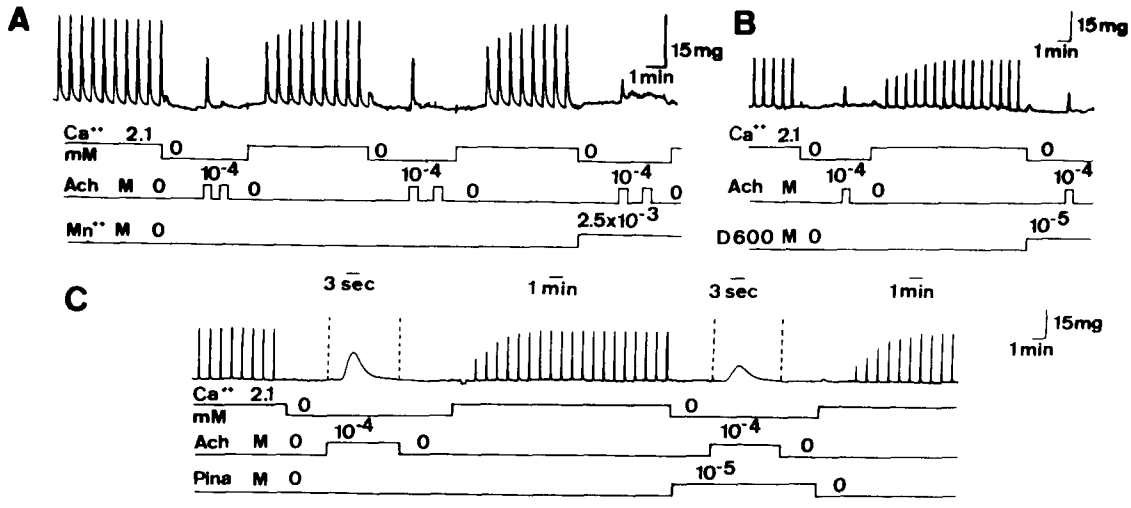

Fig. 3. Effects of Mn ions (2.5×10^{-3} M, A), D600 (10^{-5} M, B) and pinaverium bromide (10^{-5} M, C) on the amplitude of the Ach-induced contraction after 3 min in Ca-free EGTA-containing solution. In Ca-containing solution, repetitive contractions were induced by electrical stimulation. A maximal response to 10^{-4} M Ach was evoked only once in Ca-free solution (A). Total filling of the Ca store was obtained by re-exposure of the preparation to a Ca-containing solution before application of the 3 substances. It must be noted that the speed of the pen recorder was increased during the Ach-induced contraction shown in C (between vertical broken lines). Mn ions and pinaverium bromide reduced the Ach-induced contraction while D600 had no effect.

concentrations of Ach and that this contraction may persist long after the response to high K solutions was abolished. More recently, intracellular Ca stores have been proposed to play a role in smooth muscle contractions induced by membrane depolarizations (Mironneau, 1973; Mangel et al., 1982) and by muscarinic receptor activation (Casteels and Raeymaekers, 1979; Brading and Sneddon, 1980).

In rat myometrium a contractile response to a supramaximal concentration of Ach (10^{-4} M) was evoked only once in Ca-free EGTA-containing solution (fig. 3A). The response reappeared after refilling of the internal store by exposure of the preparation to a Ca-containing solution for 5 min.

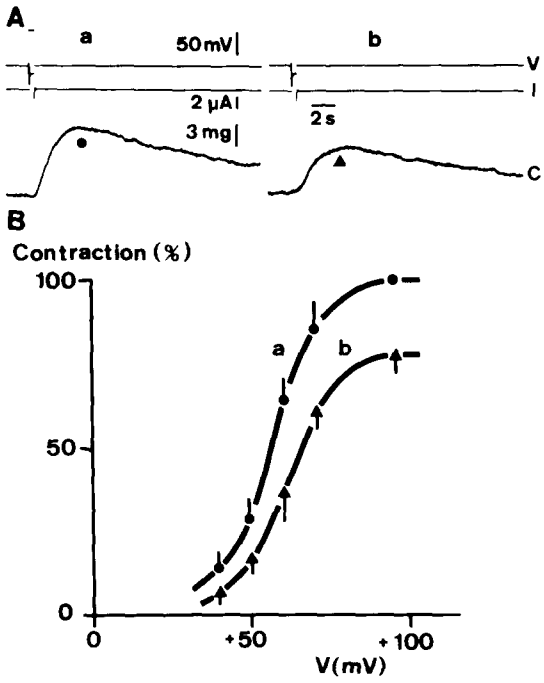

Fig. 4. (A) Outward K current and contraction recorded in Ca-free EGTA-containing solution in response to a depolarization of 400 ms in duration and +70 mV in amplitude (a) and after addition of pinaverium bromide (10^{-5} M) (b). The contraction was decreased by the drug but the K current was unaffected. (B) Peak contractions in Ca-free solution (a) and after addition of pinaverium bromide (b), plotted against voltage. The ordinate is expressed as a percentage of the maximal contraction obtained for a depolarization of +95 mV in Ca-free solution. Data points are means ±S.E.M. of 3 individual experiments.

