Bladder smooth muscle organ culture preparation maintains the contractile phenotype

Tanchun Wang,1* Derek M. Kendig,1* Shaohua Chang,3 Danielle M. Trappanese,1 Samuel Chacko,3 and Robert S. Moreland1,2,3

1Department of Pharmacology and Physiology, Drexel University College of Medicine, Philadelphia, Pennsylvania; 2Department of Pathology and Laboratory Medicine, Drexel University College of Medicine, Philadelphia, Pennsylvania; and 3Division of Urology, Department of Surgery, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania

Submitted 12 May 2011; accepted in final form 13 August 2012

Wang T, Kendig DM, Chang S, Trappanese DM, Chacko S, Moreland RS. Bladder smooth muscle organ culture preparation maintains the contractile phenotype. Am J Physiol Renal Physiol 303: F1382–F1397, 2012. First published August 15, 2012; doi:10.1152/ajprenal.00261.2011.—Smooth muscle cells, when subjected to culture, modulate from a contractile to a secretory phenotype. This has hampered the use of cell culture for molecular techniques to study the regulation of smooth muscle biology. The goal of this study was to develop a new organ culture model of bladder smooth muscle (BSM) that would maintain the contractile phenotype and aid in the study of BSM biology. Our results showed that strips of BSM subjected to up to 9 days of organ culture maintained their contractile phenotype, including the ability to achieve near-control levels of force with a temporal profile similar to that of noncultured tissues. The technical aspects of our organ culture preparation that were responsible, in part, for the maintenance of the contractile phenotype were a slight longitudinal stretch during culture and subjection of the strips to daily contraction-relaxation. The tissues contained viable cells throughout the cross section of the strips. There was an increase in extracellular collagenous matrix, resulting in a leftward shift in the passive length-tension relationship. There were no significant changes in the content of smooth muscle-specific α-actin, calponin, h-caldesmon, total myosin heavy chain, protein kinase G, Rho kinase-I, or the ratio of SM1 to SM2 myosin isoforms. Moreover, the organ cultured tissues maintained functional voltage-gated calcium channels and receptors, thus decreasing depolarization-induced contractions (26, 27, 67) and agonist-induced contractions (22, 68), respectively.

The contractile characteristics and smooth muscle-specific protein expression in cultured bladder smooth muscle (BSM) cells may vary depending on culturing conditions and specific smooth muscle cell lines. Cheng’s group (30) found that cultured BSM cells lose their contractility to both agonist stimulation (carbachol) and membrane depolarization (KCl) and have significant changes in the expression of the smooth muscle-specific α-actin isoform. These changes that occur following culture of BSM cells complicate the interpretation of results obtained in vitro, and in particular, from those studies analyzing changes in response to a pathophysiological state or the long-term effects of pharmacological agents. DiSanto and Chacko’s group (72) established a homogeneous smooth muscle cell line from hypertrophied rabbit bladder that stably expressed smooth muscle myosin, myosin light chain (MLC) kinase, MLC phosphatase, and protein kinase G (PKG). The cells also exhibited a contractile response to agonist (bethanechol) stimulation. A human BSM cell line which expresses the smooth muscle phenotype, including the ability to contract in response to carbachol, has been established (71). However, karyotypic analysis of both rabbit and human bladder cell lines showed that these cells are tetraploid. Thus published reports show that, unless smooth muscle cells become a stable cell line with chromosomal alterations, they lose their phenotype upon continued culturing. Three-dimensional BSM cell culture techniques with sustained unidirectional tension exhibited improved contractility and relatively less phenotypic modulation in culture (42). However, a completely representative tissue model of the physiological conditions of BSM in vivo has still not been developed. Atala’s group (1, 12) has successfully utilized stem cells in the bioengineering and reconstruction of whole bladder organ which restore and maintain normal function in diseased and injured tissues. However, there are no reports to date that describe an in vitro model of normal BSM that is capable of maintaining contractile phenotypic properties.

In contrast to classic cell culturing techniques, organ culture of smooth muscle tissues has the potential to maintain the contractile phenotype and may therefore be a useful alternative method. Organ culture of smooth muscle tissues has already been applied to several smooth muscle types, such as carotid...
and pulmonary arteries, in conjunction with genetic manipulation of specific smooth muscle-regulatory proteins (23, 30, 40, 48, 49). Early studies by Hellstrand’s (35) and Karaki’s laboratories (66) demonstrated that force could be maintained in an organ-cultured vascular preparation for up to 4 days if cultured in a serum-free media. Myosin content, however, in the Hellstrand study (35) was decreased by 50%. This is consistent, in terms of force development, with our previous work showing that a vascular preparation could be subjected to organ culture for up to 9 days in a serum-free media (21, 48, 49). More recently, Corteling et al. (16) again using a vascular preparation, showed no loss of contractility in response to UTP stimulation, no decrease in caldesmon content, but a 50% loss of calponin. Murata et al. (39) and Lopes et al. (36) subjected vascular rings to 7 or 14 days of organ culture, respectively, but did not compare their cultured results to the noncultured control tissues. Therefore, although several studies have reported the use of vascular smooth muscle in organ culture, with mixed results, to the best of our knowledge there is no literature systematically evaluating the viability of organ culture as a useful model for BSM research.

The goal of this study was to develop a novel organ culture model of BSM tissue containing smooth muscle cells close to native conditions and evaluate whether this model retains the contractile phenotype and therefore may be useful in studies of contractile regulation as well as other aspects of smooth muscle biology. Specifically, in this study, we determined the phenotype of the organ-cultured tissue in terms of the content of smooth muscle-specific isoforms of contractile proteins, contractility, viability of cells within the tissue, and functional membrane ion channels. Based on our previous studies using vascular smooth muscle subjected to organ culture (48, 49), we hypothesized that BSM strips would maintain their contractile phenotype including tissue contractility, expression of smooth muscle-specific protein isoforms, and typical morphology for up to 9 days in organ culture if cultured under stretch in a serum-free media and subjected to daily contraction-relaxation. We have provided a novel and what we believe will be a very valuable tool for the application of molecular biology techniques to study not only the basic regulation of BSM contrac-
tion but also the changes that occur in pathophysiological states such as overactive bladder or voiding dysfunction that occurs in response to benign prostatic hyperplasia or diabetic uropathy, using a simple in vitro system which would allow modification of the external milieu.

