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PREPARATION OF CULTURED SMOOTH MUSCLE CELLS FROM HUMAN MYOMETRIUM FOR X-RAY MICROANALYSIS

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Abstract

Methodological aspects of the use of X-ray microanalysis in physiological and pharmacological experiments on cultured myometrial cells were investigated. Cultured human myometrial cells were grown from biopsies after detaching the fibroblasts. Of the cultured cells, 95-98% showed desmin-like immunoreactivity. Transmission electron microscopy showed that subcultured cells were different from myometrial cells *in situ*. The effects of washing the cells to remove external salt-rich medium were investigated. All solutions removed the external medium, resulting in lower concentrations of Na and Cl. In the cells washed with 0.3 M mannitol, most of the elemental concentrations were significantly lower than in their unwashed counterparts and those washed in the other solutions. In cells washed in either 0.15 M ammonium acetate or distilled water, no significant differences in P and K compared with their unwashed counterparts were found. There were also no significant differences between cells washed in ammonium acetate and in distilled water. In subsequent experiments ammonium acetate was used. Incubation of cells in standard Ringer's solution resulted in an increase in Na and Cl, and a decrease in K, concomitantly with an increase in Ca. Although Ringer's solution *per se* can elicit changes in diffusible elements in the cells, physiological and pharmacological effects of oxytocin could still be detected in Ringer's solution. However, effects of oxytocin were different when the experiment was done in culture medium, instead of in Ringer's solution.

Key Words: X-ray microanalysis, diffusible elements, ion transport, smooth muscle, myometrium, cell culture, oxytocin.

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Introduction

The mechanism controlling myometrial activity in women is poorly understood. It is likely that myometrial contraction is preceded by electrical activity followed by an increase in intracellular Ca^{2+} (Carsten and Miller, 1987). The innervation of myometrium decreases throughout pregnancy (Cole and Garfield, 1989), therefore hormonal regulation could play a major role in labor. In the accepted theory on hormonal regulation, based in part on animal experiments, oxytocin stimulates uterine contraction (Wray, 1993), whereas progesterone inhibits contraction (Putnam *et al.*, 1991). In many animals, the progesterone concentration in maternal blood decreases at term, presumably allowing the onset of labor. However, this does not seem to be the case in monkeys, sheep, guinea pigs and women. In women, the progesterone concentration in maternal blood does not decrease prior to labor (Löfgren and Bäckström, 1990) and progesterone may enhance spontaneous contraction in myometrium *in vitro* (Fu *et al.*, 1993). Therefore, clinically relevant studies on myometrial contraction should preferably be carried out on human tissue.

Physiological and pharmacological studies on human myometrium evidently can only be carried out *in vitro*; for instance, on isolated tissue or in cell cultures. Isolated myometrium has been used for X-ray microanalysis (Hongpaisan *et al.*, 1995; Rezapour *et al.*, 1996). The advantage of cell cultures, on the other hand, is that a small amount of isolated myometrium can be multiplied and used for many experiments.

Rinsing the cultured cells with washing solutions before freezing the cells to remove the external salt rich solution that otherwise would disturb X-ray microanalysis, can induce artefacts. Washing cultured cells with distilled water has been shown to result in cell swelling; however, the diffusible elements Na, Cl and K do not cross the cell membrane (Borgmann *et al.*, 1994; Warley *et al.*, 1994). Washing with isotonic ammonium acetate does not result in volume changes (Borgmann *et al.*, 1994) but causes a loss of diffusible elements in some types of cultured cells (Borgmann *et al.*, 1994;

James-Kracke *et al.*, 1980). During freeze-drying, water and volatile ammonium acetate are removed by sublimation. Non-volatile sugars, e.g., mannitol or sucrose, are not removed by freeze-drying and therefore will interfere with imaging in the electron microscope and with X-ray microanalysis (Warley *et al.*, 1994).

In the present study, we have developed a system for culturing smooth muscle cells from human myometrium for an X-ray microanalytical study on elemental composition. An appropriate washing method for the cells and the effects of buffers during pharmacological stimulation were investigated.

Materials and Methods

Uterine biopsies were taken from term pregnant women undergoing elective cesarean section at the Department of Obstetrics and Gynecology, Uppsala University Hospital. Informed consent was obtained from all patients, and the study was approved by the Ethics Committee of Uppsala University. The biopsy was collected in cold sterile calcium-magnesium-free Hanks' buffered salt solution (Gibco BRL, Life Technologies, Täby, Sweden). After the decidua and serosa were removed, the myometrium was washed 4-5 times in a washing solution (calcium-magnesium-free Hanks' buffered salt solution containing 25 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), 100 U/ml penicillin G (Sigma, St Louis, MO) and 100 μg/ml streptomycin (Sigma). Dissected myometrium was placed in the washing medium supplemented with 1 mg/ml collagenase type IV (Sigma) and 3.3 U/ml elastase type II-A (Sigma) at 37°C in an incubator supplied with 5% CO_2/air for 30 minutes. Under the dissecting microscope, the remaining connective tissue was teased away from the myometrium with sterile needles. The myometrium was then finely chopped into 1 mm^3 pieces and returned to the incubator for 150 minutes in the enzyme solution, described above, but with an elastase content of 15 U/ml to aid complete dissociation. After centrifugation at 35 g at room temperature for 10 minutes and washing in culture medium which consisted of Dulbecco's Modified Eagle Medium (Gibco) supplemented with 25 mM HEPES, 10% fetal calf serum (Gibco), 100 U/ml penicillin G, 100 μg/ml streptomycin, 50 μg/ml gentamicin (Sigma) and 1.5 μg/ml amphotericin B (Sigma), isolated myometrial cells were resuspended and returned to culture in the incubator.

