Br. J. Pharmac. (1984), **82**, 735–743

Maintained contractions of rat uterine smooth muscle incubated in a Ca^{2+}-free solution

C. Mironneau, J. Mironneau & J.P. Savineau

Laboratoire de Physiologie Cellulaire, Institut de Biochimie Cellulaire et Neurochimie du CNRS et Université de Bordeaux II, 1 rue Camille Saint-Saëns, 33077 Bordeaux cedex, France

1 The effects of acetylcholine (10^{-4} M), prostaglandin E_2 (10^{-6} M), vanadate (5×10^{-4} M) and fluoride (10^{-2} M) have been studied on the mechanical and electrical activities of rat myometrial strips perfused in Ca^{2+}-free EGTA-containing solutions.

2 All four substances produced maintained contractions which could be initiated repeatedly after exposure to Ca^{2+}-free solution for more than 1 h, without a significant decrease. The largest contractions were obtained with vanadate and the smallest ones with acetylcholine. The tension was usually 7–30% of the control contraction triggered by an action potential in Ca^{2+} containing solution.

3 Maintained contractions induced by fluoride were unaffected by isoprenaline while those induced by acetylcholine, prostaglandin E_2 and vanadate were completely relaxed.

4 Prostaglandin E_2 -and vanadate-induced contractions were slightly reduced by Na^+ removal or by adding Ca^{2+} antagonists. In contrast, contractions induced by acetylcholine were suppressed in Na^+-free solution and largely inhibited in the presence of Ca^{2+} antagonists.

5 The depolarization induced by acetylcholine in Ca^{2+}-free solution was strongly dependent on the external Na^+ concentration. The relationship between the size of the acetylcholine-induced depolarization and the membrane potential (shifted by constant currents) was linear, giving an apparent reversal potential for acetylcholine close to zero potential.

6 In Ca-free solutions and in the presence of atropine, Na^+ action potentials of long duration can be evoked which produced contractions of the same order of magnitude as those initiated by acetylcholine-induced depolarizations.

7 These results are consistent with the hypothesis that the maintained contractions in Ca^{2+}-free solutions induced by several stimulants could be related to Ca^{2+}-independent mechanisms (fluoride) or Ca^{2+} release from an intracellular store. This latter mechanism would include both pharmacomechanical (prostaglandin E_2, vanadate) and electromechanical (acetylcholine) coupling.

Introduction

It is generally accepted that acetylcholine produces a contraction of visceral smooth muscles by mobilizing Ca^{2+} ions through three different mechanisms (Brading *et al.*, 1980): (i) an increased Ca^{2+}-inward current through voltage-dependent channels (Bolton, 1979); (ii) a Ca^{2+}-influx through receptor-operated channels (Bolton & Kitamura, 1983); (iii) a release of Ca^{2+} ions from a limited intracellular Ca^{2+} store producing only one transient contraction (Casteels & Raeymaekers, 1979; Brading & Sneddon, 1980; Lalanne *et al.*, 1984). However, it has also been reported that, during the same Ca^{2+}-free perfusion, maintained contractions of myometrium can be repeatedly produced by different stimulant substances without diminution in size for 1 h or longer (Sakai *et al.*, 1982; Lalanne *et al.*, 1984).

The underlying mechanisms for the maintained contractions to stimulant substances in Ca^{2+}-free solution have not been well analyzed, essentially because measurement of the changes in ionized Ca^{2+} concentration inside smooth muscle cells remains difficult (Fay *et al.*, 1979; Neering & Morgan, 1980). It has been proposed that, during maintained contractions, Ca^{2+} is mobilized by neurotransmitters from an intracellular pool which may be different from that responsible for the initial transient contraction (Heaslip & Rahwan, 1982; Ashoori & Tomita, 1983) whereas Casteels *et al.*, (1981) envisaged the

possibility that noradrenaline could also affect the contractile proteins by a mechanism which does not include a change in cytoplasmic Ca^{2+} concentration.

In the present experiments, it was found that, in the absence of external Ca^{2+}, several substances (vanadate, fluoride, prostaglandin E_2 and acetylcholine) were able to induce maintained contractions in rat myometrium which could be repeatedly elicited on the same preparation. Only the acetylcholine-induced contraction was dependent on both membrane depolarization and Na^+ gradient, and thus may be dissociated from the other maintained responses.

Methods

Experiments were performed on longitudinal muscle, free of both endometrium and circular muscle, isolated from pregnant rat uterus at the end of pregnancy (18–20 days). Strips (60–80 μm wide, 2–3 mm long) were used for mechanical and electrical recordings respectively. After a stabilizing period (30–60 min) in the reference solution, the preparation was ready for experimental recordings.

Physiological solutions had the following composition: (a) Reference solution (mM): NaCl 130, KCl 5.6, $CaCl_2$ 2.1, $MgCl_2$ 0.24 and glucose 11. The solution was aerated with O_2 and was buffered by Tris-HCl (8.3 mM) at pH 7.4. (b) In Ca^{2+}-free solution, $CaCl_2$ was omitted and EGTA was added at 0.5 mM. (c) High-K^+ solution was obtained by substituting NaCl for KCl in equimolar amounts. (d) Manganese chloride and methoxyverapamil (D600) were used as inhibitors of the inward current (Hagiwara & Nakajima, 1966; Fleckenstein, 1977). (e) Sodium orthovanadate (Na_3VO_4) was used as an inhibitor of Ca^{2+}-ATPases (Varecka & Carafoli, 1982).

Acetylcholine, prostaglandin E_2, isoprenaline, sodium orthovanadate, sodium fluoride and atropine were obtained from Sigma Chemicals Co. (St Louis, MO).