**MATERIALS AND METHODS**

**BSM tissue preparation.** Male New Zealand White rabbits, weighing 2–2.5 kg, were used in this study. All animal studies and procedures were approved by the Drexel University College of Medicine’s Institutional Animal Care and Use Committee. Rabbits were euthanized by a pentobarbital sodium overdose administered via the ear vein followed by exsanguination. The bladders were then quickly removed and placed in ice-cold physiological salt solution (PSS). PSS contained (in mM) 140 NaCl, 4.7 KCl, 1.2 MgSO4, 1.6 CaCl2, 1.2 Na2HPO4, 2 MOPS (pH 7.4), 5 d-glucose, and 0.02 EDTA. The bladder neck, trigone, and base region were removed, leaving only the midportion of the bladder body for experimentation. The bladder body was dissected in ice-cold PSS under a dissecting microscope. The mucosal and serosal layers were carefully removed, and detrusor smooth muscle strips (~1.5 x 6 mm) were cut along the central axis of the bladder in the longitudinal orientation as previously described (55) and stored in PSS at 4°C until used. Storage was never longer than 24 h.

**BSM organ culture.** Muscle strips (~1.5 x 6 mm) were mounted onto polymerized silicon elastomer (Dow Corning, Midland, MI) in six-well culture dishes, using 0.2-mm stainless steel pins. The pins were positioned so that the BSM strips were slightly stretched longitudinally while raised 2–3 mm above the surface of the elastomer. Each well held up to two muscle strips in a volume of 5 ml of serum-free medium composed of DMEM/F-12 (1:1 ratio, Cellgro, Mediatech, Manassas, VA), 100 U/ml penicillin, 100 μg/ml streptomycin, 250 mg/ml amphotericin B, 35 μg/ml l-ascorbic acid, 5 μg/ml transferrin, 3.25 ng/ml selenium, 2.85 μg/ml insulin, and 200 μg/ml l-glutamine. The muscle strips were maintained at 37°C, 5% CO2, and 100% relative humidity in an incubator. The medium was changed daily for up to 9 days. The medium-changing process included a 10-min wash with sterile PSS, then stimulation with 110 mM sterile KCl-PSS (equimolar substitution for NaCl) for 5 min, and a wash in sterile PSS for 10 min, before the strips were incubated in 5 ml of fresh serum-free medium overnight. The 5-min stimulation in 110 mM sterile KCl-PSS followed by a wash in sterile PSS constitutes the daily contraction-relaxation cycle. Selected organ-cultured tissues were either not stretched but were subjected to daily contraction-relaxation, slightly stretched but not subjected to daily contraction-relaxation, or neither slightly stretched nor subjected to daily contraction-relaxation. Tissues not subjected to daily contraction-relaxation were simply washed in sterile PSS, while the other tissues were subjected to contraction-relaxation before being returned into fresh serum-free medium overnight.

**Measurement of isometric force.** The bladder tissue strips were removed from culture and allowed to equilibrate in 37°C PSS for 10 min. Tissue strips were then mounted between a Grass FT.03 force transducer and a stationary clip in water-jacketed muscle organ baths containing 37°C PSS and aerated with 100% O2. The strips were stretched to a force of ~2 g and allowed to stress-relax until a passive force of ~1 g was achieved. This passive force approximates the optimal length for maximal active stress development (L0) (55, 61). The strips were then allowed to equilibrate for at least 40 min until a stable basal force recording was obtained.

After the equilibration period, the strips were stimulated with 110 mM KCl-PSS then relaxed with PSS; this contraction-relaxation cycle was repeated four times. The strips were also subjected to 30 μM carbachol stimulation for 5 min and then relaxed by rinsing with PSS for 5 min. Peak force during carbachol and KCl-PSS contractions was recorded. The peak force in each equilibrated muscle strip was expressed as grams force or active stress (stress = force/cross-sectional area), and cross-sectional area was determined using tissue length and calculated wet weight as previously described (38).

Concentration-response curves to carbachol were generated by the cumulative addition of carbachol from 0.01 to 100 μM. Concentration-response curves to KCl were generated by the cumulative addition of KCl from 4.7 to 110 mM. Similar osmolarity was maintained in the higher KCl concentration solutions by the equimolar substitution of KCl for NaCl. All responses were normalized to the maximal response of each individual bladder strip. EC50 values were calculated for each bladder strip using GraphPad Prism software. The average EC50 ± SE values were determined using the EC50 values from each individual concentration-response curve.

**Inhibition of membrane large-conductance calcium-activated potassium channels with paxilinize.** After the equilibration period, the strips were incubated with the large-conductance calcium-activated potassium channel (BKCa) inhibitor paxilinize (10 μM) for 5 min. The paxilinize concentration was chosen based on data from a previous study (17). After the inhibitor was added to the muscle bath, the frequency and magnitude of spontaneous contractions were recorded using a Grass FT.03 force transducer and AD Laboratory Chart 5 software for Windows.
Innovative Methodology

Measurement of passive length-tension curve. To measure the passive length-tension relationship, the strips were stretched in 1- to 2-mm length increments and then allowed to stress-relax until a stable recording of passive force was reached. This was repeated several times until high levels of passive force were achieved. The experimental points for each strip were plotted as passive stress produced as a function of length.

Histology. BSM strips were cultured as described above and then removed, placed in Histochoice, and sent to AML Labs (Rosedale, MD) for paraffin embedding and sectioning. Cross sections of 5-μm thickness were taken from the paraffin-embedded BSM strips and stained with Masson’s trichrome.

Immunohistochemistry. Paraffin slides of thin sections of BSM strips (organ cultured for 1 or 9 days) were placed in xylene and subjected to descending concentrations of alcohol (100, 95, 70, and 30% and PBS) to remove the paraffin and rehydrate the samples. Citrate buffer (10 mM citric acid, 0.05%, Tween 20, pH 6.0) was used for antigen-retrieval treatment (10 min at 90°C). The tissue sections were blocked in a 3% bovine serum albumin solution for 30 min and then incubated with anti-collagen I antibody (Abcam, Cambridge, MA) at 1:300 overnight at 4°C. After three washings with PBS, the tissue sections were incubated in a secondary anti-mouse FITC antibody (1:400; Sigma-Aldrich, St. Louis, MO) for 1 h at room temperature. A drop of mounting medium (Vector Laboratory, Burlington, ON) was added to each slide after another three washings in PBS. The slides were viewed under a Nikon Eclipse E800 Fluorescence Microscope, and images were captured using Image-Pro software. A negative control to confirm the specificity of the primary antibody was performed using nonimmune mouse serum in place of the primary antibody.