Fibroblasts can attach to the substrate relatively quickly (about 1 hour) (Polinger, 1970). After isolation, the cells were therefore left in a Petri dish in the incubator which allowed fibroblasts and macrophages to attach to the substrate. One hour later, the supernatant with non-attached cells was transferred to culture in a new Petri dish. After two days, the culture medium was replaced, and then changed every 2-3 days. Before confluence, the cultured cells were treated with low concentrations of trypsin (0.02%; Sigma) in the incubator at 37°C for 1-3 minutes. This selective detachment for fibroblasts was repeated 3-4 days later. In some cases, clones of epithelioid cells observed among cultured cells were removed with a sharp knife or detached with 1% dispase for 10-15 minutes. When confluence was reached, cultured cells were passaged by treatment with 0.05% trypsin using standard cell culture techniques.

In this study, subcultured cells at the second passage were used. The monolayer cell culture was incubated in a serum-free culture medium for one day before the experiment, allowing the cells to differentiate.

Cultured cells were seeded out on glass coverslips sterilized under ultraviolet light before use. The cells were allowed to attach and spread in the culturing chamber. After initial rinses {3 times in 440 mOsm phosphate buffered saline (PBS) at pH 7.4 to remove the culture medium}, the cultures were fixed in a cold mixture (1:1) of methanol and acetone (10 minutes), and air dried (30 minutes). The cultures were washed in PBS (3 x 5 minutes), kept in PBS + 10% normal swine serum + 0.1% triton X-100 (30 minutes) at room temperature (RT), and incubated with a desmin antibody (1:100; D-8281; Sigma) in PBS + 10% normal swine serum + 0.1% triton X-100 + 0.1% NaN_3 (overnight, RT). After rinses in PBS + 0.1 triton X-100 (3 x 10 minutes), incubation with tetramethyl rhodamine isothiocyanate-conjugated swine anti-rabbit antibodies (1:20; R-156; Sigma) in PBS was performed (1 hour, RT). The cell cultures were rinsed in PBS (3 x 10 minutes) and mounted with fluoromount-G (Southern Biotechnology Association, Birmingham, AL). As controls, the primary or secondary antibodies were omitted; in this case no immunolabeling was found.

For transmission electron microscopy, cell cultures grown in a Petri dish were fixed with glutaraldehyde (2.5%) in 0.1 M cacodylate buffer pH 7.2 for 30 minutes and washed in 0.1 M cacodylate buffer. Then tissue was postfixed in osmium tetroxide (1%) for 30 minutes, dehydrated in an ethanol series, and infiltrated in absolute ethanol containing increasing concentrations of Agar 100 Resin (Agar Scientific, Stansted, UK). The resin was polymerized in the Petri dish for two days at 60°C. Sections were cut, contrasted with uranyl acetate and lead citrate, and examined in a Hitachi (Tokyo, Japan) H-7100 transmission electron microscope (TEM).

Cultured cells were seeded out on 75 mesh titanium grids (Agar Scientific). The grids had been covered with a Formvar (Merck, Darmstadt, Germany) film coated with a thin carbon layer. The grids were sterilized under ultraviolet light before use. The cells were

allowed to attach and spread in the culturing chamber. Initially, a confluent cell culture grown on the grids covered with Formvar film was washed by dipping the grids into 0.3 M mannitol, 0.15 M ammonium acetate (adjusted to pH 7.4 with NaOH) or distilled water (all solutions at 4°C), and agitating the grids for 10 seconds. After washing, the grids were cryofixed (see below). To compare the effect of the washing solution, unwashed grids were measured. In later experiments, ammonium acetate was employed as a washing solution.

The cell-covered grids were incubated at RT for 2 and 5 minutes in standard Ringer's solution, containing 140 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 5 mM HEPES, 1 mM $MgCl_2$ supplemented with 5 mM D-glucose at pH 7.4, which had been aerated with 95% O_2 and CO_2 for 30 minutes. The cell-covered grids were also stimulated with oxytocin (Syntocinon; Sandoz, Basel, Switzerland) either in Ringer's solution supplemented with 0.02% bovine serum albumin at room temperature or in the serum-free culture medium at 37°C in a humidified atmosphere containing 5% CO_2 in air for 5 minutes. As controls, cultured cells incubated for 5 minutes in those solutions without oxytocin were used.

After washing, the grids were blotted on filter paper to drain excess fluid from the surface and frozen in liquid propane cooled by liquid nitrogen. Frozen cell-covered grids were freeze-dried at -80°C in vacuum (10^{-5} torr) overnight. The freeze-dried cell-covered grids were coated with a conductive carbon layer before analysis. X-ray microanalysis was performed at 100 kV in the scanning transmission electron microscopic mode of a Hitachi 7100 with an Oxford Instruments (Oxford, UK) ISIS energy-dispersive X-ray microanalysis system. Spectra were acquired for 100 seconds (live time). The cells were analyzed in the spot mode, with the electron beam placed on the central part of the cultured cells. Due to scattering of beam electrons in the specimen, the spatial resolution of analysis can be estimated to be 1-2 μm. Quantitative analysis was carried out based on the peak-to-continuum ratio after correction for extraneous background (Roomans, 1988) and by comparing the spectra from the cells with those from a standard. Only one spectrum was acquired from each cell. For the study of washing effects, statistics with confidence (Gardner and Altman, 1989) were used. For other experiments, Student's t-test was used to evaluate the statistical significance of differences between means.