Isometric contractions were recorded in an experimental chamber which consisted of an open-topped channel, 3 × 3 × 20 mm, connected at one end to a four way-tap opening directly into the channel (Mironneau *et al.*, 1980). The solution entered the channel at a rate of 15 ml min^{-1}. The different solutions were maintained at 35°C by means of a heating bath. 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

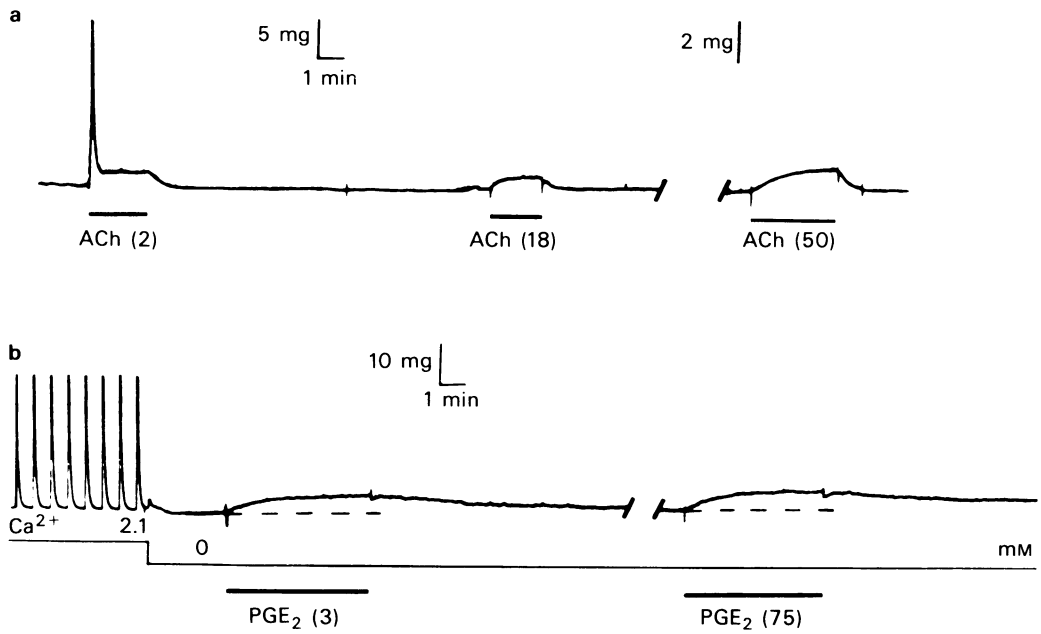

Figure 1 Contractile responses of the rat myometrium during perfusion in Ca^{2+}-free 0.5 mM-EGTA-containing solution and induced by supramaximal doses of acetylcholine (10^{-4} M ACh, a) and prostaglandin E_2 (10^{-6} M PGE_2, b). In parentheses: times in Ca^{2+}-free solution (min). The first addition of ACh (2 min) induces a response which consists of a large transient and a small maintained component. Further application of ACh induces only the small maintained contraction. Successive applications of PGE_2 induce maintained contractions of similar amplitude for times of perfusion in Ca^{2+}-free solution as long as 75 min.

fixed 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 (Akers 801, Norway) with a very low drift, good linearity and high sensitivity. The muscle was stimulated either electrically by single electrical pulses (10 ms, 2–5 V) through platinum electrodes located on each side of the chamber or by perfusion of stimulant substances. Control contractions to application of the stimulant substances in reference solution were established at the beginning of each experiment.

Electrical activity was recorded with both conventional microelectrodes filled with 3 M KCl (resistance 40–50 MΩ) and the double sucrose gap method (Mironneau, 1973). With this latter method an estimate of the gap potential was obtained before each experiment. The preparation in the test gap was perfused with a 135 mM K^+ solution 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 potential recorded by intracellular microelectrodes, this gap potential may be accepted as representing the average resting potential of the cells in the test compartment. The mechanical tension of the myometrial strip was also measured in the test compartment of the double sucrose gap with an Akers-type transducer. A similar arrangement has been applied previously to striated muscle fibres (Vassort & Rougier, 1972) and discussed in detail (Potreau & Raymond, 1983).

Results

Maintained contractile responses of rat myometrium induced by acetylcholine, prostaglandin E_2, vanadate and sodium fluoride in Ca^{2+}-free solution

In Ca^{2+}-free EGTA-containing solution, short applications (10 s) of supramaximal doses of acetylcholine (10^{-4} M) induced contractile responses which consisted of a transient component whose amplitude decreased as a function of time of exposure to the Ca^{2+}-free solution (Lalanne *et al.*, 1984). For longer applications of acetylcholine (2 min or more), the transient contraction was followed by a maintained response which lasted as long as the preparation was exposed to the agonist (Figure 1a). Only a monophasic slow contraction reappeared on readmitting acetylcholine without any additional exposure to a Ca^{2+}-containing solution. In the absence of the transient component, the maintained tension was slightly reduced (usually 5–10% of the control maintained contraction obtained after 2 min of perfusion

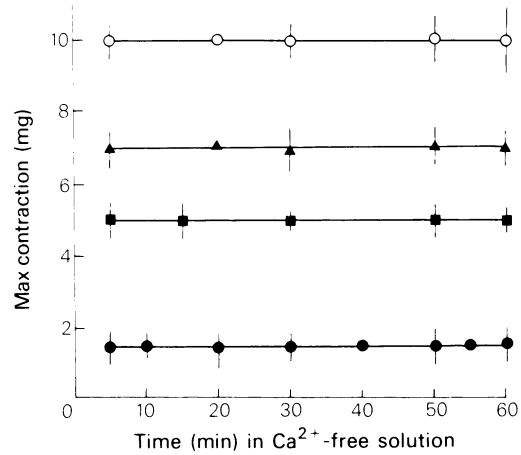

Figure 2 The amplitudes of maintained contractions of the rat myometrium induced by vanadate (○, 5×10^{-4} M), fluoride (▲, 10^{-2} M), prostaglandin E_2 (■, 10^{-6} M) and acetylcholine (●, 10^{-4} M) in Ca^{2+}-free solution. For each substance, the contractions remain constant up to 1 h. Points with vertical lines represent the mean and s.e.mean of 3 to 5 preparations. Other points correspond to an individual experiment.

