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Isolation and contractile responses of single pregnant rat myometrial cells in short-term primary culture and the effects of pharmacological and electrical stimuli

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1 A modified method for enzymatically isolating myometrial cells from the pregnant rat has been developed and the mechanical properties of single cells in short-term primary culture have been studied in response to various stimuli.

2 The dissociation method produced a high proportion of fully relaxed cells and these cells shortened and subsequently relaxed completely in response to successive applications of acetylcholine, angiotensin II, high K^+ solution or depolarizing current.

3 In single cells, the contractions induced by acetylcholine and high K^+ solution were concentration-dependent. Maximal contractions were obtained with 135.6 mM K^+ and 5×10^{-4} M acetylcholine.

4 In single myometrial cells, the time course of contractions induced by acetylcholine, high K^+ solution or depolarizing current was similar, suggesting that the rate of shortening was determined by limits of the contractile mechanism.

5 Scanning electron microscopy revealed a smooth surface to the relaxed cells which contrasted with the numerous evaginations present on fully contracted cells.

6 These results demonstrate the retention of structural integrity, acetylcholine and angiotensin II receptors, and potential-dependent Ca channels in myometrial single cells in short-term primary culture. Cells produced by this technique may provide a useful model for detailed electrophysiological studies.

Introduction

Single adult myocytes offer several advantages over multicellular smooth muscle preparations for the study of electrophysiological properties and excitation-contraction coupling mechanisms. (1) Control of membrane potential in voltage clamp experiments is better achieved in a single cell than in a multicellular preparation with electrically coupled cells (Walsh & Singer, 1981). (2) Interpretation of experimental results in multicellular strips is complicated by the existence of intercellular clefts, where the ionic composition may change due to accumulation or depletion phenomena (Bolton *et al.*, 1981). (3) The presence of terminal and preterminal neurones throughout the tissue may contribute to the release of transmitters during extracellular current stimulation or ion replacement (Bengtsson, 1977). (4) Application of the patch-clamp technique requires an identifiable cell with a particularly clean surface (Hamill *et al.*, 1981). Despite these benefits of the single cell model, there is a need for caution because it is possible that disaggregation procedures, such as treatment with proteolytic enzymes, could affect membrane ionic channels, membrane receptors and mechanical properties.

The aim of the present study was to develop a method for the isolation and short-term culture of cells from the myometrium of the pregnant rat. The mechanical responses of single cells to applications of acetylcholine, angiotensin II, high K^+ solutions, or depolarizing current were measured. The kinetics and concentration-dependence of the responses of isolated myometrial cells to these agents were determined and compared with those found for multicellular uterine smooth muscle strips. Structural changes occurring during contraction of single cells were also observed.

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Methods

Preparation of dispersed cells from rat myometrium

Uterine horns from pregnant rat myometrium at the end of gestation (18–19 days) were dissected in Hank's solution containing (mM): NaCl 136.9, KCl 5.36, $CaCl_2$ 1.26, KH_2PO_4 0.44, Na_2HPO_4 0.26, $NaHCO_3$ 4.5, $MgCl_2$ 0.4, $MgSO_4$ 0.4, glucose 5.5 at pH 7.4. Penicillin (20 u ml^{-1}, Gibco, Paisley, U.K.) and streptomycin (20 $\mu g\,ml^{-1}$, Gibco) were added. Strips of myometrium were excised from the longitudinal muscle layer at room temperature and cut into pieces 2–3 mm long and 0.5 mm wide. The muscle pieces were transferred to a 25 ml siliconized glass flask containing 3 ml of Hank's solution supplemented with 0.1% bovine serum albumin (Fraction V, Sigma Chemical, St Louis, U.S.A.) and rinsed by shaking them for 3 successive 5 min periods in a 35°C water bath at 40 cycles min^{-1}. The muscle segments were incubated in the dissociation medium for 4 successive 20 min periods and shaken continuously. The dissociation medium was Hank's solution supplemented with 0.1% collagenase (CLS 1, 131 u mg^{-1}, Cooper Biochemical, Paris, France), 0.01% soybean trypsin inhibitor (Cooper), and 0.1% bovine serum albumin (Sigma). The Ca concentration was reduced to 30 μM and Mg ions were removed during the cell dissociation. Between each dissociation period, the incubation solution was pipetted off, and the fragments were transferred to an enzyme-free solution and agitated by aspirating them with a wide-bored siliconized Pasteur pipette. The enzyme-free solution was then removed, and the fragments were re-incubated in fresh collagenase-containing solutions. The first two incubation solutions were discarded since these frequently contained debris or damaged cells. Subsequent cell suspensions were rinsed with 50 ml of M 199 medium (Flow Laboratories, Puteaux, France) containing 5% foetal bovine serum (Gibco), placed in centrifuge tubes, and centrifuged at 20 *g* for 15 min at room temperature. Finally, the cells were resuspended in M 199 medium (containing 10% foetal bovine serum, 20 u ml^{-1} penicillin, and 20 $\mu g\,ml^{-1}$ streptomycin) at a density of about 10^4 cells ml^{-1}. At each step of the dissociation protocol, cell viability was controlled using trypan blue dye. At least 60–80% of the cells excluded the trypan blue in the last two incubation solutions. The cells were counted in a haemocytometer, one uterine horn yielded about 10^6 cells from the longitudinal muscle layer. The entire isolation procedure was carried out under sterile conditions in a laminar flow cabinet.

