BIOCHEMICAL MARKERS OF CONTRACTION IN HUMAN MYOMETRICAL SMOOTH MUSCLE CELLS IN CULTURE

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SUMMARY

Phosphorylation of a light chain subunit of myosin by Ca$^{2+}$ and calmodulin-dependent myosin light chain kinase is believed to be essential for smooth muscle contraction. The biochemical properties of the myosin phosphorylation system in human myometrial smooth muscle cells in monolayer culture were compared with those of human myometrial tissue and nonmuscle cells in culture. Native myosin was isolated from other cellular proteins of crude homogenates by polyacrylamide gel electrophoresis (in the presence of pyrophosphate) and quantified by densitometry. The myosin content of myometrial smooth muscle cells in culture and that of myometrial tissue were similar and four- to five-fold greater than that of human endometrial stromal cells or skin fibroblasts in culture. The specific activities of myosin light chain kinase in homogenates of myometrial smooth muscle cells that were maintained in culture and in myometrial tissue were similar (2.05 ± 0.18 and 1.60 ± 0.37 nmol phosphate incorporated per min per mg protein, respectively). On the other hand, enzyme activity in skin fibroblasts was only 5% of that in myometrial smooth muscle cells. Myosin light chain kinase activity in myometrial smooth muscle cells was dependent upon Ca$^{2+}$ and was inhibited reversibly by the calmodulin antagonist, calmidazolium. The intracellular Ca$^{2+}$ concentration measured by quin2 fluorescence was 0.12 μM in resting cells and increased in a concentration-dependent manner with KCl to a maximal value of 0.47 μM. These results indicate that biochemical processes important for smooth muscle contraction are retained in human myometrial smooth muscle cells in culture.

Key words: calcium; myosin phosphorylation; calmodulin; myometrial smooth muscle cells.

INTRODUCTION

Contraction of smooth muscle can be initiated by the phosphorylation of the 20,000 molecular weight light chain subunit (P-light chain) of myosin (16). Phosphorylation is catalyzed by myosin light chain kinase, an enzyme that is activated by Ca$^{2+}$ and calmodulin. Although this mechanism is not believed to be essential in contraction of skeletal muscle, it is considered to be obligatory for contraction of smooth muscle with marked increases in actin-activated myosin ATPase activity dependent on myosin phosphorylation (16). In isolated tracheal smooth muscle, correlations have been established between the development of isometric tension, shortening velocity, and the extent of light chain phosphorylation in response to neural stimulation (17), carbachol (27), relaxation in response to isoproterenol (27), or fluphenazine (28). In myometrial smooth muscle, spontaneous contractions are preceded by P-light chain phosphorylation (15), whereas spontaneous and relaxin-mediated muscle relaxation are accompanied by decreases in the extent of phosphorylation (20).

The timely onset of synchronous contractions of uterine musculature is of unquestionable importance in both pregnant and nonpregnant women. Although many investigators have evaluated the effects of various physiological and pharmacological agents on uterine contractility, little is known of the biochemical processes that serve to regulate myometrial contractions, and analogies have been made to other types of smooth muscles (14). The cellular heterogeneity of uterine tissue (9) poses difficulties in analyses of biochemical mechanisms involved in contraction; in addition, substances released from adjacent cells may influence myometrial cell contractility.

Previously, we developed a method for the isolation and establishment of human myometrial smooth muscle cells in monolayer culture (6). These cells retained the morphological characteristics of smooth muscle cells and produced prostaglandins characteristic of uterine tissue (1, 24). The rate of formation of prostaglandins by these cells is affected by agents that modulate prostaglandin production in other tissues (22).
Of considerable importance is the question as to whether the contractile elements of the native muscle tissue are retained by isolated cells established in culture. Chamley-Campbell et al. (8) concluded that, with time in culture, the myosin content of smooth muscle cells decreased. In addition, others have demonstrated a loss of the characteristic Ca\(^{2+}\)-calmodulin-dependence of myosin light chain kinase activity in cells in culture (7,25). Therefore, we examined some of the biochemical properties that pertain to the contractile system in human myometrial smooth muscle cells and compared these properties with those of human myometrial tissue and other cell types in culture.