in Ca^{2+}-free solution). When the same concentration of acetylcholine (10^{-4} M) was applied at regular intervals in Ca^{2+}-free solution, the response remained constant and there was no indication of any decrease for times of perfusion in Ca^{2+}-free solution as long as 50 ± 10 min ($n = 35$). Similar slow contractions were obtained with maximal concentrations of prostaglandin E_2 (10^{-6} M, Figure 1b), vanadate (5×10^{-4} M) and sodium fluoride (10^{-2} M, Figure 3). The amplitude of these contractions was constant for a given concentration of substances and was neither affected by the time of exposure to the Ca^{2+}-free solution (75–80 min) nor by the number of preceding stimulations in the same Ca^{2+}-free solution. The largest force development during the maintained contractions in Ca^{2+}-free solution was induced by vanadate while the smallest contractions were produced by applications of acetylcholine (Figure 2). The value of tension was usually 7–30% of the contraction triggered by an action potential in Ca^{2+}-containing solution. The maintained contractions were completely relaxed by washing out the substances. However, it is noticeable that the onset and rate of relaxation were very slow after removal of prostaglandin E_2 and sodium fluoride (Figure 3).

In order to clarify the mechanisms which may be responsible for the maintained contractions induced by the different stimulant substances, we investigated the effects of isoprenaline, Na^+ removal and Ca^{2+} antagonists on the development of these contractions.

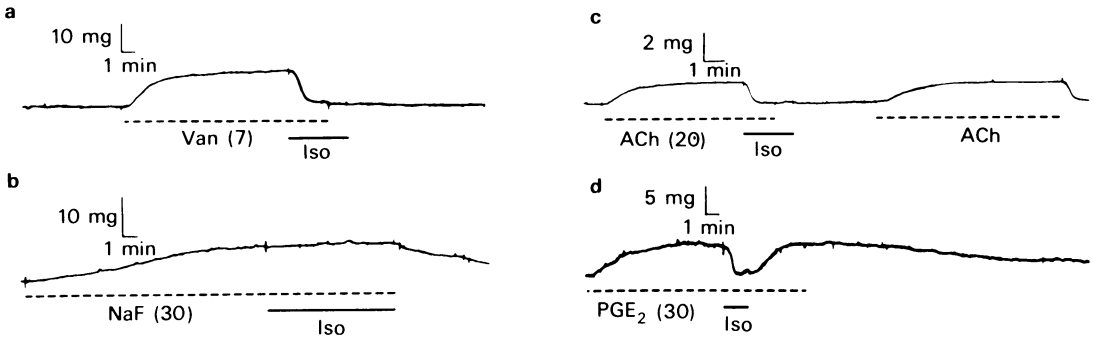

Figure 3 Effect of isoprenaline (Iso, 5×10^{-7} M) on maintained contractions induced by vanadate (Van 5×10^{-4} M, a), fluoride (NaF, 10^{-2} M, b), acetylcholine (ACh, 10^{-4} M, c) and prostaglandin E_2 (PGE_2, 10^{-6} M, d). The fluoride-induced contraction is not affected by isoprenaline while the other contractions are completely relaxed. In parentheses: time in Ca^{2+}-free solution (min).

Effects of isoprenaline, Na^+ removal and Ca^{2+} antagonists on maintained contractions in Ca^{2+}-free solution

Figure 3 shows that the maintained contractions caused by vanadate, acetylcholine, prostaglandin E_2 in Ca^{2+}-free solution were completely abolished in the presence of isoprenaline (5×10^{-7} M; 15 preparations). The relaxation was fast and had no inhibitory action on subsequent acetylcholine-induced contractions (Figure 3c). Moreover, when isoprenaline was removed in the continued presence of prostaglandin E_2 (Figure 3d) the maintained contraction was rapidly restored. Even at higher concentrations (10^{-5} M), isoprenaline was ineffective in reducing the fluoride-induced contractions (Figure 3b, 5 different preparations).

The acetylcholine-induced contraction was suppressed in the presence of atropine (10^{-4} M). It was also inhibited by partial (13 mM) or total removal of external Na^+ ions (Figure 4a) when Tris or Li^+ ions were used as Na^+ substitutes (7 preparations). On the other hand, both vanadate -and prostaglandin E_2-induced contractions were rather insensitive to Na^+-free solution (Figure 4b, c) since the reduction in maximal amplitude was respectively $7 \pm 3\%$ ($n = 9$) and $5 \pm 2\%$ ($n = 5$).

The effects of Ca^{2+} antagonists such as D600 and manganese chloride were studied on the drug-induced contractions which were relaxed by isoprenaline. Mn^{2+} ions (10^{-3} M) and D600 (10^{-6} M) were added 3–6 min before drug admission and during drug perfusion. Vanadate-and prostaglandin E_2-induced contractions (Figure 5a) were slightly reduced by Ca^{2+} antagonists ($8 \pm 2\%$, $n = 15$) while acetylcholine-induced contractions were largely inhibited ($90 \pm 4\%$, $n = 5$, Figure 5b). When the Ca^{2+} antagonists were withdrawn from the Ca^{2+}-free medium, normal acetylcholine-induced contractions returned within 5–10 min. Addition of 10^{-4} M acetylcholine to the solution during vanadate-or prostaglandin-induced contractions caused a further increase of tension (Figure 5c) which was similar in amplitude to that obtained before or after the drug application (7 preparations). Prostaglandin E_2 (10^{-6} M) was also able to induce an additional increase of the tension when added during the maintained contraction evoked by vanadate (4 preparations). Each substance seems therefore to act additively on the force-development.

