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Authors: Alcock, Joelle, Warren, Averil Y., Goodson, Yvonne J., Hill, Stephen J., Khan, Raheela N., et al.

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Inhibition of Tissue Transglutaminase 2 Attenuates Contractility of Pregnant Human Myometrium[1]

Joelle Alcock,[3] Averil Y. Warren,[4] Yvonne J. Goodson,[5] Stephen J. Hill,[3] Raheela N. Khan,[4] and Joanne S. Lymn[2,5]

Institute of Cell Signalling,[3] School of Biomedical Sciences, University of Nottingham, Queens Medical Centre, Nottingham, United Kingdom
Graduate Entry, Medicine and Health,[4] University of Nottingham, Royal Derby Hospital, Derby, United Kingdom
Nursing, Midwifery and Physiotherapy,[5] University of Nottingham, Queens Medical Centre, Nottingham, United Kingdom

ABSTRACT

Premature delivery remains a serious risk factor in pregnancy, with currently licensed tocolytics unable to offer significant improvement in neonatal outcome. Further understanding of the regulators of uterine contractility is required to enable the development of novel and more effective tocolytic therapies. The transglutaminase family is a class of calcium-dependent, transamidating enzymes, of which tissue transglutaminase 2 is a multifunctional enzyme with roles in cell survival, migration, adhesion, and contractility. The aim of the present study was to investigate the role of this enzyme in regulating the contractility of pregnant human myometrium. Tissue strips from biopsy samples obtained at elective cesarean section were either allowed to contract spontaneously or induced to contract with oxytocin, phenylephrine, or bradykinin. Activity integrals, used to measure contractile activity, were taken following cumulative additions of the reversible, polyamine transglutaminase inhibitors cystamine and mono-dansylcadaverine and the irreversible, site-specific transglutaminase inhibitors *N*-benzyloxycarbonyl-L-phenylalanyl-6-dimethylsulfonium-5-oxo-L-norleucine and 1,3-dimethyl-2[(oxopropyl)thio]imidazolium. The ability of cystamine and mono-dansylcadaverine to affect oxytocin-mediated calcium mobilization within primary cultured myometrial cells was also measured utilizing a calcium indicator. All inhibitors attenuated myometrial contractions in a concentration-dependent manner independent of the method of contraction stimulus. Similarly cultured myometrial cells preincubated with cystamine and mono-dansylcadaverine displayed an altered calcium response to oxytocin stimulation. Our findings demonstrate a potential role for tissue transglutaminase 2 in regulating uterine contractility in pregnant human myometrium that may be associated with the calcium signaling cascade required for contraction.

cystamine, mono-dansylcadaverine, myometrium, polyamine, tissue transglutaminase 2, tocolysis, uterus

INTRODUCTION

Premature delivery (<37 completed weeks of gestation) is a significant risk factor for perinatal mortality and long-term health problems, with 75% of all perinatal deaths presenting in premature infants [1]. Surviving infants suffer from a wide range of both short- and long-term health problems, with the risks of medical and social disabilities in adulthood increasing with decreasing gestational age at birth [2]. An additional gestational week can have significant benefits for infant survival and morbidity [3] as well as financial benefits for the National Health Service, with each preterm infant needing £22,885 more than a term birth over the first 18 yr of life [4]. Current tocolytics are only used over a 72-h period, which provides the opportunity to relocate the mother to a suitable facility and be able to administer drugs to develop fetal organs sufficiently to survive postutero [5]. Therefore, clear economic and medical needs exist for the identification and development of novel tocolytic therapies, the success of which lies in a greater understanding of the signaling pathways that control the balance between the quiescent and contracting uterus.

Tissue transglutaminase 2 (TGM2) is one of eight mammalian transglutaminase enzymes (TGM1, TGM2, TGM3, TGM4, TGM5, TGM6, TGM7, and F13A1) that catalyze the calcium-dependent formation of covalent bonds between protein-bound glutamine residues and primary amino groups, resulting in protein-protein cross-linking or amine incorporation into proteins [6]. Among this transglutaminase superfamily, most enzymes exhibit restricted tissue expression, with TGM1, TGM3, and TGM5 being expressed in hair and skin [7], TGM4 expressed specifically in prostate tissue [8], and F13A1 being a blood coagulation factor [7]. Little information currently is available in the literature regarding TGM6 and TGM7 [9]. Unlike the other transglutaminases, TGM2 is expressed in a number of tissues, exists in both intracellular and extracellular forms, and possesses both additional GTPase and intrinsic kinase activities [10, 11]. As a result of this multiplicity of activities, the functions of TGM2 remain a complex and intriguing conundrum, although roles in cell survival, motility, invasion, adhesion, and contractility have been demonstrated [12–16]. Links with vascular smooth muscle contractility also have been made [13], but to our knowledge, a role in myometrial contractility has not been investigated despite gene expression studies observing dramatically higher expression of TGM2 within the placenta and uterus [17]. Moreover, both the mRNA and protein expression of TGM2 are up-regulated in rat myometrium throughout pregnancy, reaching a peak at term [18]. Through utilizing transglutaminase inhibitors with isometric tension recording

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[2]Correspondence: FAX: 0115 8231208;
e-mail: joanne.lymn@nottingham.ac.uk

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and a live-cell calcium-signaling model, we sought the present study to investigate the potential role of TGM2 in the regulation of pregnant human myometrial contractility.