Results

Approximately 95-98% of cultured cells from human myometrium (tissue from 4 women; 200 randomly selected cells were counted from each biopsy) showed desmin-like immunoreactivity (Fig. 1). Transmission

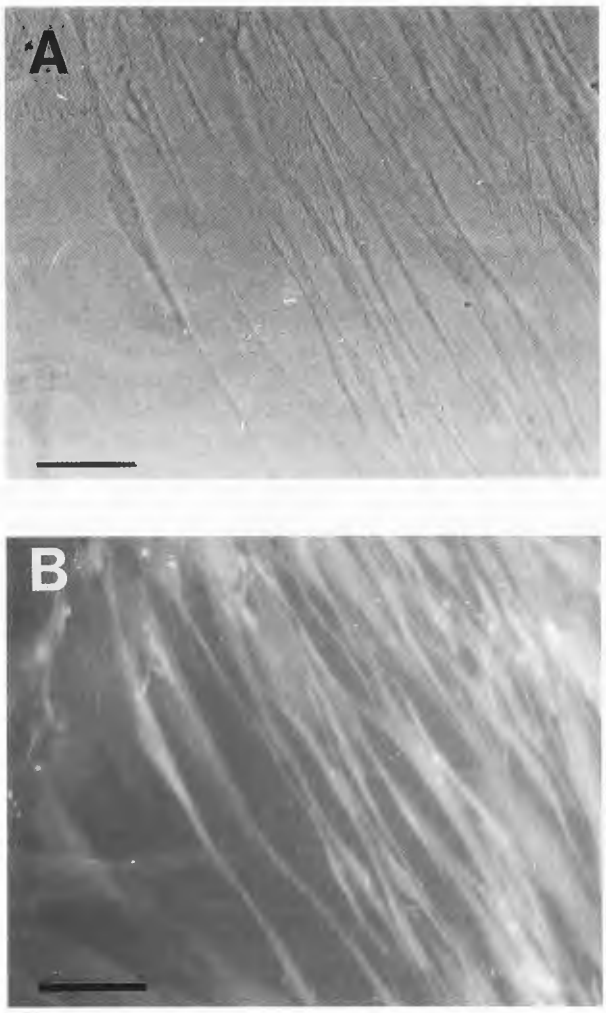

Figure 1. Micrograph of cultured myometrial cells. **(A)** light microscopy; **(B)** fluorescence microscopy of the same area of cells as (A) showing fluorescent desmin-like immunoreactivity. Bar = 15 μm.

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electron microscopy of cultured cells fixed by conventional methods and embedded in resin showed that the ultrastructural appearance of subcultured cells was different from that of myometrial cells *in situ*. The cultured cells (Fig. 2) were elongated and flattened with a central oval nucleus containing several nucleoli. They contained thin filament bundles interspersed with large areas containing free ribosomes and polysomes, dilated rough endoplasmic reticulum, mitochondria, and prominent Golgi complexes. Large numbers of dense bodies were found throughout the bundles of thin filaments. Plasmalemmal vesicles were occasionally observed. In contrast to the cultured myometrial cells, approximately 80 to 90% of the volume of myometrial cells *in situ* is occupied by myofilaments, intermediate filaments, and dense bodies (Broderick and Broderick, 1990).

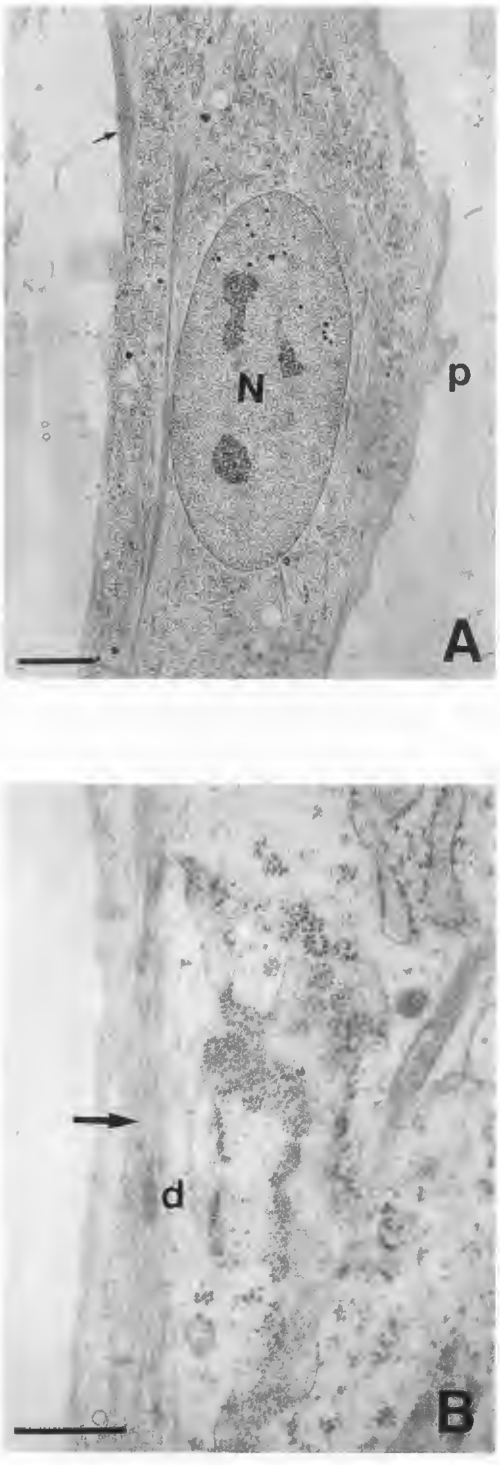

Figure 2. Transmission electron micrographs of cultured myometrial cells fixed with aldehyde and embedded in resin. N: nucleus, d: dense body, p: finger-like projection. The arrows indicate bundles of contractile filaments. (A) Bar = 5 μm; (B) higher magnification from the same cell as in (A), Bar = 500 nm.