Effects of acetylcholine, prostaglandin E_2 and vanadate on membrane potential in Ca^{2+}-free solution

The membrane potentials of isolated myometrial strips were measured by both intracellular microelectrodes and the double sucrose gap method. In reference solution, the mean membrane potential measured with microelectrodes was -48 ± 6 mV ($n = 65$). In Ca^{2+}-free solution containing 0.5 mM EGTA, the membrane was depolarized to -38 ± 5 mV ($n = 25$). With these thin myometrial strips, spontaneous potentials were rarely recorded, and when seen stopped rapidly within a few minutes. This observation was different from previous data showing that larger muscle segments were able to produce rhythmic electrical activity for several hours (Mironneau *et al.*, 1982). Application of vanadate (5×10^{-4} M) induced a slight depolarization of the membrane (3 ± 1 mV, $n = 7$). The depolarizing effect of prostaglandin E_2 (10^{-6} M) appeared to be more pronounced (6 ± 2.5 mV, $n = 11$) but was similar to that previously obtained with prostaglandin E_1 (Grosset & Mironneau, 1977). A supra-maximal dose of acetylcholine (10^{-4} M) resulted within 1 min in a rapid depolarization (34.1 ± 4.5 mV, $n = 11$), the membrane reaching a

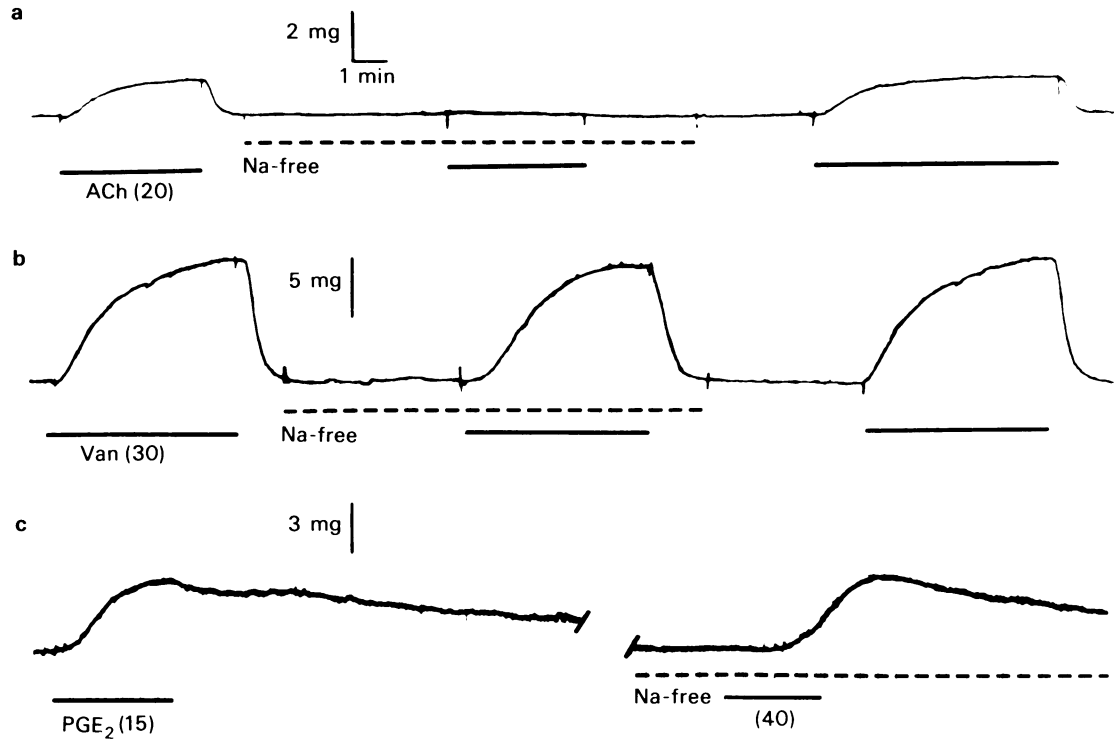

Figure 4 Effect of Na^+-free solution (Tris as Na substitute) on maintained contractions induced by acetylcholine (ACh, 10^{-4} M, a), vanadate (Van, 5×10^{-4} M, b) and prostaglandin E_2 (PGE_2, 10^{-6} M, c). The ACh-induced contraction is totally inhibited by perfusing with Na^+-free solution whereas vanadate -and PGE_2-induced contractions are slightly reduced, by $7 \pm 3\%$ ($n = 9$) and $5 \pm 2\%$ ($n = 5$) respectively. In parentheses: times in Ca^{2+}-free solution (min).

steady-state value (-4.9 ± 0.6 mV, $n = 11$). When acetylcholine was removed from the solution, complete repolarization occurred within 2–3 min (Figure 6a). Atropine (10^{-4} M) selectively blocked the acetylcholine-induced depolarization (5 preparations). On the same preparation, similar acetylcholine-induced depolarizations were observed at regular intervals for 50–55 min in Ca^{2+}-free solutions (15 preparations). For longer periods in Ca^{2+}-free EGTA-containing solution, the acetylcholine-induced depolarizations were generally lost. Figure 6b shows the relationship of the size of the depolarization to the concentration of acetylcholine in Ca^{2+}-free solution. It is noticeable that 10^{-7} M acetylcholine depolarized the membrane slightly (5 ± 2 mV, $n = 5$) while 10^{-6} M depolarized the membrane maximally (35 ± 2 mV, $n = 5$). Manganese chloride (10^{-3} M) and D600 (10^{-6} M) did not induce noticeable variation in the resting potential measured in Ca^{2+}-free solution (Mironneau *et al.*, 1982). However, in the presence of Mn^{2+} ions, the depolarizations induced by various doses of acetylcholine were strongly reduced, so that with concentrations higher than 10^{-6} M, the acetylcholine-induced depolarization reached a maximal value of 6.8 ± 1.5 mV ($n = 5$).