Myometrial cells in primary culture

A 2 ml suspension was transferred into 35 mm tissue culture dishes by means of a Pasteur pipette and plated on collagen-coated glass coverslips (Bornstein, 1958). The cells were kept in an incubator gassed with 95% O_2 plus 5% CO_2 at 37°C. They spontaneously adhered to the collagen gel within 1–3 h of culture, thus immobilizing themselves sufficiently to allow experimentation, but still showing clearly observable movements. The cells were maintained in primary culture for 36 h in M 199 medium supplemented with 2 mM glutamine (Gibco). They were transferred to a saline solution (reference solution) just before mechanical or electrophysiological measurements.

Measurement of contractile response

Contractile responses were expressed as the decrease in length or planar area of a single myometrial cell evoked by a stimulus. Photomicrographs of the cell were taken at × 400 magnification, and for kinetic measurements at a rate of 2 frames s^{-1}. The enlarged projection of the negative was traced on cardboard, and a cut-out of the tracing was weighed. The planar projection of the cell was then estimated against the weight of a calibrated surface (Singer & Walsh, 1980). Both length and planar area were determined for each cell used.

Measurement of membrane potential

Membrane potentials were recorded with conventional microelectrodes filled with 3 M KCl. The potential difference between the intracellular microelectrode and a bath reference electrode was measured with a Dagan 8100 system (Dagan, Minneapolis, U.S.A.) using the switched mode (switch frequency between 3 and 15 kHz). Currents were injected into the cell through the recording microelectrode. Microelectrodes with resistances ranging from 30 to 50 $M\Omega$ were pulled from borosilicate capillary tubes (Clark Electromedical Instruments, Reading, U.K.), and insulated with nail polish. To adjust the leakage current flowing through the recording circuit, a 300 $M\Omega$ resistor (1%, Victoreen), in parallel with a 100 pF capacitor, was connected between the input stage and the ground. The recorded potential was then adjusted to less than ± 1 mV, indicating leakage current of less than ± 4 pA (Mollard *et al.*, 1985, 1986).

Electron microscopy

Glass coverslips with the attached cells were immersed in 2% glutaraldehyde (in a 1 mM Mn-containing cacodylate buffer), post-fixed in osmium tetraoxide, dehydrated in alcohol and then transferred into isoamylacetate. The coverslips were dried using the critical-point method and coated with a layer of Au. The cells were observed with a scanning electron

microscope (JSM-35 J.E.O.L., Japan).

Application of substances

Test solutions was applied to the cells by pressure ejection from micropipettes of tip diameter 3–5 μm. The tip of such application pipettes were positioned at about 100 μm from the cell surface and pneumatic pressures of 80–100 mbar were applied (Corson & Fein, 1983). Such experimental conditions were necessary to avoid non-specific contractions in response to the ejection flow. Solutions were prepared by adding agonists to the reference solution and pressing solutions through filters of 0.22 μm pore size before use. In these experiments we have stated the concentrations of mediators in the pipette, so that the mediator concentration at the cell surface was necessarily lower. In several experiments, continuous perfusion of the experimental chamber with agonist-containing solutions or high K^+ solutions was obtained by means of a constant-flow pump (0.75 ml min^{-1}).