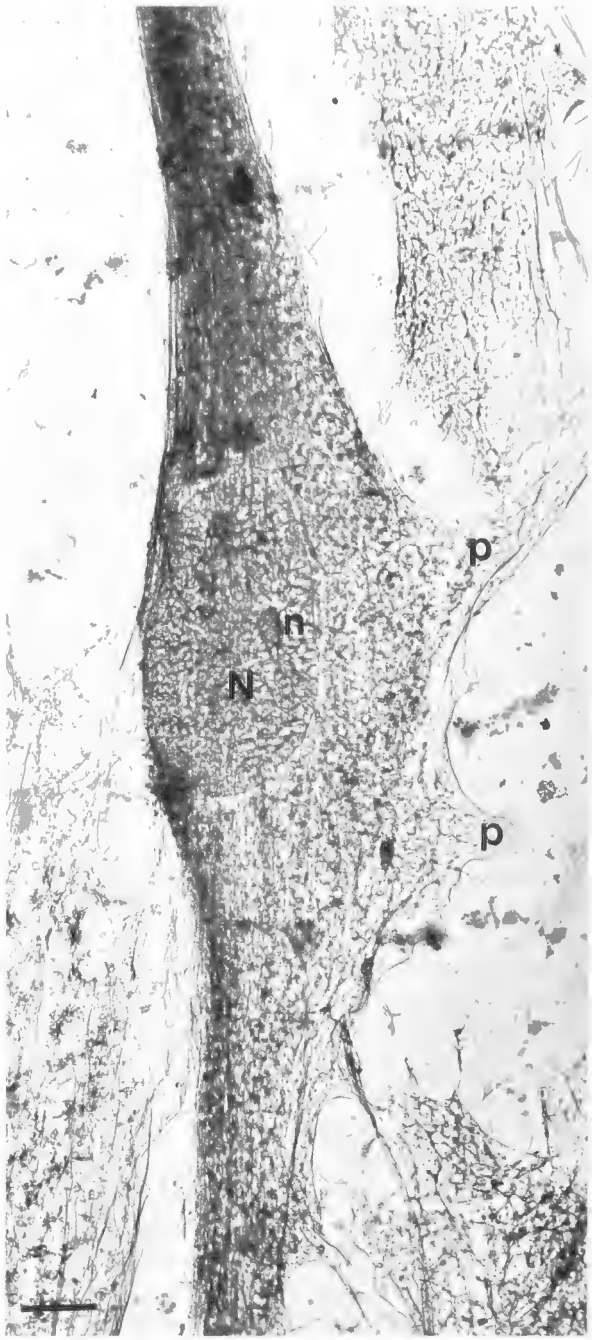

Figure 3. Transmission electron micrograph of cultured myometrial cells rinsed with distilled water and freeze-dried (unstained). N: nucleus, n: nucleolus, p: finger-like projection. Bar = 5 μm.

The morphology of cultured cells after different washing procedures was studied by TEM. Unwashed cells showed remnants of overlying medium. Cells washed with mannitol showed traces of the washing solution remaining on the cell surface obscuring the