Infection with adenovirus encoding green fluorescent protein to determine viability of organ-cultured BSM. A recombinant adenoviral vector encoding green fluorescent protein (GFP) was purchased from Stratagene (La Jolla, CA). The viral vector was introduced into BSM strips subjected to organ culture for 7 days. The titer of adenovirus encoding GFP was 2 × 10⁷/ml. After coculture in the culture medium described above for an additional 2 days, resulting in 9 days of total organ culture, muscle strips were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek USA, Torrance, CA) for cryostat sectioning. The frozen sections (5-μm thick) were mounted onto slides with a drop of mounting medium containing 4,6-diamidino-phenylindole (DAPI) to stain nuclei (Vector Laboratory). The fluorescence of the frozen sections was detected using a Nikon eclipse E800 microscope. Images were captured using Image-Pro Plus software (Media Cybernetics, Bethesda, MD).

Immunoblots and quantitation of smooth muscle contractile phenotype marker proteins. BSM strips were removed from culture at days 3, 5, 7, or 9 and incubated in 37°C PSS for 10 min, rinsed in acetic acid, and then completely air dried. The strips were homogenized in a 1% SDS, 10% glycerol, 1 mM DTT, and Protease Inhibitor Cocktail solution (1:100; Sigma-Aldrich) using glass/glass homogenizers. Samples were centrifuged at 12,000 rpm for 5 min at 4°C and assayed for protein concentration using a Bio-Rad DC assay (Hercules, CA). All reagents for solutions described above, unless otherwise specified below, were purchased from Fisher Scientific (Pittsburgh, PA) and were of analytic grade or better. All electrophoretic and blotting reagents were obtained from Bio-Rad Laboratories. Carbachol and paxilline were purchased from Sigma-Aldrich. Statistical significance between means was determined using one-way ANOVA followed by the Tukey post hoc test or a Student’s t-test when appropriate. A P value <0.05 was taken as significant. All “n” values refer to the number of bladder muscle strips; each strip was taken from a different animal.

RESULTS

Effect of organ culture on BSM isometric contraction. BSM strips were dissected and subjected to organ culture as described in MATERIALS AND METHODS. At 3, 5, 7, or 9 days of organ culture, the BSM strips were removed from culture, mounted for isometric force recording, and stimulated by either 110 mM KCl-PSS or 30 μM carbachol. Isometric force was recorded at the peak of contraction. Stimuli of 110 mM KCl and 30 μM carbachol were used to reduce diffusional delays and produce uniform activation of the bladder strip. Lower concentrations of stimuli can result in activation of the outer layers of a multicellular preparation with an unstimulated inner core. This will then be followed by activation of the inner core while the outer cells are in a state of decreasing activation. We have previously shown that repeated stimulation with either 110 mM KCl or 30 μM carbachol has no deleterious effect on our preparation of BSM (55, 61). The responses to 110 mM KCl-PSS and 30 μM carbachol were calculated as stress (force/cross-sectional area) to normalize for differences in tissue size, and are shown in Fig. 1A. There was a significant decrease in stress development as early as 3 days after the start of organ culture, and this decrease continued over the 9 days of organ culture. Because a decrease in stress could be due to either a decrease in contractile force or an increase in tissue mass, which would result in an increase in cross-sectional area and decreased calculated stress, the cross-sectional area of all tissues was calculated, and the results are shown in Fig. 1B. The cross-sectional area increased after 3 days in culture to a maximum after 5–7 days in culture, a time course similar to the decrease in calculated stress. We then analyzed the amount of force developed in terms of grams force in response to carbachol and KCl-PSS, as shown in Fig. 1C. This analysis was independent of any potential increase in tissue mass. Grams force in response to KCl-PSS stimulation decreased by ~20% after 5 days of organ culture, with a maximal decrease of 30% after 9 days of organ culture. Grams force in response to carbachol was slightly but significantly decreased at 7–9 days.
of organ culture. These results suggest that organ culture increased tissue weight but did not have a large effect on force development.

The suggestion that the decrease in stress in the organ-cultured tissues was due to a significant increase in tissue mass while still maintaining contractile viability is supported by the results shown in Fig. 1B. An increase in cross-sectional area would have a significant effect on stress, possibly accounting for the decreases shown in Fig. 1A. Therefore, in terms of contractile viability, grams force as shown in Fig. 1C is more representative of the state of the tissues.

The maintenance of stress at high, albeit lower than noncultured values and the maintenance of force to near noncultured values were due in part to the stretch imposed on the tissues and daily contraction-relaxation during culture. The far right bars in Fig. 1, A and C, show the stress and force values for tissues not subjected to stretch, the daily contraction-relaxation, or either stretch or contraction. Removal of the tissue stretch or removal of both stretch and contraction resulted in significantly lower levels of stress or force development, suggesting that stretch and a daily contraction-relaxation are critical for the maintenance of contractility over the course of organ culture.

Effect of organ culture on BSM contractile profile. BSM is categorized as a typical phasic-like smooth muscle based on its contractile pattern. Upon membrane depolarization or agonist stimulation, the contractile response is characterized by a rapid increase to peak force followed by a slow decrease to a lower level of quasi-maintained force. To investigate whether organ culture changes the time course of a contractile event in BSM, we recorded force development at several time points during KCl-PSS or carbachol stimulation. Figure 2A shows typical force tracings in response to carbachol and KCl from bladder tissues not cultured (day 0) and those subjected to 9 days of organ culture. The results of several experiments such as those shown in Fig. 2A are provided in Fig. 2, B (carbachol) and C (KCl), expressed as a percentage of the maximal force attained. Tissue strips subjected to organ culture stimulated by carbachol exhibited significantly slower decreases from peak force during the first 90 s; later time points were not significantly different between noncultured and cultured tissues (Fig. 2B). Tissue strips subjected to 3, 5, 7, or 9 days of organ culture showed no significant differences compared with noncultured tissues in the time course of a contraction in response to KCl-PSS (Fig. 2C).