Solutions

Physiological solutions used during the experiments had the following compositions: (a) reference solution

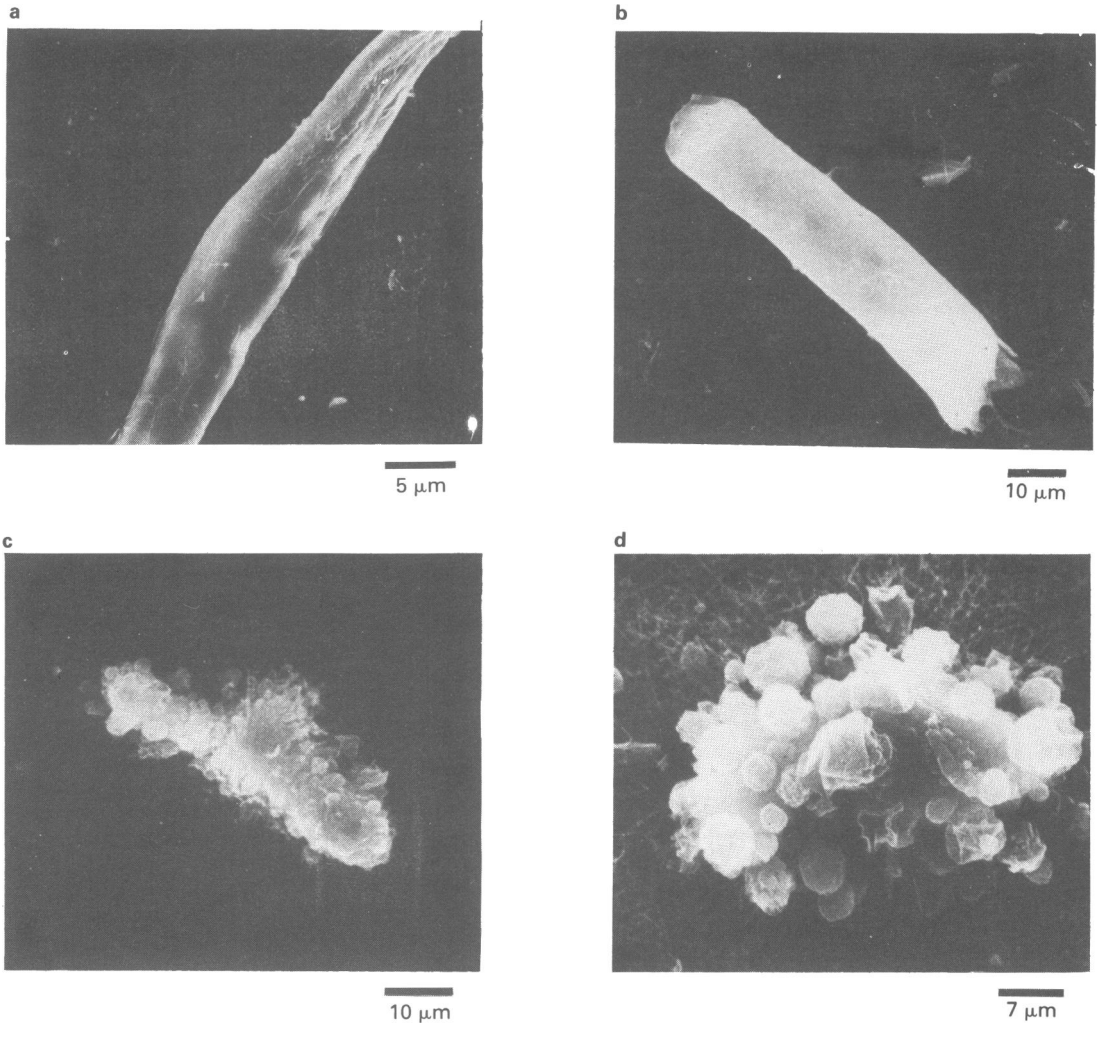

Figure 1 Scanning electron micrographs of isolated smooth muscle cells from pregnant rat myometrium. (a and b) Single cells fixed in relaxed state just after the enzymatic dissociation (a) and after 5 h of culture (b). (c and d) Single cells fixed after shortening induced by the application of 60.6 mM external K^+ solution; (c) shows cylindrical cells and (d) round cells.