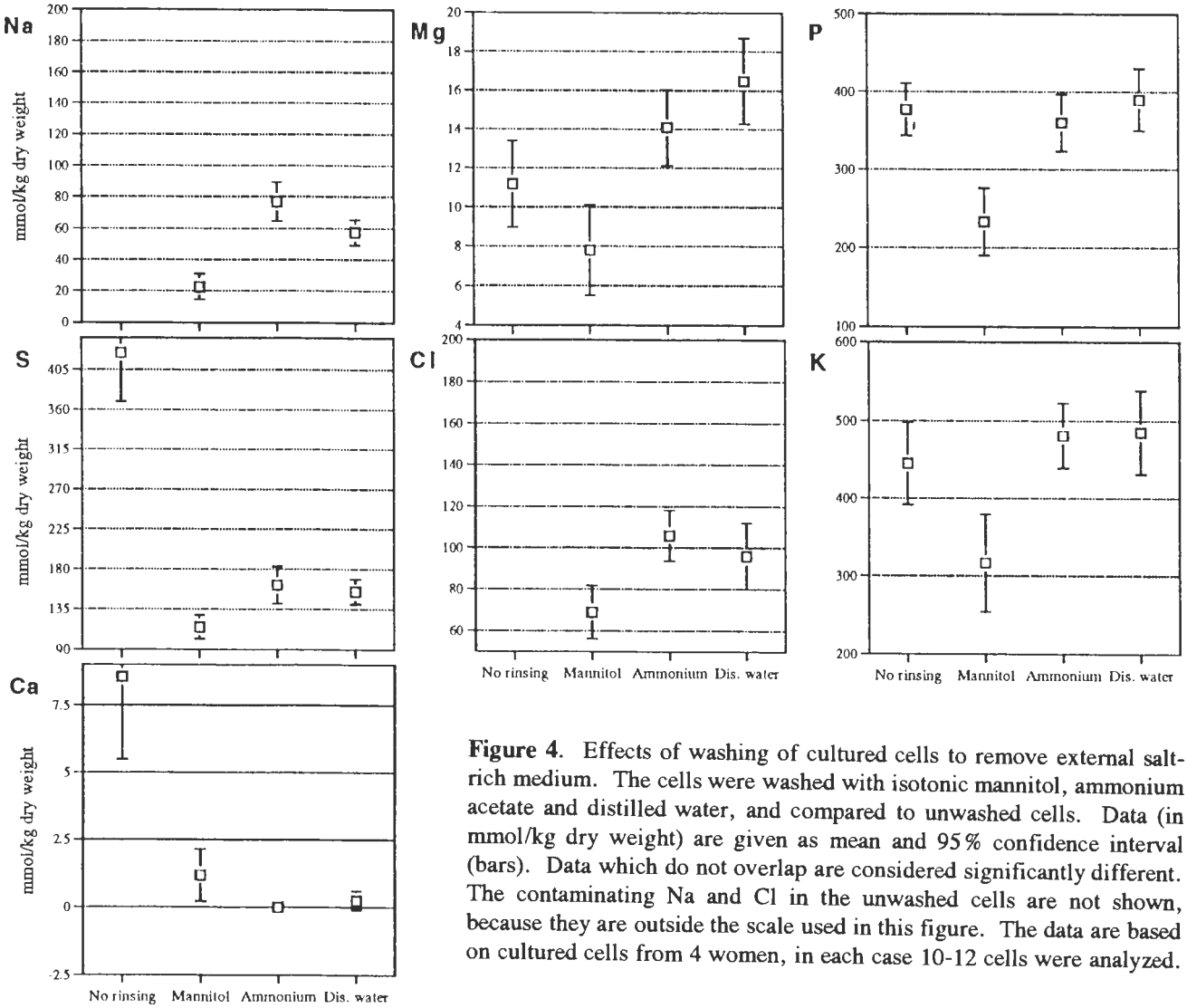

Figure 4. Effects of washing of cultured cells to remove external salt-rich medium. The cells were washed with isotonic mannitol, ammonium acetate and distilled water, and compared to unwashed cells. Data (in mmol/kg dry weight) are given as mean and 95% confidence interval (bars). Data which do not overlap are considered significantly different. The contaminating Na and Cl in the unwashed cells are not shown, because they are outside the scale used in this figure. The data are based on cultured cells from 4 women, in each case 10-12 cells were analyzed.

Table 1. Effects of rinsing solutions on the elemental content of human myometrial cells in culture.

Element	Not washed	Mannitol	Ammonium acetate	Distilled water
Na	1188 ± 114	23 ± 4¤	77 ± 6	57 ± 4
Mg	11 ± 1	8 ± 1¤	14 ± 1	17 ± 1*
P	377 ± 17	233 ± 21*¤	360 ± 18	390 ± 20
S	424 ± 27	115 ± 7¤	162 ± 10	155 ± 7
Cl	1157 ± 115	69 ± 6¤	106 ± 6	96 ± 8
K	445 ± 26	317 ± 31¤	480 ± 21	485 ± 27
Ca	9 ± 2	1 ± 0.5	0 ± 0	0.3 ± 0.2

Data were collected from 10-12 cells from each of 4 women. Asterisks (*) indicate significant differences (from unwashed cells) with 95% confidence interval, only tested for the elements Mg, P and K. Significant differences between mannitol and ammonium acetate or distilled water are indicated by ¤ (all elements).

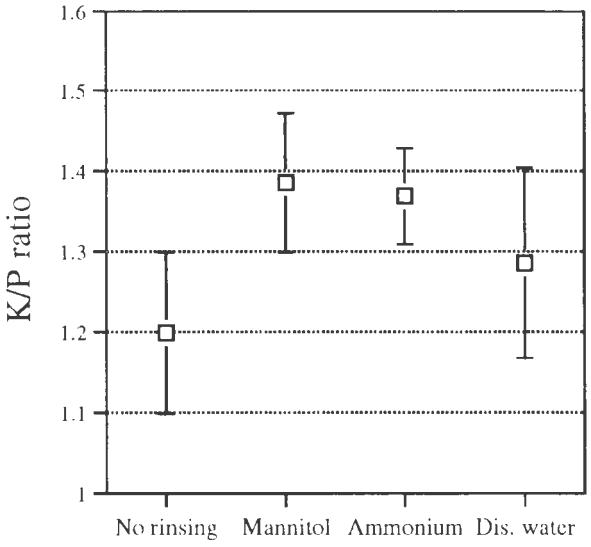

Figure 5. Effects of washing of cultured cells to remove external salt-rich medium. The cells were washed with isotonic mannitol, ammonium acetate and distilled water, and compared to unwashed cells. The K/P ratio is given as mean and 95% confidence interval (bars). Data which do not overlap are considered significantly different. The data are based on cultured cells from 4 women, in each case 10-12 cells were analyzed.

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cells. Ammonium acetate and distilled water are volatile buffers and disappear during freeze drying; thus no remnants of these solutions were present. After freeze drying, cultured myometrial cells showed an oval nucleus with prominent nucleoli. In some cells, finger-like projections were visible at the edges of the cells (Fig. 3).

The effect of washing the cells to remove the salt-rich medium, which would otherwise disturb X-ray microanalysis, was investigated (Fig. 4). The statistical analysis, except for contaminating Na, S, Cl and Ca in unwashed cells, is summarized in Table 1. All solutions removed the external medium, resulting in lower concentrations of Na, S, Cl and Ca in washed cells than in unwashed controls. In the cells washed with mannitol, most elemental concentrations were significantly lower than in their unwashed counterparts and those washed in distilled water or ammonium acetate. Cells washed with ammonium acetate or distilled water did not show significant differences in P and K concentrations compared to their unwashed counterparts; Mg in cells washed with distilled water was higher than in unwashed cells. No significant differences were observed between cells washed with ammonium acetate and with distilled water.