MATERIALS AND METHODS

Tissue Collection

Myometrial biopsy specimens taken from the upper margin of the lower uterine section were obtained during elective cesarean section (at term but not in labor) between 38 and 41 wk of gestation. Indications for surgery were maternal request, breech delivery, or previous cesarean section. Biopsy specimens were either stored in physiological salt solution (PSS; NaCl, 119 mmol/L; KCl, 4.69 mmol/L; $MgSO_4$, 1.17 mmol/L; KH_2PO_4, 1.18 mmol/L; $NaHCO_3$, 25 mmol/L; D-glucose, 5.5 mmol/L; and $CaCl_2$, 2.5 mmol/L) at 4°C overnight for contractility studies or prepared immediately for cell culture [19]. All biopsies were performed with written, informed patient consent and following the protocol approved by the Derbyshire Research Ethics Committee.

Isometric Tension Recording

Longitudinal myometrial tissue strips were mounted in an organ bath, and contractility was recorded as a measure of tensile force as previously described [20]. Contractile activity was stimulated by the addition of oxytocin (100 pmol/L; Sigma-Aldrich), phenylephrine (10 nmol/L; Sigma-Aldrich), or bradykinin (10 nmol/L; Sigma-Aldrich) or was spontaneously generated. Once rhythmic contractions were established, cumulative additions of the transglutaminase inhibitors cystamine (1 nmol/L to 10 mmol/L; Sigma-Aldrich), monodansylcadaverine (MDC; 10 nmol/L to 100 μmol/L; Sigma-Aldrich), *N*-benzyloxycarbonyl-L-phenylalanyl-6-dimethylsulfonium-5-oxo-L-norleucine (R281; 10 nmol/L to 100 μmol/L), and 1,3-dimethyl-2[(oxopropyl)thio]imidazolium (R283; 10 nmol/L to 100 μmol/L; both R281 and R283 synthesized and kindly donated by Professor Martin Griffin, Aston University, Birmingham, UK) were made at 25-min intervals. Activity integrals taken for 20-min time windows were used as a measure of contractile activity of the tissue strip as a percentage of that seen initially (i.e., before drug or vehicle addition). Control tissue strips were kept under the same conditions but with vehicle additions (PSS). Viability of tissue strips was assessed at the end of experiments by addition of KPSS (equimolar replacement of Na^+ in PSS for 20 mmol/L of K^+). Inhibitor-response curves of the activity integral were analyzed by fitting the data to the following equation using GraphPad Prism (Version 4.03 for Windows; GraphPad Software):

$$\text{response} = \text{bottom} + \frac{(\text{top} - \text{bottom})[x]}{[x] + [IC_{50}]}$$

where x is the drug concentration, IC_{50} is the median inhibitory concentration, and top and bottom are defined by the upper and lower plateaus of the curve, respectively. If the lower plateau was difficult to discern, the log IC_{50} was recorded as greater than the highest concentration used.

Calcium Assay

Human myometrial cells were harvested from tissue biopsy specimens as described previously [19]. Cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 2 mmol/L of L-glutamine, 10% fetal calf serum (FCS), 20 U/ml of penicillin, and 20 μg/ml of streptomycin at 37°C in a 5% CO_2 atmosphere. Once confluent, cells (passage 0–1) were plated in 96-well, black-walled plates; left overnight; and then incubated at 37°C for 45 min in 100 μl of DMEM containing 10% FCS, 2.5 mmol/L of probenecid, 2.5 μM Fluo 4AM (calcium indicator; Invitrogen), and 0.023% pluronic acid. Probenecid blocks active transport of the Fluo 4AM out of the cells, whereas pluronic acid stops Fluo 4AM breakdown by external esterases. Cells were then washed twice with PBS (Lonza) and incubated in 100 μl of PSS containing 2.5 mmol/L of probenecid at 37°C for 10 min with or without between 100 nmol/L and 10 μmol/L of cystamine or 1 μmol/L of MDC. Fluorescence was then measured on a FLEXstation (Molecular Probes) for 200 sec with or without the addition of oxytocin (10 pmol/L to 10 μmol/L) at 15 sec. Control cells had an equivalent volume of PSS added at the 15-sec time point. Concentration-response curves were determined by fitting data to a sigmoidal dose-response curve described by the following equation using GraphPad Prism (Version 4.03 for Windows):

$$\text{response} = \text{bottom} + \frac{(\text{top} - \text{bottom})}{(1 + 10^{((\log EC_{50} - x)\text{HillSlope})})}$$