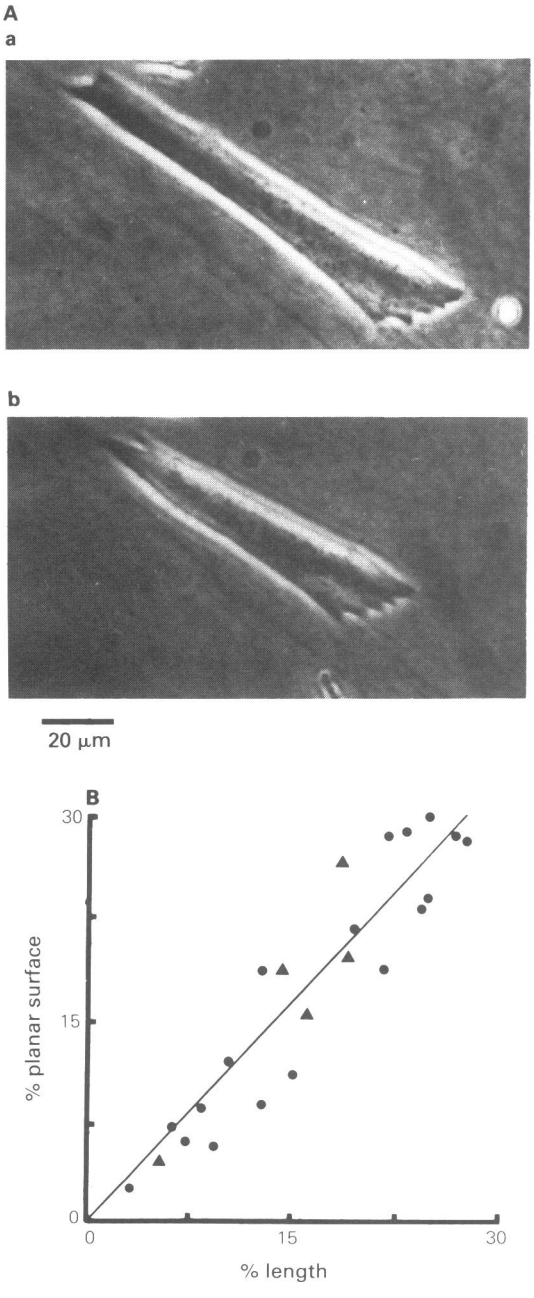

Figure 2 (A) Phase contrast light micrographs showing the contraction of a single myometrial cell, in culture for 12 h, to the application of 5×10^{-5} M acetylcholine. (a) Cell before contraction, (b) after addition of acetylcholine. (B) Relationship between the decrease in planar surface and the decrease in length of 23 single cells which were exposed to different concentrations of acetylcholine (●) and angiotensin II (▲). The straight line was calculated by linear regression and has a slope of 1.092.

(mM): NaCl 130, KCl 5.6, $CaCl_2$ 2.1, $MgCl_2$ 0.24, and glucose, 11; this solution was buffered with Tris-HCl (8.3 mM) at pH 7.4 (b) High K^+ solution was obtained by substituting NaCl in the reference solution with KCl in equimolar amounts. (c) In Ca^{2+}-free solution, $CaCl_2$ was omitted and 0.1 mM EGTA was added; (d) D600 was used as an inhibitor of the calcium inward current (Fleckenstein, 1977). The different solutions were maintained at 35°C by means of a heating bath. Acetylcholine chloride and atropine free base were obtained from Sigma. Angiotensin II (Hypertensin) was obtained from Ciba (Paris, France) and D600 (methoxyverapamil) from Knoll AG (Ludwigshafen, West Germany).

Results

Morphological profile of isolated myometrial cells

Freshly dispersed cells varied widely in length. Most of them were fully relaxed and appeared as long, thin cylinders, ranging from 80 to 300 μm in length and from 6 to 12 μm in width. Figure 1a shows a typical scanning electron micrograph of a freshly isolated cell with a smooth contour to the cell surface. When the cells adhered to the collagen gel in primary culture, they modified their morphology. Most single cells remained cylindrical (Figure 1b) with a diameter of 10–20 μm and longitudinal dimension of 60–90 μm. Other cells were round with diameters between 25 and 50 μm. Figure 1c,d shows typical micrographs of cylindrical (c) and round (d) cells fixed at the state of maximal contraction induced by a 60.6 mM K^+ solution. The cell surface of both cells appeared to be covered by vesicles and bulbous evaginations. It is worthy of note that removal of the K^+ rich medium allowed the smooth appearance of relaxed cells to reappear.