**MATERIALS AND METHODS**

**Tissue preparation and culture techniques.** Myometrial tissues were obtained at the time of hysterectomy from the uteri of premenopausal women who before surgery gave informed consent for the use of these tissues. The protocol used for obtaining tissues was approved by the Institutional Review Board of this institution. Endometrium was separated from myometrium by sharp dissection. Sections of myometrial tissue were frozen in liquid N\(_2\) and stored at \(-60^\circ\)C for later use. Myometrial smooth muscle cells and endometrial stromal cells were prepared by limited enzymatic digestion by methods described previously (6,10). Tissue minces were incubated with agitation at 37\(^\circ\)C for approximately 4 h for myometrium or 30 min for endometrial stroma in Hanks' balanced salt solution that contained collagenase (1.5 and 0.7 mg/ml, respectively), deoxyribonuclease I (0.15 and 0.07 mg/ml, respectively), HEPES buffer (25 mM, pH 7.4), and antibiotic-antimycotic solution (2%, vol/vol). At the end of the incubation period the dispersed myometrial cells were separated from tissue fragments by filtration through gauze. Endometrial glands and stroma were separated by filtration of the mixture through a stainless steel sieve with a pore size of 75 \(\mu\)m. The dispersed stromal cells (in the filtrate) were collected by centrifugation at 600 \(<\) g for 10 min and then suspended in Waymouth's enriched culture medium, which was comprised of Waymouth's MB 752/1 medium, fetal bovine serum (10% vol/vol), HEPES buffer (25 mM, pH 7.4), penicillin (200 U/ml), streptomycin (0.2 mg/ml), kanamycin (0.2 mg/ml), sodium pyruvate (1%, vol/vol), minimal essential medium (MEM) nonessential amino acids (1%, vol/vol), MEM amino acids (1%, vol/vol), and MEM vitamins (1%, vol/vol) (Irvine Scientific, Santa Anna, CA). The suspension of cells was centrifuged a second time and the cell pellets were suspended again in culture medium.

The cells in suspension were transferred to plastic culture flasks that were maintained at 37\(^\circ\)C in air 95%;CO\(_2\), 5% until cell confluence was attained. Trypsin was used to disperse confluent cells for collection and passage to 60-mm culture dishes or 75-mm flasks. After confluence was attained, cells were frozen by immersion in dichlorodifluoromethane precooled in liquid nitrogen and used for the conduct of studies. No differences were noted in experimental results if frozen or nonfrozen cells were used. All cells used for these studies had been passed 1 to 3 times. No differences were noted in the properties with 1 vs. 3 passages.

Fibroblasts (CCD-55SK) from human fetal skin were obtained from the American Type Culture Collection, Rockville, MD. Trachealis muscle was obtained from mature steers at slaughter, frozen in liquid N\(_2\) and stored at \(-60^\circ\)C. In some experiments, samples of this tissue were used for comparison because the contractile and biochemical properties of the tissue have been studied previously (16,17,27) and because the cell type is approximately 95% smooth muscle cells (9).

**Quantification of myosin.** Native myosin was isolated from homogenates prepared from cells in culture and from tissues by use of pyrophosphate-polyacrylamide gel electrophoresis as described originally by Hoh et al. (12) and modified for smooth muscle (2,17). Frozen sections of bovine trachealis and human myometrium were homogenized in 60 vol (vol/wt) of a buffer that consisted of 100 mM sodium pyrophosphate (pH 8.8), 5 mM ethyleneglycol bis (\(\beta\)-aminoethyl ether)-\(N,N,N',N'\)-tetraacetic acid (EGTA), 50 mM sodium fluoride, 10% glycerol, 15 mM \(\beta\)-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 100 U/ml aprotinin (Trasyloil), and 0.1 mM leupeptin to facilitate extraction of the myosin. Human myometrial smooth muscle cells, endometrial stromal cells, and skin fibroblasts were collected from culture dishes and homogenized in the same buffer. The homogenates were centrifuged at 700 \(\times\) g for 20 min. Potassium iodide (4 M stock solution) was added to the supernatant fluid to achieve a final concentration of 0.6 M, and the mixture was allowed to incubate at 0\(^\circ\)C for 20 min. After incubation, a saturated sucrose solution was added (20% vol/vol, final concentration), and aliquots of the mixture were layered immediately onto pyrophosphate-polyacrylamide gels (6.5 \(\times\) 0.5 cm tubes) that were comprised of 20 mM sodium pyrophosphate (pH 8.8), 5 mM EGTA, 100 mM sodium fluoride, 10% glycerol, 3.3% acrylamide, 0.16% bis-acrylamide, and 0.20% N,N,N',N'-'tetramethylethylene-diamine. Electrophoresis was conducted at 100 V for 4.5 h in a refrigerated chamber with recirculation of the electrophoresis buffer to maintain temperature between 1\(^\circ\) and 3\(^\circ\)C. After electrophoresis, the gels were stained with Coomassie blue (0.15%, R-250) in methanol (50%) and acetic acid (10%) for 16 to 18 h, followed by destaining in methanol and acetic acid. The protein of the bands corresponding to myosin were quantified by soft laser densitometry and expressed as arbitrary integration units. The fraction of protein in homogenates that consisted of myosin was also determined by densitometry of the entire gel. Protein content was determined by the method of Bradford (4). Statistical differences were evaluated by use of the Student-Newman-Keuls multiple comparisons and Student's t-tests.

**Myosin light chain kinase activity.** The activity of myosin light chain kinase extracted from smooth muscle tissue and cells in culture was measured by incorporation of \(^{32}\)P from \([\gamma-\(^{32}\)P]ATP into purified myosin P-light chains (3). Cells were collected from culture dishes (60 mm) and homogenized in 0.3 ml of buffer that contained 50 mM postassium phosphate (pH