The size of the contraction was largely dependent on the time interval between removal of Ca and application of Ach. The response was lost after 13-15 min of perfusion in Ca-free EGTA-containing solution (unpublished experiments). We have investigated the effects of pinaverium bromide, D600 and manganese chloride on the myometrial contractions induced in Ca-free solution by Ach or, in voltage-clamp experiments, by membrane depolarization. When manganese chloride (2.5×10^{-3} M, fig. 3A) or pinaverium bromide (10^{-5} M; fig. 3C) were applied for 3 min in Ca-free solution, the amplitude of the Ach-induced contraction was reduced by $55 \pm 5\%$ ($n = 4$) and $35 \pm 6\%$ ($n = 5$) respectively while the contraction was unaffected during application of D600 (10^{-5} M; fig. 3B). Such a lack of effect of D600 on carbachol-induced contraction has also been observed in intestinal muscle perfused with Ca-free K-rich solution (Ohashi et al., 1975). Using the double sucrose gap apparatus, the application of long-lasting depolarizations (150-500 ms) in Ca-free solution produced slow contractions of reduced amplitude (Mironneau, 1973). The magnitude of these contractions increased with the depolarization. A typical experiment is shown in fig. 4A. The contraction induced by a depolarization of 400 ms duration and +70 mV amplitude was decreased by about 30% after pinaverium bromide treatment (10^{-5} M). It must be noted that pinaverium bromide did not change the outward K current measured in Ca-free solution suggesting that a limited reduction in cytoplasmic Ca concentration was not effective in producing a noticeable reduction in Ca-activated K current. Since membrane depolarizations are a necessary condition for the appearance of the Ca-sensitive K current in uterus (Mironneau and Savineau, 1980), the internal Ca binding site that activated K conductance is certainly located through the plasma membrane, as suggested for neurons by Gorman and Thomas (1980), and Lux and Hofmeier (1982). Despite definite signs of decreased cytoplasmic Ca concentration in the vicinity of the contractile proteins during pinaverium bromide application, there was no clear indication of a reduced activation of K conductance measured in Ca-free solution. The relationship between contraction and voltage (fig.

4B) indicates that the contraction was decreased at each potential in pinaverium bromide-containing solution while no significant effect of D600 and manganese chloride was observed in similar experimental conditions.

3.3. Effects of pinaverium bromide on action potentials

Table 1 summarizes the effects of pinaverium bromide (10^{-5} M) on the resting and action potentials. It can be observed that the resting potential was not modified by the drug. The amplitude of the action potential as well as the maximal rate of rise and of repolarization were significantly reduced. Moreover, the action potential duration (measured when repolarization had reached 80% of its value) was increased. Under our experimental conditions the action potential parameters were restored within 10-15 min. D600 (5×10^{-7} M) and Mn ions (4×10^{-4} M) showed qualitatively similar behaviour.

3.4. Effects of pinaverium bromide on ionic currents

It has been shown that the inward and outward currents develop almost simultaneously in rat myometrium (Mironneau et al., 1981), so that a large outward current can hide the inward current. To simplify the recording of a net Ca inward current, TEA ions (20 mM) were used to reduce the K outward current. In uterus, TEA is the most effective K conductance blocker since it reduces the outward rectification measured during a depolarization of +100 mV, by 87 ± 5%, n = 5 (Mironneau, 1974). Moreover, the peak inward