Effect of organ culture on BSM sensitivity. Organ culture had minimal effects on maximal force generation by BSM strips subjected to stretch and daily contraction-relaxation to KCl. To determine the effect of 9 days of organ culture on the
sensitivity of the BSM strips, we subjected control, noncultured BSM and BSM organ cultured for 9 days to the cumulative addition of either carbachol or KCl. Moreover, to determine the influence of the slight stretch and daily KCl contraction-relaxation, we also subjected BSM strips that were organ cultured for 9 days with stretch but without daily contraction-relaxation, without stretch but with daily contraction-relaxation, or without stretch and daily contraction-relaxation to the cumulative addition of carbachol or KCl. The results of these studies are shown in Fig. 3. The calculated EC_{50} values for carbachol and KCl concentration-response curves for all five conditions are shown in Table 1.

Figure 3A shows the averaged cumulative concentration-response curves to KCl. Neither organ culture nor any of the altered organ culture conditions resulted in a significant change in sensitivity to KCl compared with control, noncultured BSM.
subjected to daily contraction-relaxation (Table 1). In fact, the KCl concentration-response curves of BSM strips were either not cultured (●), organ cultured for 9 days while stretched and subjected to daily contraction-relaxation (△), organ cultured for 9 days not stretched but subjected to daily contraction-relaxation (■), or organ cultured for 9 days neither stretched nor subjected to daily contraction-relaxation (▲). A: concentration-response relationship to KCl was unaffected by any of the various organ culture conditions. B: carbachol sensitivity of BSM strips organ cultured with stretch alone or stretch and daily contraction were not significantly different from control, noncultured BSM strips. Strips placed in organ culture in the absence of stretch and daily contraction-relaxation or organ cultured in the absence of both manipulations significantly shifted the concentration-response curve to carbachol to the right. Values are means ± SE from 3–4 bladder strips; each strip was taken from a different animal.

Figure 3B shows the averaged cumulative concentration-response curves to carbachol. Subjecting the BSM strips to 9 days of organ culture with stretch and daily contraction-relaxation or with just stretch alone had no significant effect on the sensitivity of the tissues to carbachol compared with control, noncultured tissue strips (Table 1). BSM organ cultured for 9 days in the absence of stretch was significantly less sensitive to carbachol than control noncultured tissues (Table 1). Qualitatively, however, it appears that organ culture alone shifted the carbachol concentration-response curve to the right, but the shift was not statistically significant.

Effect of organ culture on morphology and viability of BSM. To further investigate the potential effects of organ culture on BSM morphology and extracellular matrix content, we subjected tissues to Masson’s trichrome stain. Representative BSM tissues subjected to organ culture for 0, 5, or 9 days were sectioned and then stained with Masson’s trichrome, as shown in Fig. 4. Noncultured BSM tissues (day 0) showed red staining of the smooth muscle cells with a dense extracellular matrix in blue. BSM tissues subjected to 5 or 9 days of organ culture showed an apparent increase in extracellular matrix surrounding the smooth muscle layers and between the bundles of smooth muscle within the longitudinal and circular muscle layers, defined by orientation of the tissue sectioning. These findings are consistent with our hypothesis that organ culture increased the weight of the tissues, resulting in an increase in cross-sectional area and a decrease in calculated stress development, with little effect on grams force.

Due to the fact that organ-cultured alterations in the Masson’s trichrome-stained sections were noted, we were interested in determining more precisely whether this was due to an increase in collagen. Therefore, we performed immunohistochemistry on the organ-cultured BSM strips to examine collagen content as described in MATERIALS AND METHODS. Representative images are shown in Fig. 5. Figure 5A shows the collagen content in tissues organ cultured for 1 day, and Fig. 5B shows the collagen content in tissues organ cultured for 9 days. Collagen is shown in green. Tissues organ cultured for 9 days show a qualitative increase in collagen content compared with those organ cultured for only 1 day. These results support our conclusion that organ culture increases matrix content, and specifically collagen. Figure 5C shows the negative control to confirm the specificity of the primary antibody using nonimmune mouse serum in place of the primary antibody in tissues cultured for 9 days.

We investigated the potential effects of organ culture on BSM tissue viability using adenoviral infection of GFP as an indicator of live cells. GFP will be expressed only in the smooth muscle cells which take up the adenovirus and therefore retain viability (29). The results of these studies are shown in Fig. 6B. Sections of BSM organ cultured for 9 days (7 days of organ culture followed by 2 additional days of organ culture in the presence of GFP-adenovirus) expressed green fluorescent signals, which appear to be evenly distributed across the tissue. In addition, we used DAPI staining to determine whether the cultured bladder strips contained intact nuclei. Figure 6A shows a representative DAPI-stained cultured BSM strip. The DAPI staining shows even distribution of the nuclei in BSM cells within the cultured tissue.
Effect of organ culture on the passive length-tension curve of BSM. Bladder tissue strips were removed from organ culture after 3, 5, 7, or 9 days and subjected to passive length-tension experiments as described in MATERIALS AND METHODS. The results are shown in Fig. 7. Tissues organ cultured for 3, 5, 7, or 9 days showed no significant differences in the passive length-tension curve compared with each other. However, the passive length-tension relationship of all tissues subjected to organ culture was shifted to the left compared with noncultured tissues. The viscoelastic properties of the bladder are primarily dependent on the elements contained within the extracellular matrix (4, 64). Elastin, for example, is very compliant compared with collagen (32, 45). Elastin contributes mainly to the foot of the passive length-tension curve, while the sharp rising portion reflects the collagen component. As the content of extracellular matrix proteins increases, the passive force curve shifts to the left (53). Consistent with our immunohistochemical results showing an increase in collagen content, these results provide mechanical data consistent with an increase in collagen content in the organ-cultured tissues.