Contraction of isolated myometrial cells

Cell contractions were studied between 3 and 36 h of culture. After 36 h of culture, the contraction amplitude was strongly decreased and, finally, mechanical activity disappeared after 3–4 days. Figure 2A shows a typical contraction obtained after ejection of 5×10^{-5} M acetylcholine. The single cell appeared to be shortened in all areas. Film records were analysed to determine if planar surface changes accompanied the contractile responses of the isolated muscle cells. Area and length measurements were made during contractions which were induced by acetylcholine or angiotensin II at various concentrations. As shown in Figure 2B, a good correlation was seen between the decrease in planar surface and the

decrease in cell length for 23 different cells. The reduction in either cell surface or cell length was used to estimate the contraction of isolated cells.

Effects of acetylcholine

The contractile response to acetylcholine (5×10^{-6} M for 5 s) was prompt, rising to a peak within 3–4 s (Figure 3a). The duration of the contraction ranged between 10 and 15 s ($n = 8$). Similar acetylcholine-induced contractions could be recorded repetitively on the same cell in response to 10 successive applications of acetylcholine. Most of the single myometrial cells examined contracted in the presence of acetylcholine; 30–40% of the cells were unresponsive to acetylcholine although their morphology was quite similar to that of active cells.

The size of the contractile response of isolated cells to acetylcholine was concentration-dependent. The decrease in length or surface area of 11 different cells was plotted against acetylcholine concentration. As shown in Figure 3b, the maximal contraction induced by acetylcholine was 27 ± 4%. The concentrations producing half and maximal responses were 2×10^{-6} and 5×10^{-4} M, respectively. Addition of 5×10^{-8} M atropine specifically inhibited the response to acetylcholine (5×10^{-6} M) within 3–5 min. Complete recovery was observed on the same cell after removal of atropine within 10–12 min.

Effects of high K^+ solutions

When isolated cells were superfused with 60.6 mM K^+ solutions, cyclic contractions were recorded which were characterized by a duration of 5–7 s and a time-to-peak of 2–3 s (Figure 4). Spontaneous contractile responses were observed as long as the cells were superfused in 60.6 mM K^+ solution. D600 (1 μM) abolished the K^+-induced contractions within 2–3 min. The reversibility was very slow since normal contractions were obtained after about 20–30 min of perfusion in D600-free solution. The contractile response to high K^+ solution was dependent on the K^+ concentration. With 60.6 mM external K^+, the maximal amplitude of the contraction expressed as a decrease in cell length or surface area was 12 ± 4% ($n = 4$). With a higher K^+ concentration (135.6 mM K^+), a maintained contractile response was recorded within 5–7 min. The maximal amplitude of the maintained contraction was higher than that of cyclic responses obtained in 60.6 mM K^+ with a mean value of 25 ± 9% ($n = 3$).

Removal of external Ca^{2+} in the presence of EGTA (0.1 mM) produced a complete suppression of the external K^+-induced contractions within 3 min. Complete recovery was obtained after 10–12 min of perfusion in Ca^{2+}-containing solution.