Using the double-sucrose gap technique, the depolarizations induced by 10^{-4} M acetylcholine were determined when the membrane potential was displaced positively and negatively by constant current injection. Figure 7a shows the relationship between the amplitude of the acetylcholine-induced depolarization (abscissa scale) as a function of the membrane potential (ordinate scale) shifted by constant currents. There was a linear relationship between acetylcholine-induced depolarization and membrane potential. Similar observations have been made in guinea-pig intestine (Bolton & Kitamura, 1983). By extrapolating the straight line, the reversal potential for acetylcholine was 38 mV depolarized to the resting potential (which was about -40 mV). This value was close to the maximal depolarization induced by 10^{-4} M acetylcholine in Ca^{2+}-free solution (35 ± 2 mV, $n = 5$).

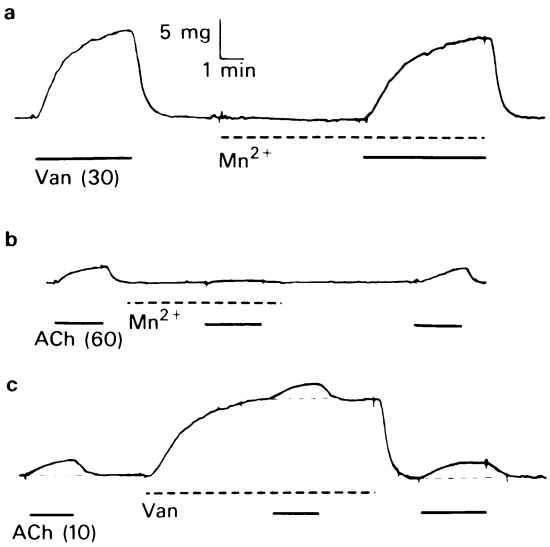

Figure 5 (a and b) Effect of manganese ions (Mn^{2+}, 10^{-3} M) on maintained contractions induced by vanadate (Van, 5×10^{-4} M, a) and acetylcholine (ACh, 10^{-4} M, b). The vanadate- and acetylcholine-induced contractions are reduced by $8 \pm 2\%$ ($n = 10$) and $90 \pm 4\%$ ($n = 5$) respectively. (c) Addition of acetylcholine (10^{-4} M) during a vanadate-induced contraction causes a contraction similar in amplitude to those obtained before and after vanadate-application. In parentheses: times in Ca^{2+}-free solution (min).

To find out whether acetylcholine-induced depolarization in Ca^{2+}-free solution was dependent on the external Na^+-concentration, we measured the amplitude of the depolarizations induced by 10^{-4} M acetylcholine in Ca^{2+}-free solutions when the external Na^+ concentration was progressively reduced and replaced with Tris. It has been previously shown that there was no variation in resting potential when the external Na^+ concentration varied between 130 and 13 mM (Mironneau *et al.*, 1982). In figure 7b, the maximal acetylcholine-induced depolarizations are plotted as a function of the log of the external Na^+ concentration. The experimental points were closely distributed along a straight line representing the theoretical variation of the Na^+ equilibrium potential for a ten fold reduction in external Na^+ concentration (60 mV). This finding indicates that the Na^+ ions seem to be necessary for the generation of the acetylcholine-induced depolarization. Moreover, in Na^+-free Ca^{2+}-free solution, the membrane repolarized to its normal resting value (about -50 mV) and acetylcholine did not induce any variation in membrane potential (5 preparations). When Li^+ ions were used as Na^+ substitute, there was a continuous depolarization of the membrane reaching a steady-state value at about -5 mV (Mironneau *et al.*, 1982). Addition of 10^{-4} M acetylcholine did not produce any subsequent depolarization (4 preparations).

Relationships between action potential and mechanical activity in Ca^{2+}-free solution

It is now accepted that, in myometirum, Ca^{2+} action potentials trigger the development of phasic contractions which last 15–25 s (Mironneau, 1973 and Figure 8a). Removal of external Ca^{2+} (in the presence of 0.5 mM EGTA) rapidly suppressed both Ca^{2+} action potential and contraction. After a 2–3 min Ca^{2+}-free perfusion, brief depolarizing current injections were able to induce action potentials which were characterized by a long duration (several seconds) and the absence of overshoot. It is believed that these durable action potentials resulted largely from prolonged inward Na^+ currents occurring through the membrane channels that normally have a high selectivity for Ca^{2+} ions (Mironneau *et al.*, 1982). As shown in Figure 8b, contractions of low amplitude were recorded simultaneously. Both electrical and mechanical activities were not substantially different in the