The K/P ratios of unwashed cells were significantly lower than those of the cells washed with mannitol or ammonium acetate (Fig. 5). The K/P ratio of cells washed with mannitol was not significantly different

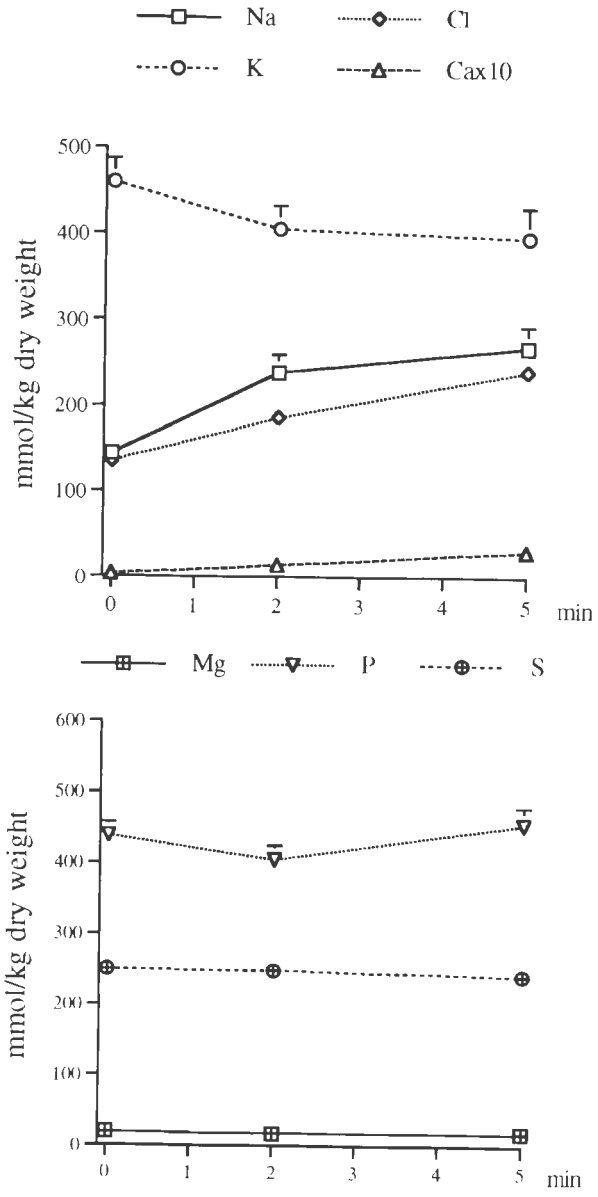

Figure 6. Effect of standard Ringer's solution on element composition of myometrial cell cultures. Cells incubated in culture medium served as control. Data (in mmol/kg dry weight) are given as mean and standard error (bars). Ca concentration is multiplied with 10. The data are based on cultured cells from 3 women, in each case about 15 cells were analyzed.

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from those washed in ammonium acetate and in distilled water (Fig. 5). In subsequent experiments, ammonium acetate was therefore employed as a washing fluid.

When cell-covered grids were incubated at room temperature in drops of standard Ringer's solution, an increase in Na and Cl, and a decrease in K concentration was observed, concomitantly with an increase in Ca concentration, at 2 minutes after the start of the incubation

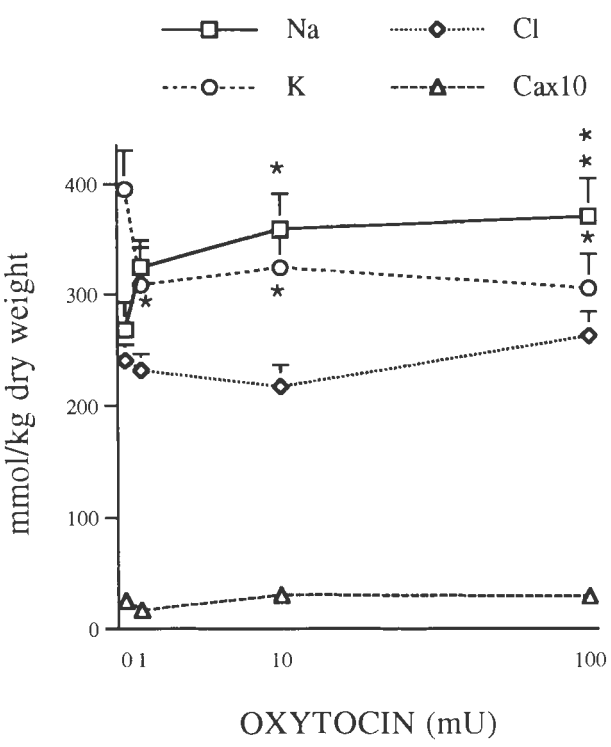

Figure 7. Effects of oxytocin, tested in standard Ringer's solution on myometrial cell cultures. Controls were cells not exposed to oxytocin. Data (in mmol/kg dry weight) are given as mean and standard error (bars). Ca concentration is multiplied with 10. The data are based on cultured cells from 4 women, in each case about 10-12 cells were analyzed. * $P < 0.05$; ** $P < 0.01$ (compared to control).

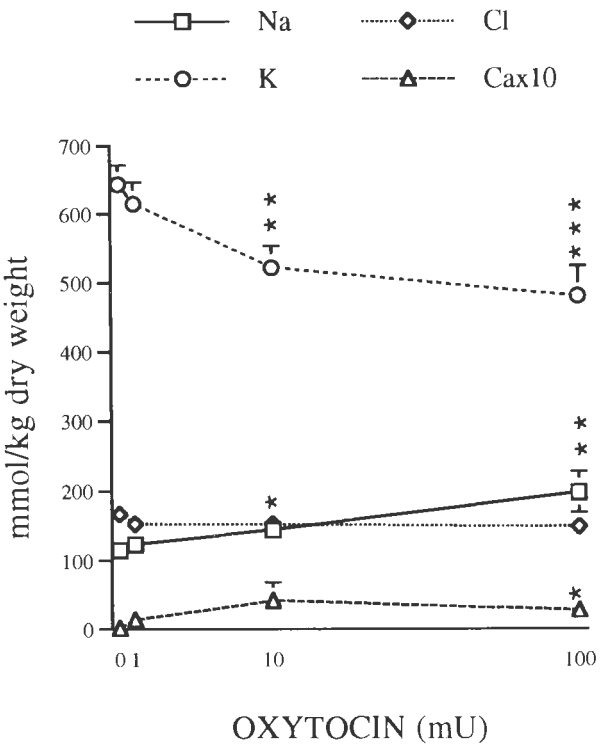

Figure 8. Effects of oxytocin, tested in culture medium for each oxytocin concentration on myometrial cell cultures. Controls were cells not exposed to oxytocin. Data (in mmol/kg dry weight) are given as mean and standard error (bars). Ca concentration is multiplied with 10. The data are based on cultured cells from 3 women, in each case about 15 cells were analyzed. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