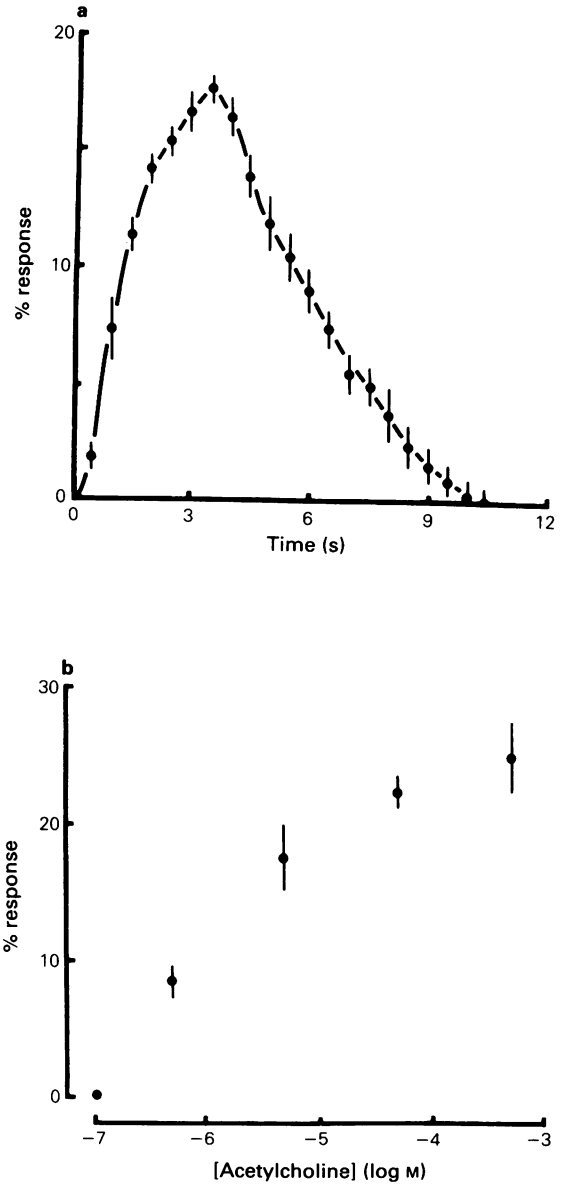

Figure 3 (a) Time course of the contraction of isolated myometrial cells in response to the application of 5×10^{-6} M acetylcholine for 5 s. (b) Contractile responses induced by different doses of acetylcholine on several single myometrial cells. Acetylcholine was applied for 5 s. Points represent the mean with vertical lines indicating s.e.mean of (a) 8 and (b) 11 single cells. The contractile response was expressed as a percentage decrease in cell length or planar surface.

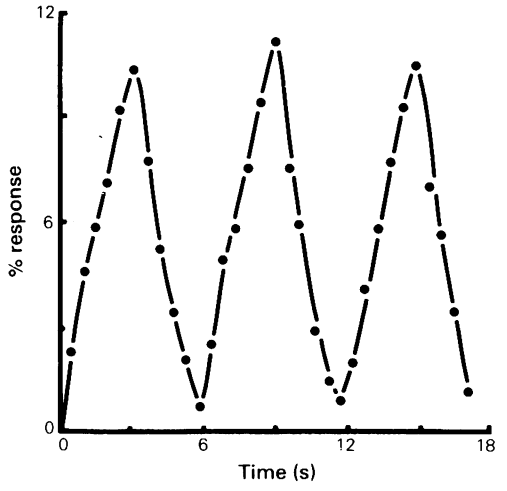

Figure 4 Typical cyclic contractions of a single myometrial cell during superfusion with a 60.6 mM external K^+ solution. These contractions were obtained as long as the cell was superfused with high K^+ solution. Similar results were obtained in 4 single cells. The contractile response was expressed as a percentage of decrease in planar surface or cell length.

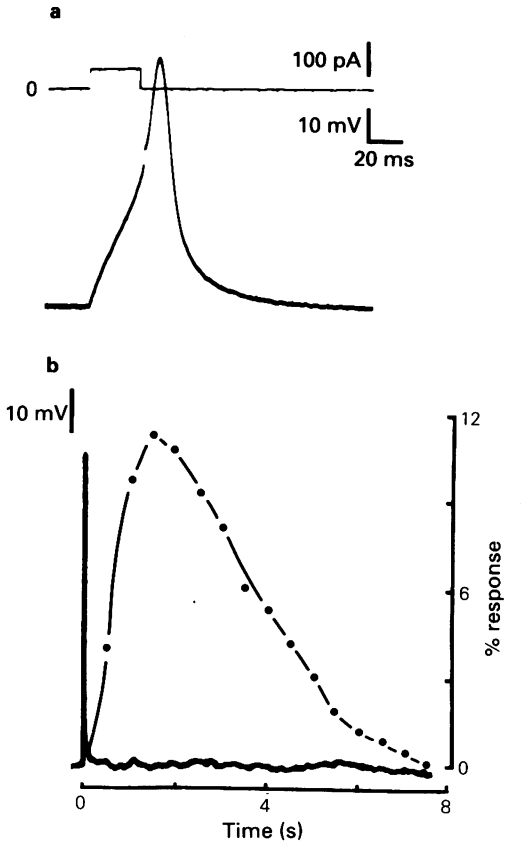

Figure 5 (a) Action potential, recorded with an intracellular microelectrode in a single myometrial cell, in response to the injection of a depolarizing current pulse of low amplitude and short duration. (b) Simultaneous recordings of action potential and contraction in a single myometrial cell. The contractile response (●) was expressed as a percentage of decrease in planar surface or cell length.