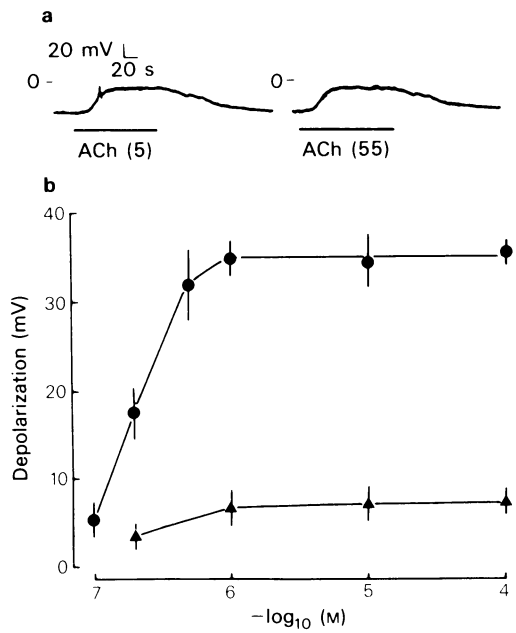

Figure 6 (a) Depolarization of rat myometrial strip by acetylcholine (ACh, 10^{-4} M) recorded at two different times (number in parentheses) in Ca^{2+}-free solution by microelectrodes. (b) Relationships between depolarization (mV) and concentration of acetylcholine (M) in Ca^{2+}-free solution (●) and in the presence of manganese (10^{-3} M, ▲). Points with vertical lines represent the mean and s.e.mean of 5 preparations.

presence of atropine (10^{-4} M). The maximal contraction induced by a Na^+ action potential (2–2.5 mg) was of the same magnitude as that induced after application of 10^{-4} M acetylcholine (1–2 mg). Replacing external Na^+ by Tris caused the suppression of both electrical and mechanical events.

Discussion

The above results indicate that, in Ca^{2+}-free EGTA-containing solution, maintained contractions of rat myometrium could be induced by stimulant substances such as acetylcholine, prostaglandin E_2, vanadate and fluoride. All these contractions were independent of the duration of the exposure to the Ca^{2+}-free solution (up to 1 h) and could be elicited many times without exposure to external Ca^{2+} ions. In so far as isoprenaline interacted with Ca^{2+} movements, either by enhancing Ca^{2+} extrusion to the outside (Bülbring & Den Hertog, 1980), or by stimulating Ca^{2+} re-uptake into sarcoplasmic reticulum (Casteels & Raeymaekers, 1979), the complete inhibition of the contractions induced by vanadate, prostaglandin E_2 and acetylcholine in the presence of isoprenaline supports the idea that Ca^{2+} ions were involved in these contractile responses. The amplitude of responses was not modified after isoprenaline treatment (Lalanne *et al.*, 1984 and Figure 3) suggesting that the loss of Ca^{2+} from the cell through β-adrenoceptor activation may be a limited mechanism in myometrium. As no effect of isoprenaline on the fluoride-induced contraction was observed, a release of Ca^{2+} from an intracellular store seemed unlikely. So, fluoride might replace intracellular chloride leading to a direct activation of contractile proteins (Fermum *et al.*, 1977; Casteels *et al.*, 1981). Thus, we can assume that vanadate, prostaglandin E_2 and acetylcholine could displace Ca^{2+} ions from an intracellular store which might be different from that involved in the transient contractions induced by acetylcholine and angiotensin II in rat myometrium (Lalanne *et al.*, 1984). Several possible mechanisms

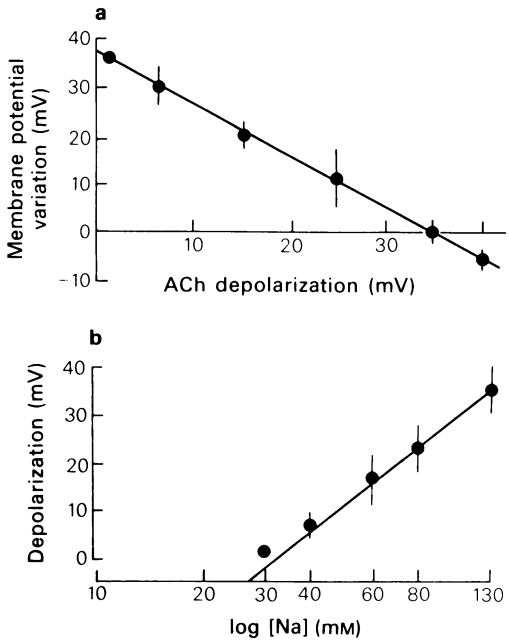

Figure 7 (a) Relationship between 10^{-4} M acetylcholine(ACh)-induced depolarization in Ca^{2+}-free solution (abscissa scale) and the membrane potential shifted by constant currents (ordinate scale) using the double sucrose gap technique (resting potential = 0). The experimental points are closely distributed along a straight line which allows estimation of the reversal potential for acetylcholine (38 mV from the resting potential equal to about −40 mV in Ca^{2+}-free solution). (b) Maximal depolarizations induced by 10^{-4} M ACh in Ca^{2+}-free solution are plotted as a function of the log of the external Na^+ concentration. The straight line represents the theoretical variation of E_{Na^+} for a tenfold variation in external Na^+ concentration (slope of 60 mV). Points with vertical lines represent mean and s.e.mean of 4 preparations (a) and 7 preparations (b).