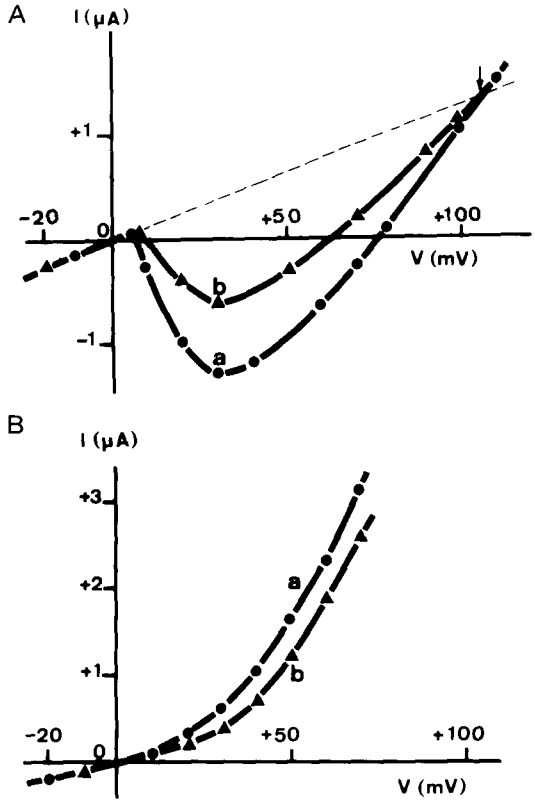

Fig. 5. (A) Example of current-voltage relationships for the Ca current obtained in TEA-containing solution (a) and after the addition of pinaverium bromide (10^{-5} M, b). There was no appreciable shift of the reversal potential (arrow, obtained by assuming that the leakage current, broken line, was a linear function of the potential) while the inward current was decreased over a wide range of membrane potential. Similar results were obtained in 5 preparations. (B) Example of current-voltage relationships for the K current measured at the end of a 300 ms pulse in reference solution (a) and after adding pinaverium bromide (10^{-5} M, b). At +50 mV, the K current was decreased by 25 ± 4% (n = 4).

TABLE 1

Effects of pinaverium bromide (10^{-5} M) on action parameters. Mean value ± S.E. (8 preparations).

	Resting potential (mV)	Action potential (AP) amplitude (mV)	AP duration (ms) 80% repolarization	Max. rate of rise (V/s)	Max. repolarization (V/s)
Reference solution	45 ± 2.3	48.6 ± 6.9	53.2 ± 6.9	2.84 ± 0.3	2.01 ± 0.4
Pinaverium bromide (10^{-5} M; 5 min)	44.2 ± 2.7	26.2 ± 3.9	82.7 ± 9.2	1.08 ± 0.2	0.33 ± 0.1
	n.s.	P < 0.01	P < 0.01	P < 0.001	P < 0.001

current was measured during the first 10-20 ms of depolarizations while the outward current was determined at its steady state value, i.e. at the end of depolarizing steps lasting 300 ms. These different precautions were used to provide an experimental separation of inward and outward currents. The effects of pinaverium bromide were studied at a supra-maximal dose (10^{-5} M). As shown in the current-voltage relationship illustrated in fig. 5A, the amplitude of the inward current was decreased over a wide range of depolarizing voltages. It must be pointed out that the maximal inward current intensity was decreased by $56 \pm 5\%$ ($n = 5$). In smooth muscles, a large outward leakage current was observed for depolarizations and the reversal potential of the inward current was difficult to measure. If it is assumed that the leakage current is a linear function of voltage (Anderson et al., 1971), a possible correction was made by subtracting the linear current-voltage relationship (broken line in fig. 5A) from the U-shaped curves. This correction resulted in a shift of the current-voltage relationships to more positive voltage values, the apparent reversal potential in reference solution being obtained at about +105 mV. As shown in fig. 5A, the control and the drug current-voltage relationships converge to a similar potential indicating little or no change in the apparent reversal potential. The steady state inward currents measured during the application of hyperpolarizing pulses were unaffected in the presence of the drug indicating that the resting properties of the membrane were not modified.

The complicated pattern of the outward current in rat myometrium has been explained by two pharmacologically distinct sets of K channels (Mironneau and Savineau, 1980). A first set of voltage-dependent K channels is inhibited by 4-aminopyridine and TEA ions. A second set of K channels is sensitive to TEA ions and is activated by an increase in the internal Ca concentration which is related to the Ca inward current. To determine whether or not both components of K current were sensitive to pinaverium bromide, the effects of the drug were analyzed in Ca-free and Ca-containing solution. As shown in the current-voltage relationships (fig. 5B), the K outward current measured in Ca-containing solution, at the end of a 300 ms pulse, was reduced by $26 \pm 4\%$ ($n = 4$) at +50 mV. On the contrary, the K-current measured in Ca-free solution was not significantly affected in the presence of pinaverium bromide (fig. 4A). In this respect, it can be proposed that pinaverium bromide reduced essentially the K current through inhibition of the K component which dependent on the Ca influx. D600 (5×10^{-7} M) and Mn ions (4×10^{-4} M) displayed similar effects on ionic currents.