Effects of electrical stimulation

Depolarizing current pulses of low amplitude (40–60 pA) and short duration (15–30 ms) triggered regenerative action potentials, the amplitude of which depended on the external Ca^{2+} concentration (Mollard *et al.*, 1985, 1986). A typical action potential triggered from a resting potential of − 60 mV is shown in Figure 5a. The amplitude and the duration at half repolarization were 71 mV and 40 ms, respectively. Figure 5b shows a contractile response induced by a single action potential. The peak contraction was reached in about 2 s and the relaxation phase was clearly slower than the activation phase. The duration of contractions ranged between 7 to 10 s ($n = 5$).

Discussion

This study describes a method for the isolation of single smooth muscle cells from pregnant rat myometrium. The myometrial cells in primary culture shortened and subsequently relaxed completely in response to various agonists, high K^+ solutions and action potentials induced by depolarizing currents. The contractile responses of single cells were expressed as a reduction in cell length or planar surface, as previously used for other smooth muscle cells (Bagby *et al.*, 1971; Fay & Delise, 1973; Momose & Gomi, 1978; Bitar *et al.*, 1979; Bitar & Makhlouf, 1982; Obara, 1984). The contraction of single myometrial cells appears to occur via normal physiological mechanisms, as judged by its complete relaxation on washout of spasmogens. The calcium requirement of K^+-induced contractions and their inhibition by D600 adds weight to the argument.

Scanning electron microscopic observations illustrated the ultrastructural changes associated with contraction. The relaxed smooth muscle cell was generally long and was characterized by a smooth cell surface. In contrast, the fully contracted cell exhibited

evaginations of the plasma membrane. The formation of cellular evaginations may represent the application of inwardly directed forces at several plasma membrane points. Thus, these regions may have been pulled inward while the remaining plasma membrane was forced outward to form blebs (Fay & Delise, 1973). When the cells were superfused in relaxant solutions, the formation of blebs was rapidly reversed suggesting that these blebs were correlated to the active process of contraction.

The shortening of isolated myometrial cells induced by acetylcholine and high K^+ solution was concentration-dependent. In single cells, the maximal contractile response was obtained with 135.6 mM external K^+ (present study) but with 60.6 mM external K^+ in intact strips (Gabella, 1978; Mironneau *et al.*, 1980). In intact strips, it has been suggested that the release of intramural noradrenaline by high K^+ solution may explain the decrease in contraction observed in 135.6 mM external K^+ (Bengtsson, 1977). The sensitivity of single cells to acetylcholine appeared to be lower than that obtained in intact strips. In single cells, the acetylcholine concentrations producing half-maximal and maximal contractions were 2×10^{-5} and 5×10^{-4} M, respectively. In intact myometrial strips, the acetylcholine concentrations producing half-maximal and maximal contractions were 2×10^{-6} and 10^{-5} M, respectively (Mironneau, unpublished data). This could be explained by the fact that acetylcholine increased the frequency of action potential discharge in intact tissue (Marshall, 1962; Bolton, 1979), producing a summation of elementary contractions to a fused tetanus (Mironneau, 1973). These results differ from the data of Fay & Singer (1977) on isolated cells of the stomach and Obara (1984) on taenia caeci smooth muscle cells which show a greater sensitivity of the isolated cells to acetylcholine than that of intact tissues. The acetylcholine-induced contractions were reduced by atropine, at low concentrations, in both intact myometrium and single isolated myometrial cells.

Our results reveal a similar time course of contraction in isolated cells to various stimuli, such as acetylcholine, high K^+ solution and depolarizing current. The time-to-peak of contraction in single cells ranged between 1.5 and 3 s. In intact strips, the time-to-peak of contractions was greater, ranging from 3.5 to 12 s (Mironneau, 1973; Kato *et al.*, 1982). As discussed by Fay & Singer (1977), it is clear that the rate of shortening of myometrial cells is determined by limits of the contractile mechanism and not by the rate of agonist-receptor interactions. From these considerations, it is likely that the large time-to-peak for contraction of intact strips may reflect diffusion limitations. After a maximal contraction, the single myometrial cells completely relaxed and successive contraction-relaxation cycles could be obtained without noticeable change in the contraction time course. Such complete contraction-relaxation cycles are not generally observed with single smooth muscle cells (Van Dijk & Laird, 1984; De Feo & Morgan, 1985), except in rabbit aorta cells (Ives *et al.*, 1978).

In conclusion, these data demonstrate the retention of structural integrity, acetylcholine and angiotensin II-receptors, and potential-dependent calcium channels in primary cultured myometrial myocytes. Such cells may prove useful for detailed studies of ionic membrane channels on smooth muscle.

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