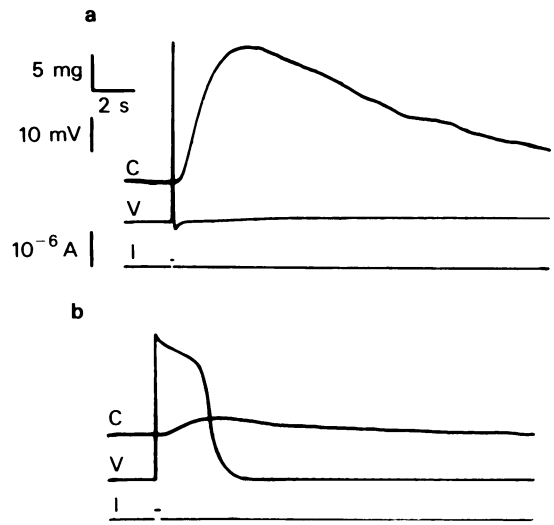

Figure 8 Simultaneous recordings of action potential and contraction using the double sucrose gap in reference solution (a) and after 5 min of perfusion in Ca^{2+}-free EGTA-containing solution (b). Long-lasting Na^+ action potential induces a contraction which represents 10 ± 3% ($n = 6$) of the maximal response obtained in reference solution (C = contraction, V = membrane potential, I = stimulating current).

could be responsible for the development of maintained contractions in Ca^{2+}-free solution: (i) Ca^{2+} ions could be mobilized (by a membrane depolarization) from an intracellular store able to resequester the same Ca^{2+} with very little loss from the cell; (ii) despite the loss of Ca^{2+} from the cell, this intracellular Ca^{2+} store is large enough to produce maintained contractions for prolonged periods of time without Ca^{2+} filling from the external medium; (iii) cytoplasmic Ca^{2+} concentration may be dependent both on continuous release of subeffective amounts of Ca^{2+} from intracellular stores, and removal of this released Ca^{2+} by efflux from the cell and by intracellular resequestration; contractions could be elicited either by inhibiting efflux and resequestration of the released Ca^{2+}, or by enhancing Ca^{2+} release from intracellular stores. Alternatively, a combination of the above mechanisms might be occurring. Our results are not sufficient to establish one of the above possibilities as a definitive mechanism for each of the substances used. However, vanadate has been described as a potent inhibitor of both Na^+-K^+-ATPase (Grover *et al.*, 1980) and Ca^{2+}-ATPases (Di Polo *et al.*, 1979; Varecka & Carafoli, 1982). These data suggest that a possible mechanism for the maintained contraction induced by vanadate could involve inhibition of both Ca^{2+} efflux and Ca^{2+} accumulation through ATP-dependent mechanisms. The E prostaglandins stimulate the release of Ca^{2+} from isolated sarcoplasmic reticulum (Carsten, 1973) and mitochondria (Malmström & Carafoli, 1975), and may also inhibit ATP-dependent Ca^{2+} binding (Carsten, 1974). Since the contractions evoked by vanadate and prostglandin E_2 are additive, it is likely that the contraction induced by prostaglandin E_2 is mainly produced by a release of Ca^{2+} from intracellular stores. Further support for the intracellular action of these substances comes from experiments showing that: (i) neither vanadate nor prostaglandin E_2 induced important depolarizations of the membrane; (ii) the vanadate-and prostaglandin-induced contractions were unaffected by Na^+ removal and Ca^{2+} antagonists; (iii) the contractions evoked by acetylcholine, vanadate and prostaglandin E_2 were additive suggesting different mechanisms of Ca^{2+} translocation.

In contrast, acetylcholine induced a maintained contraction in Ca^{2+}-free solutions which is associated with a membrane depolarization, as confirmed by the following observations upon acetylcholine-induced depolarization and contraction: (i) an inhibition in Na^+-free solution; (ii) a similar reduction in the presence of Mn^{2+} ions or D600. Triggered Na^+ action potentials (in the presence of atropine) also induced contractions of the same order of magnitude as those induced by acetylcholine depolarizations. This observation suggests that the membrane depolarizations evoked by the two stimuli could displace Ca^{2+} ions from the same intracellular store. As experimental points representing the decrease in acetylcholine-induced depolarization by a reduction in external Na^+ concentration were well fitted by the theoretical change of the Na^+ equilibrium potential, a link between depolarization and Na^+ influx can be proposed. The importance of the transmembrane Na^+ gradient on acetylcholine-induced depolarizations has been previously presented in smooth muscles (Bolton, 1972; Szurszewski & Bülbring, 1973). However, the position of the equilibrium potential for acetylcholine (close to zero potential) presumably reflects the balance between Na^+-inward and K^+-outward movements (Bolton & Clark, 1981).

In conclusion, the present investigation has shown that, in Ca^{2+}-free solution, maintained and repetitive contractions induced by different substances depended on several mechanisms, one of which was Ca^{2+}-independent (fluoride-induced contraction) and the other, which depended on Ca^{2+} displacements from an internal store. This latter mechanism would include both a pharmacomechanical coupling mechanism (vanadate-and prostaglandin-induced contractions) and an electromechanical coupling mechanism (acetylcholine-induced contraction).

This work was supported by grants from CNRS (ATP Neuromédiateurs) and INSERM (PRC Reproduction et Développement).

References

ASHOORI, F. & TOMITA, T. (1983). Mechanical reponse to noradrenaline in calcium-free solution in the rat vas deferens. *J. Physiol.*, **338**, 165–178.

BOLTON, T.B. (1972). The effects of varying the concentrations of ions in the external solution on the oscillations of the membrane potential (slow waves) produced by carbachol in longitudinal ileal muscle. *Pflügers Arch.*, **335**, 85–96.

BOLTON, T.B. (1979). Mechanisms of action of transmitters and other substances on smooth muscle. *Physiol. Rev.*, **59**, 606–718.

BOLTON, T.B. & CLARK, J.P. (1981). Effects of histamine, high potassium and carbachol on ^{42}K efflux from logitudinal muscle of guinea-pig intestine. *J. Physiol.*, **320**, 347–361.

BOLTON, T.B. & KITAMURA, K. (1983). Evidence that ionic

channels associated with the muscarinic receptor of smooth muscle may admit calcium. *Br. J. Pharmac.*, **78,** 405–416.

BRADING, A.F. & SNEDDON, P. (1980). Evidence for multiple sources of calcium for activation of the contractile mechanism of guinea-pig taenia coli on stimulation with carbachol. *Br. J. Pharmac.*, **70,** 229–240.