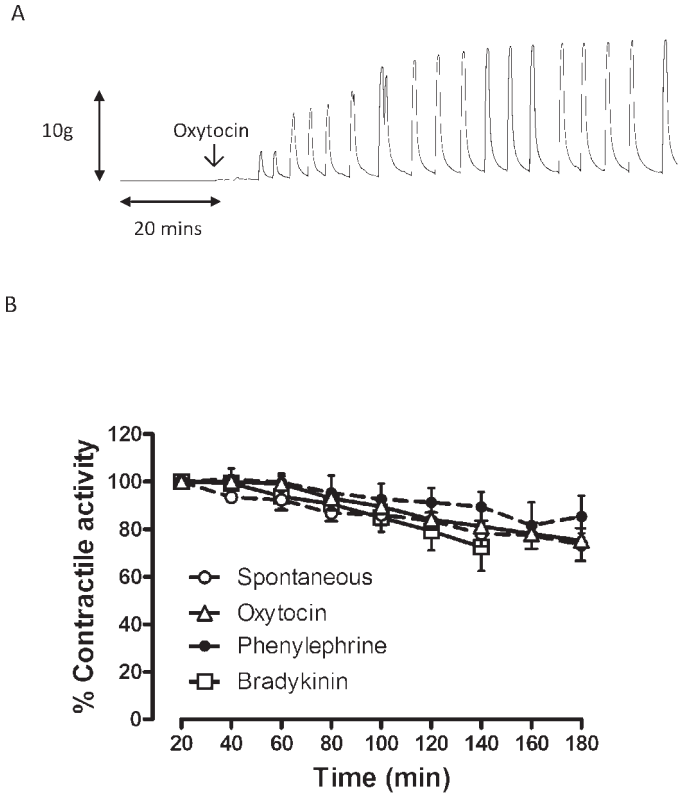

FIG. 1. Stimulation and maintenance of myometrial contractility. **A**) Representative trace recording of myometrial contractions, in this case induced by initial addition of 100 pmol/L of oxytocin (arrow). **B**) Grouped activity integrals of control strips, measured as a percentage of that observed initially, allowed to contract spontaneously (n = 6) or induced to contract by oxytocin (100 pmol/L, n = 14), phenylephrine (10 nmol/L, n = 6), or bradykinin (10 nmol/L, n = 6) over 140–180 min. Activity integral is used as a measure of contractile activity.

where x is the drug concentration, EC_{50} is the median effective dose, curves were fitted to a HillSlope of 1, and top and bottom are defined by the upper and lower plateaus of the curve, respectively.

Statistical Analysis

All data are presented as the mean ± SEM. Analysis of IC_{50} values, excluding those for which a lower plateau was difficult to discern, was compared between the different methods of contraction stimulus and the different inhibitors using a Kruskal-Wallis test followed by the Dunn multiple-comparison test if $P < 0.05$. For analysis of contractile activity, frequency, and amplitude, the effect of compound on these parameters was compared with that of the vehicle (PSS) using a repeated-measures, two-way ANOVA followed by the Bonferroni post hoc multiple-comparison test if $P < 0.05$. Analysis of contraction amplitude following restimulation of contractions was analyzed using a Mann-Whitney test. The characterization of the response of cultured myometrial cells to oxytocin and the effect of cystamine preincubation on calcium mobilization was analyzed by a repeated-measures, one-way ANOVA. The effect of MDC preincubation on calcium mobilization was analyzed using a paired *t*-test. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Initiation of Contraction and Time-Matched Controls

Agonist-induced and spontaneously evoked contractions reached stable rhythmic contractions within 60–90 min of mounting (Fig. 1A). In control tissue strips, equivalent additions of PSS reduced contractile activity by 15–26% at the end of the experimental time period dependent on stimulus of contraction (Fig. 1B), although neither contractile amplitude