Quantification of smooth muscle-specific marker proteins with organ culture. Similar to other types of smooth muscle cells, BSM cells phenotypically modulate when subjected to cell culture (30, 42). Following exposure to culture media, BSM cells undergo a transition from a contractile to a migratory/secretory state. Biochemically, expression levels of smooth muscle specific-isoforms of contractile and regulatory proteins as well as cell adhesion proteins, ion channels, and receptors decrease when smooth muscle cells are placed in culture, while concurrently nonmuscle isoforms of

<table>
<thead>
<tr>
<th>KCl</th>
<th>Day 0</th>
<th>Day 9</th>
<th>Contraction Only</th>
<th>Stretch Only</th>
<th>Neither</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50</td>
<td>20.4 ± 1.4 mM</td>
<td>19.8 ± 2.6 mM</td>
<td>41.0 ± 12.6 mM</td>
<td>17.6 ± 1.7 mM</td>
<td>27.3 ± 1.4 mM</td>
</tr>
<tr>
<td>Carbachol</td>
<td>0.12 ± 0.03 µM</td>
<td>0.65 ± 0.19 µM</td>
<td>1.40 ± 0.20 µM*#</td>
<td>0.51 ± 0.11 µM</td>
<td>0.79 ± 0.20 µM*</td>
</tr>
</tbody>
</table>

Values are means ± SE for 3–4 determinations, each from a different bladder. EC50 values were calculated from individual cumulative concentration-response curves to KCl or carbachol. *P < 0.05 compared with day 0, uncultured bladder smooth muscle strips. #P < 0.05 compared with day 9, cultured bladder smooth muscle strips.

Fig. 4. Masson’s trichrome staining of BSM strips subjected to 0, 5, or 9 days of organ culture (>40 magnification). Left: longitudinal BSM; left: circular BSM; images. Red: smooth muscle; blue: extracellular matrix. These slides are representative of an n of at least 4 experiments. Each experiment was performed on a BSM strip taken from a different animal.
several of the same proteins increase (28). To verify that the BSM cells in our organ culture model retained the contractile phenotype, we measured the expression levels of several smooth muscle-specific proteins. α-Smooth muscle actin, h-caldesmon, calponin, smooth muscle myosin heavy chain, and the ratio of SM1 to SM2 myosin isoforms are specifically downregulated in dedifferentiated or synthetic smooth muscle cells (2, 29, 30, 44, 65). The results shown in Fig. 8, A–E, are from experiments to quantify expression levels of these proteins in noncultured and organ-cultured smooth muscle strips. To normalize for any potential loading differences, we first measured the expression levels of several commonly used housekeeping proteins, e.g., GAPDH, β-actin, and α-actin. The results suggested the most stably expressed proteins during 9 days of organ culture are α-actin and β-actin (data not shown). We used α-actin as the loading control in all experiments. Quantified and averaged data based on several Western blots and gels are shown below each respective blot or gel.

h-Caldesmon levels were not significantly changed during the 9 days of organ culture (Fig. 8A). This is in contrast to the significant decreases to near zero expression levels noted in smooth muscle cells subjected to cell culture (46, 59). The basic isoform of calponin is a smooth muscle-specific marker that is significantly depressed in cultured smooth muscle cells (7, 25). Figure 8B shows that subjecting bladder strips to 9 days of organ culture has no effect on calponin expressions levels.

BSM cells subjected to culture or to conditions that induce hypertrophy exhibit decreased levels of total myosin and an increase in the ratio of the SM1/SM2 myosin isoforms (9, 62). Representative Western blots for total myosin and Coomassie blue-stained gels for the ratio of SM1/SM2 myosin isoforms from bladder strips subjected to organ culture for up to 9 days are shown in Fig. 8, C and D, respectively. Neither total myosin nor the ratio of SM1 to SM2 myosin isoforms was changed by subjecting BSM strips for 9 days of organ culture.

Quantification of ROCK-I and PKG in organ-cultured BSM tissue. Several studies have demonstrated that subjecting smooth muscle cells to culture or tissues to protocols that produce hypertrophy results in changes in the expression level of ROCK (6, 41). To exclude the possibility that our organ culture model might have similar changes in ROCK-I, we examined ROCK-I protein expression levels in bladder tissues subjected to 0–9 days of culture. The results in Fig. 8E demonstrate that 3–9 days of organ culture had no significant effect on levels of ROCK-I expression.

Expression of PKG has been suggested to positively correlate with the phenotype of smooth muscle, meaning that higher expression levels of PKG correlate with the contractile phenotype and lower expression levels suggest a secretory phenotype. Vascular smooth muscle cells undergoing in vitro culture show decreased levels of PKG expression over time. Restoration of PKG expression to near physiological levels rescues the...
vascular smooth muscle cells from the secretory phenotype and reverts them back to a contractile phenotype (15, 18, 33). We investigated the expression levels of PKG in our organ-cultured BSM strips. Based on the hypothesis proposed by Lincoln’s laboratory (15, 18), if our organ-cultured BSM strips maintained the contractile phenotype, then PKG expression levels should not be different from the noncultured control tissues. The results shown in Fig. 8E demonstrate that 3–9 days of organ culture had no significant effect on PKG expression levels compared with noncultured strips of BSM, supporting the conclusion that our strips maintain their contractile phenotype.

Effect of stretch and daily contraction-relaxation on BSM protein expression. To determine whether either stretch and/or daily contraction-relaxation was important in the maintenance of the contractile phenotype during organ culture of BSM, strips were subjected to organ culture with stretch and daily contraction-relaxation, stretch only, contraction-relaxation only, or neither for 9 days. Expression levels of h-caldesmon, calponin, total myosin heavy chain, myosin SM1/SM2 ratio, ROCK-1, and PKG were measured in control, noncultured BSM, and BSM subjected to the various organ culture conditions listed above. Representative Western blots and averaged results from several such blots are provided in Fig. 9. h-Caldesmon expression was significantly decreased when organ cultured in the absence of both stretch and daily contraction-relaxation compared with both control, noncultured BSM strips, and those organ cultured for 9 days with stretch and daily contraction-relaxation (Fig. 9A). Similarly, ROCK-1 expression was also significantly decreased when organ cultured in the absence of both stretch and daily contraction-relaxation (Fig. 9E). Neither calponin, PKG, nor α-actin expression levels were decreased by organ culture in any condition (Fig. 9, B and F). Total myosin expression was increased by organ culture in the absence of stretch but subjected to daily contraction-relaxation (Fig. 9C). No change was noted in the ratio of the SM1/SM2 isoforms of myosin (Fig. 9D).