4. Discussion

The present study indicates that pinaverium bromide acts on the Ca conductance of the uterine membrane since the inward Ca current was decreased without noticeable modification of the apparent reversal potential. As the inward current is responsible for the rate of rise and the amplitude of the action potential (Anderson et al., 1971; Mironneau, 1974), and since it is reduced by pinaverium bromide, our results illustrate this phenomenon. In the same way, the outward K current was decreased by pinaverium bromide, and this depression could explain the reduction of the rate of repolarization and the prolongation of the action potential duration. However, there are two major ways in which the drug could have reduced the K current: (a) by blocking K channels and (b) by affecting the K current activated by the increase in intracellular Ca generated by the Ca influx (Meech, 1978). In most cells of several voltage-dependent K channels present, only one type is activated by Ca_i and abolished upon the removal of the external calcium or block of inward current (see for uterus, Mironneau and Savineau, 1980). Our data show that the reduction of the inward current by pinaverium bromide appears to be responsible for the decrease in K current.

The importance of the external Ca pool in the action of pinaverium bromide was also indicated by the depression of Ca-dependent contractions in polarized and K-depolarized preparations. An important observation is that the depressant action of both D600 and pinaverium bromide was increased during depolarization of the membrane with high K solution. On the contrary, there was a

similar sensitivity to manganese chloride with both types of contractions. These observations suggest that the Ca channels involved in spike generation may be different from those involved during K contracture since those involved in spike activity are known to be completely inactivated by sustained depolarization (membrane potential less negative than −30 mV) (Mironneau, 1974) and appear to be less sensitive to organic Ca inhibitors than the channels responsible for K contracture. This concept that several sets of Ca channels may reside in the plasma membrane of smooth muscles has been introduced previously (Boev et al., 1976; Bolton, 1979; Hurwitz et al., 1980; Golenhofen, 1981). Another alternative explanation is that organic blockers may reach their Ca channel blocking sites more easily during sustained depolarization and could exert a more effective inhibitory action in depolarized preparations. In cardiac cells, a common property of Ca antagonistic drugs is the dependence of their blocking effects on the membrane potential and on the stimulation frequency (Pelzer et al., 1983; Lee and Tsien, 1983; Molyvdas and Sperelakis, 1983). However, the degree of potential dependence was not similar for the two compounds. Only pinaverium bromide, at a concentration of 4×10^{-7} M, depressed the Ca-dependent contractions in K-depolarized but not in normally polarized uterine tissue.

The results of the experiments in Ca-free EGTA-containing solution are consistent with the proposal that there are cellular Ca stores in myometrium cells from which Ca ions can be released into the cytoplasm by either Ach application or prolonged membrane depolarization. Presumably, Ach released essentially the calcium distributed within the cell membrane and/or just beneath the cell membrane through the activation of muscarinic membrane receptors (Casteels and Raeymaekers, 1979; Bolton, 1979; Brading and Sneddon, 1980). On the other hand, prolonged membrane depolarizations (after suppression of the phasic Ach responses) may release the calcium stored mainly in the sarcoplasmic reticulum (Rubànyi et al., 1980). It has been shown recently that, in skinned smooth muscle, intracellular calcium may be released from the sarcoplasmic reticulum by a depolarization induced by a sudden change in the ionic composition of the solution (Saida, 1982). Pinaverium bromide depressed both contractile responses produced by release of intracellularly sequestred Ca. One possible explanation for this effect is that pinaverium bromide, at high concentrations, may penetrate into the uterine cell, the entry of the drug being facilitated by membrane depolarization. Once inside the cell, pinaverium bromide may act to inhibit Ca release from the stores. This hypothesis is supported by the finding that pinaverium bromide depressed the twitch contractions of uterus more than the Ca inward current. D600 did not exert such an effect. On the other hand, manganese chloride strongly reduced the Ach-induced contraction, and had no important effect on the contraction induced by prolonged membrane depolarization. It is suggested that Mn ions could prevent the leakage of Ca from the peripheral Ca store (sensitive to Ach) but not from sarcoplasmic reticulum.

It can be concluded that pinaverium bromide at low concentrations acts directly on the uterine membrane by depressing the Ca inward current in a manner similar to that observed for D600 (Reiner and Marshall, 1975) and manganese chloride. However, it is also reported that pinaverium bromide at high concentrations may have a second intracellular site of action such as to depress the release of Ca from internal stores.

Acknowledgements

This work was supported by grants from CNRS (ATP Neuromédiateurs) and INSERM (P.R.C. Reproduction et Développement) and by special help from L.T.M. Laboratory (Paris).

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