(Fig. 6). Incubation in Ringer's solution for 5 minutes resulted in increases in Na, Cl and Ca, parallel with a decrease in K concentration (Fig. 6). Intracellular Mg, P and S concentrations did not change throughout the period of incubation (Fig. 6).

Stimulation with oxytocin in Ringer's solution was carried out at room temperature for 5 minutes. Oxytocin caused a concentration-dependent increase in Na; at 1 mU, oxytocin caused a significant decrease in K, but the effect of oxytocin on K was not enhanced by increasing the oxytocin concentration. No significant effect on Cl was observed. Ca was slightly increased (Fig. 7).

The myometrial cell cultures were also stimulated by addition of oxytocin to the serum-free culture medium at 37°C in a humidified atmosphere containing 5% CO_2 in air, in which the cells were maintained quiescent before the experiment. The cells were exposed to oxytocin for 5 minutes. At 1 mU, oxytocin did not cause significant changes in Na, K and Ca; 10 mU oxytocin resulted in a significant increase in Na, and a significant decrease in K content (Fig. 8). At 100 mU oxytocin, a further increase in Na and Ca and a decrease in K concentration was observed (Fig. 8). Cl was not affected by oxytocin at any concentration.

Discussion

The intermediate filament desmin is present specifically in adult muscle cells (Lazarides, 1982); fibroblasts may produce desmin in cell cultures (Tuszynski *et al.*, 1979). In liver cultures, smooth muscle cells expressed desmin-like immunoreactivity, while fibroblasts did not react with an antibody to desmin (Takase *et al.*, 1988). Thus, the literature on this point is controversial and it cannot be excluded that fibroblasts present in cultures of human myometrium can produce desmin. However, most of the cultured cells used in this study did not originate from other cells which do not produce desmin, e.g., endothelial cells, nerve cells (Lazarides, 1982). After culture, the morphological appearance of myometrial cells was different from the ultrastructure of myometrium *in situ*. This is in agreement with findings on

other types of smooth muscle in culture, but cultured fibroblasts also transform and have morphological characteristics similar to subcultured smooth muscle cells (Chamley-Campbell *et al.*, 1979). However, the cultured cells used in this study responded to oxytocin stimulation indicating that most of the cultured cell population in the present study was derived from the smooth muscle cells of human myometrium.

To find an optimal washing procedure for myometrial cell cultures grown on permeable supports, measurements were performed on washed freeze-dried cells, as compared to unwashed cells. Washing the cells with mannitol resulted in significantly lower concentrations of most of the elements compared to their unwashed counterparts and to cells washed in the other solutions. The possible explanation for this is that the mannitol deposit on the cells causes absorption of characteristic X-rays of most elements, and/or increases the background (continuum) radiation.

In a comparison of concentrations in washed cells with those in unwashed cells, elements such as Mg, P and K, which are present in high concentrations intracellularly but in low concentrations in the extracellular medium, can be used as controls. However, Mg in cells washed in distilled water was higher than in unwashed cells, possibly due to absorption of Mg X-rays by contaminating NaCl in the unwashed preparation. The P content adequately represents the dry mass of the cell, whereas the K concentration may indicate the effect of the rinsing solution in washing out K from the cell (Warley *et al.*, 1994). In the present study, the K/P ratios of the cells with different rinsing solutions were compared with the unwashed counterpart. The K/P ratio of mannitol-washed cells was not significantly different from that in cells washed with ammonium acetate and distilled water. These results suggest that the absorption of characteristic X-rays by the deposited mannitol or the increased production of the continuum affects the X-ray signal for P and K in about the same way. Surprisingly, the K/P ratio of unwashed cells was significantly lower than that of the cells rinsed with mannitol or ammonium acetate. One possible explanation is that when the electrons pass the salt-rich contamination left on the cells, they are left with insufficient energy to excite K X-rays which requires a higher critical potential than excitation of P X-rays. Also, K X-rays are absorbed much more strongly by the Cl of the salt than P X-rays.

Rinsing the cultured myometrial cells with washing solutions can induce artefacts. Borgmann *et al.* (1994) have shown that washing of cultured renal A6 cells for 10 s with distilled water resulted in cell swelling of about 40% and in a decrease of cellular Na, Mg, P, Cl and K concentrations related to wet weight, whereas concentrations related to dry weight were not changed. Similarly, in cultured smooth muscle cells from airway rinsing cells with distilled water for 5, 10 and 20 seconds did not result in any significant change on Na, Mg, P, Cl and K concentrations per dry weight compared to cryosectioned cultured cells (Warley *et al.*, 1994). Ammonium acetate is (about) isotonic and no volume changes would therefore be expected, but the possibility of pH changes cannot be excluded. Washing with isotonic ammonium acetate did not cause volume changes, but it caused a loss of cell Cl already after 10 seconds in cultured renal cells (Borgmann *et al.*, 1994). A 2-second wash with ammonium acetate was accompanied by a loss of cell K in cultured vascular smooth muscle (James-Kracke *et al.*, 1980).