DISCUSSION

Our understanding of the mechanism(s) by which an increase in intracellular Ca$^{2+}$ initiates contraction of smooth muscle has increased dramatically since the discovery of MLC phosphorylation in the 1970s (3, 50). However, much more information is needed on the changes in the regulatory mechanisms during many disease states (63). Cell culture systems have been used to investigate basic cellular mechanisms that are observed in vivo in disease processes. One of the technical problems that has hindered progress in this area is the fact that smooth muscle cells when subjected to culture phenotypically modulate from a contractile to a noncontractile migratory/secretory phenotype (5, 10, 33). This presents a roadblock to progress toward understanding the biology and pathobiology of smooth muscles. In this study, we provide an extensive qualitative and quantitative characterization of a novel organ culture model of BSM. The advantage of organ culture compared with cell culture is that the contractile phenotype is maintained and the cell-to-cell associations are similar to that of intact smooth muscle.
smooth muscle. Organ culture preparations of other smooth muscle tissues have been reported (20, 40, 48, 49), but to the best of our knowledge this is the first demonstration of BSM subjected to organ culture as well as a near complete characterization of the preparation in terms of contractility, isoform protein expression, and qualitative information on ion channels. Our primary finding is that BSM tissue can be organ cultured for up to 9 days with maintenance of the contractile phenotype. With this organ culture model, grams force of the cultured tissues remained similar to the values obtained from noncultured tissues in response to stimulation, sensitivity to both membrane depolarization and agonist activation was unchanged, membrane ion channels remained functional, and all contractile proteins were retained in their smooth muscle-specific isoforms.

Fig. 8. Representative Western blot or Coomassie blue-stained electrophoretic gel of smooth muscle-specific protein isoforms in BSM strips subjected to organ culture for 0–9 days. Smooth muscle α-actin was used as the loading control in all blots. Figures showing the cumulative results of several blots or gels are shown to the right of each respective blot or gel. A: representative Western blot showing h-caldesmon content in noncultured control tissues (day 0) and tissues subjected to organ culture for 3, 5, 7, or 9 days. B: representative Western blot showing calponin content in noncultured control tissues (day 0) and tissues subjected to organ culture for 3, 5, 7, or 9 days. C: representative Western blot showing myosin heavy chain content in noncultured control tissues (day 0) and tissues subjected to organ culture for 3, 5, 7, or 9 days. D: representative electrophoretic gel stained with Coomassie blue of SM1 and SM2 myosin isoforms in noncultured tissues (day 0) and tissues subjected to organ culture for 3, 5, 7, and 9 days. E: representative Western blot showing Rho kinase (ROCK)-I (top band) and PKG (bottom band) content in noncultured control tissues (day 0) and tissues subjected to organ culture for 3, 5, 7, or 9 days. Organ culture of BSM for up to 9 days had no effect on the cellular content of any of the smooth muscle-specific isoforms shown. Values for all panels shown to the right of the respective blots or gels are means ± SE of 5–7 determinations.
specific isoform and at expression levels similar to noncultured tissues.

**Smooth muscle contractility.** The organ culture model of BSM developed in this study retained a high degree of contractility over the 9-day experimental period. This was demonstrated by the presence of robust contractions in response to KCl and carbachol. We were at first disappointed that stress values appeared to be depressed in tissues subjected to organ culture. However, we realized that if there were any secretory responses to organ culture these changes would increase the weight of the tissue, thus increasing the calculated cross-sectional area, and decrease stress values (N/m²). Therefore, we reanalyzed the contractions in terms of grams force. When presented as grams force, which does not take into account changes in tissue weight, it was apparent that the carbachol contraction was unaffected by organ culture except at 7–9 days of culture and KCl contractions were only slightly but significantly reduced. This conclusion is supported by the significant increase in cross-sectional area and increase in collagen content indicative of an increase in nonmuscle tissue weight. If nonmuscle tissue weight increased in the absence of smooth muscle hypertrophy but force levels remained similar, then it is reasonable to suggest that force as a function of smooth muscle mass remained similar in the organ-cultured compared with

---

**Fig. 9.** Representative Western blot or Coomassie blue-stained electrophoretic gel of smooth muscle proteins in BSM strips subjected to various organ culture conditions for 9 days. Protein expression was determined in BSM strips not cultured (Control); organ cultured for 9 days with stretch and daily contraction/relaxation (+K+S); organ cultured for 9 days without stretch but with daily contraction-relaxation (+K-S); organ cultured for 9 days with stretch but without daily contraction-relaxation (−K+S); or organ cultured for 9 days without either stretch or daily contraction/relaxation (−K−S). **A:** h-caldesmon. Nine days of organ culture in the absence of both stretch and daily contraction-relaxation significantly reduced h-caldesmon expression. **B:** calponin. Calponin expression levels were not affected by any of the various organ culture conditions. **C:** total myosin heavy chain. Nine days of organ culture without stretch but with daily contraction-relaxation increased myosin expression levels. **D:** myosin SM1/SM2 ratio. SM1/SM2 ratio was not affected by any of the various organ culture conditions. **E:** ROCK-1. Nine days of organ culture in the absence of both stretch and daily contraction-relaxation significantly reduced ROCK-1 expression levels. **F:** PKG. PKG expression levels were not affected by any of the various organ culture conditions. Values for all panels shown to the right of the respective blots or gel are the means ± SE of 4 determinations, each from a different animal. Statistical significance was taken as P < 0.05. *Statistically different from control, noncultured muscle strips, P < 0.05. #Statistically different from muscle strips organ cultured for 9 days in the presence of stretch and daily contraction-relaxation, P < 0.05.
both membrane depolarization (KCl) and agonist activation (carbachol) was unaffected by 9 days of organ culture. It should be noted, however, that although the EC50 for carbachol was not statistically shifted to the right after 9 days in organ culture while strips were stretched, there was a nonsignificant, qualitative decrease in sensitivity. When the BSM tissues were organ cultured in the absence of longitudinal stretch, the EC50 to carbachol was significantly higher, denoting a shift to the right in the concentration-response curve and therefore a decrease in sensitivity to the agonist.