BRADING, A.F., BURNETT, M. & SNEDDON, P. (1980). The effect of sodium removal on the contractile responses of the guinea-pig taenia coli to carbachol *J. Physiol.*, **306,** 411–429.

BÜLBRING, E. & DEN HERTOG, A. (1980). The action of isoprenaline on the smooth muscle of the guinea-pig taenia coli. *J. Physiol.*, **304,** 277–296.

CARSTEN, M.E. (1973). Sarcoplasmic reticulum from pregnant bovine uterus. Prostaglandins and calcium. *Gynec. Invest.*, **4,** 95–105.

CARSTEN, M.E. (1974). Prostaglandin and oxytocin: their effects on uterine smooth muscle. *Prostaglandins.*, **5,** 33–39.

CASTEELS, R. & RAEYMAEKERS, L. (1979). The action of acetylcholine and catecholamines on an intracellular calcium store in the smooth muscle cells of the guinea-pig taenia coli. *J. Physiol.*, **294,** 51–68.

CASTEELS, R., RAEYMAEKERS, L., SUZUKI, H. & VAN ELDERE, J. (1981). Tension response and ^{45}Ca release in vascular smooth muscle incubated in Ca-free solution. *Pflügers Arch.*, **392,** 139–145.

DI POLO, R., ROJAS, H.R. & BEAUGE, L. (1979). Vanadate inhibits uncoupled Ca efflux but not Na-Ca exchange in squid axons. *Nature*, **281,** 228–229.

FAY, F.S., SHLEVIN, H.H., GRANGER, W.C. & TAYLOR, S.R. (1979). Aequorin luminescence during activation of single isolated smooth muscle cells. *Nature*, **280,** 506–508.

FERMUM, R., MEISEL, P. & KLINNER, U. (1977). Versuche zum Wirkungsmechanismus von Gefässpamolytika. 3. Wirkung von Nitroprussid-Natrium, Nitroglyzerin, Prenylamin und Verapamil an der Fluorid-induzierten Kontraktur isolierter Koronarterien. *Acta biol. med. germ.*, **36,** 245–255.

FLECKENSTEIN, A. (1977). Specific pharmacology of calcium in myocardium, cardiac pacemakers, and vascular smooth muscle. *A. Rev. Pharmac. Tox.*, **17,** 149–166.

GROSSET, A. & MIRONNEAU, J. (1977). An analysis of the actions of prostaglandin E_1 on membrane currents and contraction in uterine smooth muscle. *J. Physiol.*, **270,** 765–784.

GROVER, A.K., JONES, T.R. & DANIEL, E.E. (1980). Effect of vanadate on rat myometrium plasma membrane enzyme activities. *Can. J. Physiol. Pharmac.*, **58,** 1247–1250.

HAGIWARA, S. & NAKAJIMA, S. (1966). Differences in Na^+ and Ca^{2+} spikes as examined by application of tetrodotoxin, procaine and manganese ions. *J. gen. Physiol.*, **49,** 793–805.

HEASLIP, R.J. & RAHWAN, R.G. (1982). Evidence for the existence of two distinct pools of intracellular calcium in the rat aorta accessible for mobilization by norepinephrine. *J. Pharmac. exp. Ther.*, **221,** 7–13.

LALANNE, C., MIRONNEAU, C., MIRONNEAU, J. & SAVINEAU, J.P. (1984). Contractions of rat uterine smooth muscle induced by acetylcholine and angiotensin II in Ca^{2+}-free medium. *Br. J. Pharmac.* **81,** 317–326.

MALMSTRÖM, K. & CARAFOLI, E. (1975). Effects of prostaglandins on the interaction of Ca^{2+} with mitochondria. *Archs Biochem. Biophys.*, **171,** 418–423.

MIRONNEAU, J. (1973). Excitation-contraction coupling in voltage-clamped uterine smooth muscle. *J. Physiol.*, **233,** 127–141.

MIRONNEAU, J., MIRONNEAU, C., GROSSET, A., HAMON, G. & SAVINEAU, J.P. (1980). Action of angiotensin II on the electrical and mechanical activity of rat uterine smooth muscle. *Eur. J. Pharmac.*, **68,** 275–285.

MIRONNEAU, J., EUGENE, D. & MIRONNEAU, C. (1982). Sodium action potentials induced by calcium chelation in rat uterine smooth muscle. *Pflügers Arch.*, **395,** 232–238.

NEERING, I.R. & MORGAN, K.G. (1980). Use of aequorin to study excitation-contraction coupling in mammalian smooth muscle. *Nature*, **288,** 585–587.

POTREAU, D. & RAYMOND, G. (1983). Existence of a sodium-induced calcium release mechanism on frog skeletal muscle fibres. *J. Physiol.*, **333,** 463–480.

SAKAI, K., HIGUCHI, K., YAMAGUCHI, T. & UCHIDA, M. (1982). Oxytocin-induced Ca-free contractions of rat uterine smooth muscle: effects of preincubation with EGTA and drugs. *Gen. Pharmac.*, **13,** 393–400.

SZURSZEWSKI, J.H. & BÜLBRING, E. (1973). The stimulant action of acetylcholine and catecholamines on the uterus. *Phil. Trans. R. Soc. Lond. B.*, **265,** 149–156.

VARECKA, L. & CARAFOLI, E. (1982). Vanadate-induced movements of Ca^{2+} and K^+ in human red blood cells. *J. biol. Chem.*, **257,** 7414–7421.

VASSORT, G. & ROUGIER, O. (1972). Membrane potential and slow inward current dependence of frog cardiac mechanical activity. *Pflügers Arch.*, **331,** 191–203.

(Received January 24, 1984.
Revised February 25, 1984.)