In the present study, the P and K concentrations after rinsing with ammonium acetate and distilled water were not significantly different from that of unwashed cells. This indicates that both solutions could not have any effect on washing out intracellular P and K. In addition, we did not see a significant difference in Cl concentrations between cells washed with ammonium acetate and distilled water. This indicates that ammonium acetate might not wash out Cl, possibly because in this study its pH was adjusted to 7.4. In stimulation experiments, it should also be taken into consideration that controls and experimentally treated cells were rinsed in the same way, and that minor artefacts caused by the rinsing procedure therefore would cancel out.

In general, an increase in Na and Cl and a decrease in K concentration in myometrial cells indicates membrane depolarization. It is unknown why cultured myometrial cells develop depolarization as well as an increase of Ca concentration when incubated in standard Ringer's solution. The elemental composition of isolated myometrium could be affected by changes in the extracellular fluid (from the culture medium to Ringer's solution) (Daniel and Lodge, 1973). Moreover, cultured myometrial cells maintained in serum-free medium for 24 hours, similar to the preparation in this study, were found to have a higher spontaneous increase in intracellular Ca^{2+} ions after being placed in a normal balanced salt solution (Morgan *et al.*, 1993).

Oxytocin can activate contraction in myometrial cells (for review see, e.g., Wray, 1993) by: (a) increasing Ca^{2+} influx via receptor-operated and voltage-operated channels; (b) G-protein-phosphoinositidase C-coupling receptors which increase inositol 1,4,5-triphosphate, which then releases Ca^{2+} ions from an internal depot; (3) suppressing Ca^{2+} extrusion at the sarcolemma and the sarcoplasmic reticulum, e.g., by inhibiting the Ca^{2+}-ATPase.

In Ringer's solution, oxytocin at any concentration induced depolarization of cultured myometrial cells, while total Ca content did not increase significantly.

This suggests that oxytocin in Ringer's solution can stimulate cultured myometrial cells by releasing Ca^{2+} from an intracellular store, e.g., the endoplasmic reticulum, by an inositol 1,4,5-trisphosphate (IP_3)-dependent mechanism (reviewed by Wray, 1993) rather than by causing Ca^{2+} influx from the extracellular medium. It has also been shown that oxytocin can cause contraction of isolated myometrium in Ca^{2+}-free medium without a measurable increase in intracellular free Ca^{2+} (Matsuo *et al.*, 1989). On the other hand, these experiments are a summation of the effects of the Ringer's solution itself and of oxytocin on the cultured cells (compare Figs. 6, 7 and 8). Both factors together enhance the changes of Na and K, whereas they do not further increase the total Ca concentration, as compared to the effects of Ringer's solution or oxytocin alone.

The cultured cells remain quiescent during incubation in the serum-free culture medium. Experiments with oxytocin in the culture medium showed a different pattern of response compared to experiments done in Ringer's solution. Oxytocin at 10 mU elicited an increase in the Na and a decrease in the K concentration, whereas total Ca in the cells was not changed. Again, this might mean that activation may be elicited by the involvement of intracellular Ca^{2+} stores (Morgan *et al.*, 1993). An increase in total Ca concentration in the cells after stimulation with 100 mU oxytocin indicates an increase in Ca^{2+} influx.

Conclusion

It seems likely that ammonium acetate and distilled water are suitable washing solutions for X-ray microanalysis of cultured smooth muscle cells from human myometrium. Standard Ringer's solution caused change in intracellular Na, Cl, K and Ca concentrations of the incubated cell cultures in a time-dependent manner. In stimulation experiments, changes in elemental concentrations induced by oxytocin can be detected in both Ringer's solution and serum-free culture medium. The pattern of elemental changes involving movement of ions from the extracellular fluid into the intracellular spaces is, however, different in the culture medium.

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Discussion with Reviewers

J. Wroblewski: According to your data distilled water and ammonium acetate are equally good as rinsing solutions before freezing. Other studies have shown that ammonium acetate may cause leakage of ions from cultured chondrocytes (Wroblewski *et al.*, 1983) or vascular smooth cells (James-Kracke *et al.*, 1980). Could you please comment on how myometrial myocytes better withstand ammonium acetate treatment than chondrocytes or other smooth muscle cells?

Authors: The unwashed cells were contaminated with Na, S, Cl and Ca from the external medium. Only the P and K concentrations in cultured cells rinsed with ammonium acetate or distilled water can therefore be compared with the unwashed control. The possibility that both rinsing solutions might cause loss of Na, S, Cl and/or Ca in the washed cells cannot be excluded. However, as mentioned in the **Discussion**, because controls and oxytocin-stimulated cells were treated in the same way, minor artefacts caused by the rinsing procedure would be negligible. Our conclusion that cultured myocytes can withstand rinsing with ammonium acetate is based on a comparison with cells rinsed with distilled water and cells rinsed with mannitol. We do not know exactly why cultured myometrial smooth muscle cells withstand ammonium acetate better than chondrocytes and other smooth muscle cells. This may be due to biological differences between cell types and/or methodological differences between studies. For instance, in the present study, the pH of the ammonium acetate solution was adjusted to 7.4, while this pH adjustment was not reported for the other studies.

A. Campos: How do you think that the ultrastructural substrate of the microanalyzed region of the cell might influence your data?

Authors: In the analysis of cultured cells grown on the titanium grids covered with Formvar film, areas close to the grid bars were avoided. Thus, the only extraneous structure in the microanalyzed region of the cell was the Formvar film. Formvar consists of polyvinyl formal resin containing only low molecular weight elements that cannot be detected by the X-ray detector used in this study. However, the Formvar film contributes to the background (continuum) radiation. In the present study, quantitative analysis was carried out based on the peak-to-continuum ratio after correction for extraneous background (Roomans, 1988). Hence we expect no effect of the substrate on the analysis.

Additional Reference

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