In our opinion, the key elements in our new organ culture protocol that contributed to the maintenance of contractility were the slight longitudinal stretch applied to the tissues during organ culture and daily contraction-relaxation protocol using a depolarizing solution of KCl. Slightly stretching the tissue is apparently the more important of the two conditions, consistent with the results of a recent study using organ-cultured vascular smooth muscle (58). However, when both stretch and daily contraction-relaxation were employed, the organ-cultured BSM tissues retained phenotypic characteristics most similar to the noncultured control tissues. Stretching the tissues may have also enhanced an integrin-mediated increase in extracellular matrix proteins (60), resulting in an increased weight of the tissue and therefore a decrease in the calculated levels of stress.

Smooth muscle contractile pattern and extracellular matrix content. BSM contraction is characterized by an initial phasic-like increase in force followed by a smaller tonic-like phase of force maintenance. A typical BSM contractile event is defined not only by the total amount of force developed but also the temporal profile of the contraction. In this study, the time course of contraction in response to both KCl and carbachol stimulation was measured. Organ culture caused no significant changes in the temporal pattern of the contraction in response to KCl-induced membrane depolarization. In response to carbachol, the transition from the phasic-like portion to the tonic-like portion of the contraction was slower in organ-cultured tissues compared with noncultured tissues. The mechanism responsible for the slower change to the tonic-like portion of the contraction is not known. This change is similar to the change seen in hypertrophied bladder, such as that which occurs in response to partial bladder outlet obstruction (6, 19, 70). The hypertrophied bladder, however, exhibits this change in response to both membrane depolarization and carbachol stimulation, whereas in our organ-cultured preparation the slower transition from the phasic-like to the tonic-like contraction occurs only in response to carbachol stimulation, suggesting that receptor activation or receptor-activated pathways are involved. It is possible that the muscarinic receptor population or subtype may be altered following organ culture; a question beyond the scope of this current study.

The decrease in stress with only a small change in grams force following culture implies an increase in tissue weight. This would suggest that organ-cultured tissues had increased extracellular matrix content or the cells have undergone hypertrophy during in vitro growth with frequent stimulation. To determine whether this resulted in a functional change, we measured the passive length-tension relationship in the organ-cultured and noncultured BSM tissues. The shape of the passive length-tension curve is dependent on the type and amount of extracellular matrix proteins (28, 39). Our results showed a leftward shift of the passive length-tension curve,

noncultured tissues. These results are important because smooth muscle cells subjected to cell culture produce little to no contraction, and several organ culture preparations have significantly depressed contractions in response to membrane depolarization (20, 26, 30). To our knowledge, these results demonstrate for the first time the ability to maintain BSM contractility using in vitro culture methods. Moreover, compared with the complete or near complete loss of contractility following standard cell culture techniques, the small losses in grams force noted in this study should be considered minimal.

Our hypothesis that the BSM strips maintained the contractile phenotype after organ culture is, we believe, strengthened by the finding that the sensitivity as determined by EC50 to

Fig. 10. Representative force tracings of the effect of the large-conductance calcium-activated potassium channel (BKCa) channel inhibitor paxilline (10 μM) on BSM strips subjected to organ culture for 0 (A), 7 (B), or 9 (C) days. The arrow denotes time of addition of paxilline to the muscle baths. The addition of paxilline induced basal spontaneous activity in unstimulated strips. The large contraction at the end of the force tracings is in response to the addition of 30 μM carbachol to ensure tissue viability.
which is suggestive of an increase in extracellular matrix content, specifically collagen content (53). Interestingly, although the passive length-tension curve was shifted to the left after 3 days of organ culture, no further changes were noted with up to 9 days of organ culture. This would suggest that the increase in extracellular matrix is a rapid response to organ culture and that maintained organ culture either does not further increase matrix content or does so very slowly.

Histological examination of the tissues subjected to organ culture was consistent with the results of the passive length-tension results. Masson’s trichrome staining of both longitudinal and circular BSM layers subjected to 0, 5, or 9 days of organ culture showed an increase in extracellular matrix proteins while the smooth muscle cells appeared to not be affected. Although Masson’s trichrome stain does not differentiate the type of extracellular matrix proteins, the overall increase in matrix shown is consistent with a decrease in stress but not grams force and our interpretations based on the passive length-tension experiments. The immunohistochemistry experiments demonstrated that the increase in matrix shown in the Masson’s trichrome-stained sections is, at least in part, collagen.

One concern of using whole tissue strips in any cultured environment is the possibility that the central core of the tissue became hypoxic and the cells in the core were dying. However, one advantage of our predominantly smooth muscle strip preparation is that due to removal of the urothelium and mucosa hypoxia should be less of a factor. To determine the viability of the smooth muscle cells within the organ-cultured strips, we introduced a recombinant adenovirus encoding GFP into our organ-cultured smooth muscle tissues and observed the fluorescence. The cells in the smooth muscle tissue strips showed a strong and evenly distributed green fluorescent signal. This confirms our hypothesis that the smooth muscle cells within the cultured strips retained viability, both in the periphery and in the core. The tissue viability also provides another confirmation of our observation that tissues subjected to organ culture for up to 9 days are fully functional.

Expression of smooth muscle-specific proteins. One of the primary changes that occur in smooth muscle cells subjected to cell culture is loss of smooth muscle-specific isoforms of several contractile-related proteins and the increase in the non-muscle-specific isoform of those same proteins (37, 51). In terms of thick-filament proteins, subjecting BSM strips to organ culture resulted in the maintenance of total myosin levels, the ratio of SM1 to SM2 myosin isoforms, and the predominance of the SMB myosin isoform (data not shown). These indices are altered in cell culture or in bladder subjected to a stimulus that induces hypertrophy (9, 19, 62). Surprisingly, the lack of either stretch or daily contraction-relaxation increased total myosin expression. Tissues that were organ cultured in the absence of stretch but subjected to daily contraction-relaxation had a statistically significant elevation in myosin expression. In terms of thin-filament proteins, the smooth muscle-specific isoform of calponin was not altered nor was the smooth muscle-specific α-smooth muscle actin isoform. The smooth muscle-specific h-caldesmon was also not significantly decreased in our model in contrast to results obtained in smooth muscle cells subjected to either cell culture or organ culture (16, 29, 69). It should be noted, however, that in one report of cultured smooth muscle cells, h-caldesmon levels were shown to be reasonably well maintained (14). Maintenance of h-caldesmon in a rabbit smooth muscle cell line required frequent stimulation of cultured cells with carbachol or transfection of the cultured cells with h-caldesmon cDNA (47). Our repeated daily stimulation of the organ-cultured tissues may therefore account for the maintained levels of h-caldesmon in our preparation. Stretch alone or daily contraction-relaxation alone did not result in a decrease in h-caldesmon expression in the organ-cultured BSM tissues. If the tissues were organ cultured in the absence of both manipulations, h-caldesmon content was significantly decreased. The loss of the contractile isoform of caldesmon is a good indicator of the phenotypic modulation from a contractile to a noncontractile phenotype, suggesting again that stretch and daily contraction are important in maintaining the contractile phenotype. Overall, these results support our hypothesis and interpretation that our organ-cultured BSM maintains the contractile phenotype.

The organ-cultured BSM model was further evaluated by quantification of the expression levels of the signaling protein PKG. The fact that PKG levels were not affected by 9 days of organ culture is one of the best lines of evidence supporting our hypothesis that this tissue model retains the contractile phenotype. This is based on the work from Lincoln’s laboratory (18, 34), which clearly demonstrated that loss of PKG correlates with the loss of the smooth muscle contractile phenotype while increasing expression levels of PKG rescues the cells from the secretory phenotype back to the contractile phenotype. Based on this work from Lincoln’s laboratory, we were surprised that subjecting BSM tissues to organ culture in the absence of stretch, daily contraction, or both had no effect on PKG expression.

Potential for smooth muscle hypertrophy. One concern related to smooth muscle hypertrophy is the possibility of inducing smooth muscle cell hypertrophy. If the smooth muscle cells undergo hypertrophy, it could complicate the interpretation of experimental results in terms of signaling and contractility. Based on the literature, there are several characteristics seen after in vitro cell culture suggestive of smooth muscle hypertrophy, such as increased expression levels of ROCK-I (6), a shift of the ratio of the myosin SMA to SMB isoforms from a predominance of SMB to a predominance of SMA (9), overexpression of l-caldesmon levels (8, 70), and a shift in contractile pattern from a phasic-like to a more tonic-like contraction (8). Our results showed that organ culture did not produce any significant change in the expression levels of either l-caldesmon (data not shown) or ROCK-I. The absence of both stretch and daily contraction decreased ROCK-I expression after 9 days in organ culture. ROCK-I expression in BSM tissues not stretched but subjected to daily contraction was also lower than noncultured tissues, albeit not significantly, suggesting that stretch is an important parameter in maintaining ROCK-I expression. Moreover, preliminary data not presented here demonstrated that organ culture did not alter the SMB/SMA ratio of myosin isoforms. The SMB isoform remained the predominant isoform present in the organ-cultured tissues. While organ culture did alter the phasic component of a contraction in response to carbachol, no change in contractile profile was evident in response to KCl-induced membrane depolarization. Based on those observations, we suggest that our organ culture technique did not
induce a significant hypertrophic response in the strips of BSM.

Ion channels. Several groups have presented evidence that smooth muscle subjected to culture, even organ culture, showed significant loss of membrane-associated ion channels (5, 20, 24). The results presented in this study suggest that our BSM organ culture model maintains functional membrane-associated ion channels. The fact that membrane depolarization-induced contractions are maintained at near control levels clearly demonstrates the presence of voltage-gated calcium channels. Another important smooth muscle ion channel is the BKCa channel. The BKCa channel is a key regulator of BSM contractility. It functions through connecting membrane depolarization and local increases in cytosolic free Ca2+ to hyperpolarizing K+ outward currents, thereby limiting smooth muscle contractility (24, 31, 54). Our results showed that inhibition of BKCa channels with the specific BKCa channel inhibitor paxilline had similar effects in both organ-cultured and noncultured tissues. In both cultured and noncultured tissues, inhibition of the BKCa channel increased spontaneous basal activity, which is consistent with the loss of a hyperpolarizing outward K+ current that limits contractility. This finding is consistent with a recent report from Nelson’s group (31) showing that activation of the BKCa channel decreased contractility of BSM. Taken together, these results support the idea that our organ culture model maintains at least these two ion channels in terms of functionality.

Summary. In conclusion, the results of this study provide a near complete characterization and verification of a novel organ culture model of BSM. In contrast to most cell culture models for BSM, our new organ-cultured model maintains near control levels of contractility, normal sensitivity to both membrane depolarization and agonist stimulation, functional ion channels, and control levels of smooth muscle-specific isoforms of contractile and signaling proteins. Our results also demonstrate that this model maintains cell viability across the entire cross section of the tissue. It is evident that some secretory function is generated due to the increase in extracellular matrix, specifically collagen, and a decrease in calculated stress values most likely due to increased tissue weight. However, this increase in secretory activity does not appear to be deleterious to the overall contractile functionality of this model of BSM. The ability to culture strips of BSM while maintaining the contractile phenotype will now allow the use of molecular biology techniques such as small interfering RNA or lentiviral knockdown of specific proteins, which should provide new information concerning the regulation of both normal and diseased BSM.

ACKNOWLEDGMENTS

The authors acknowledge the excellent technical assistance of Stephanie Costa, Hillevi K. Ets, and Bailey Sylvester.

GRANTS

This study was supported, in part, by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK 57252 and DK 85734 (to R. S. Moreland), DK 69898 (to S. Chacko), and O'Brien Urology Center Grant PS0 DK 52620 to the University of Pennsylvania. D. M. Kendig was supported by a predoctoral fellowship from the Drexel University College of Medicine Aging Initiative.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: T.W., D.M.K., S. Chang, and R.S.M. provided conception and design of research; T.W., D.M.K., S. Chang, and D.M.T. performed experiments; T.W., D.M.K., S. Chang, D.M.T., S. Chacko, and R.S.M. analyzed data; T.W., D.M.K., D.M.T., S. Chacko, and R.S.M. interpreted results of experiments; T.W., D.M.K., S. Chang, D.M.T., S. Chacko, and R.S.M. prepared figures; T.W., D.M.K., D.M.T., S. Chacko, and R.S.M. drafted manuscript; T.W., D.M.K., S. Chang, D.M.T., S. Chacko, and R.S.M. edited and revised manuscript; T.W., D.M.K., S. Chang, D.M.T., S. Chacko, and R.S.M. approved final version of manuscript.

REFERENCES