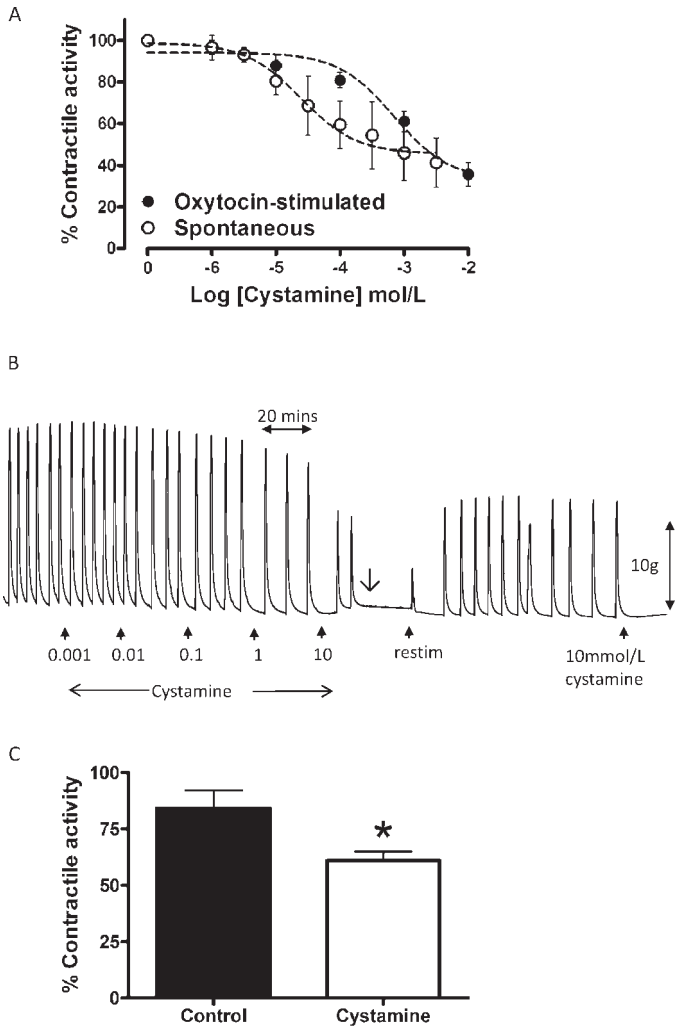

FIG. 2. Cystamine addition affects oxytocin-stimulated and spontaneously generated contractions. **A**) Cumulative additions of cystamine attenuated oxytocin-induced contractions (closed circles; n = 10) and spontaneously generated contractions (open circles; n = 9). **B**) Representative trace recording following stimulation with 100 pmol/L of oxytocin and cumulative cystamine addition (range, 1 μmol/L to 10 mmol/L; units shown are in mmol/L) and subsequent flushing with PSS and restimulation (restim) with 100 pmol/L of oxytocin. Baseline tension is increased by 0.5 g (downward arrow) after addition of 10 mmol/L of cystamine. Addition of 10 mmol/L of cystamine following resumption of contractions causes complete cessation. **C**) Contractile activity, as a percentage of that observed initially, of resumed contractions following flushing with PSS and restimulation with 100 pmol/L of oxytocin is significantly reduced compared to control strips (*$P < 0.05$, n = 9).

nor frequency alone was significantly affected. Because of the inherent variability in contractile performance, control tissues that either failed to respond to KPSS or showed greater than 40% reduction in activity (5 of 37 tissue strips) were excluded from analysis. Our choice of 40% as a cutoff was based on the average reduction expected in control strips over the duration of the experiment.

Effect of Cystamine on Spontaneous and Oxytocin-Stimulated Myometrial Contractions

Cystamine exhibited a concentration-dependent effect on oxytocin-stimulated contractility as measured by the activity integral, which was significantly reduced at 100 μmol/L ($P < 0.01$, n = 5); fitting data to a sigmoidal dose-response curve gave a log IC_{50} of −3.6 ± 0.3 mol/L (Fig. 2A). With cumulative cystamine additions, no significant effect on contraction frequency or amplitude was found except at the point of complete abolition of contraction. At the highest dose of cystamine tested (10 mmol/L), baseline tension was increased by 0.5 g, and complete cessation of contractions was observed for four of eight biopsy specimens (Fig. 2B). Following washout of cystamine from the organ bath with PSS and subsequent restimulation with oxytocin, contractions were recovered, although these were attenuated compared to initial contractile activity (60.9% ± 4.0% of initial contractile activity, n = 9) and significantly reduced compared to those observed with control strips following flushing with PSS (84.2% ± 7.9% of initial contractile activity; $P < 0.05$, n = 6) (Fig. 2C). Interestingly a final addition of cystamine following resumption of contractions resulted in a complete cessation in 47% of strips tested (Fig. 2B).

Cumulative additions of cystamine also significantly attenuated the contractile activity of spontaneous contractions at 100 μmol/L ($P < 0.05$, n = 9), and data fitted to a sigmoidal dose-response curve gave a log IC_{50} of −4.1 ± 0.3 mol/L (Fig. 2A). No significant effect on frequency or amplitude of contractions was found except at the point of complete abolition of contractions, which occurred for three of nine tissue strips.

Effect of MDC on Spontaneous and Oxytocin-Stimulated Myometrial Contractions

Cumulative additions of MDC significantly attenuated oxytocin-stimulated contractile activity of pregnant myometrial tissue strips at 10 μmol/L ($P < 0.01$, n = 7), with a log IC_{50} of greater than −4 mol/L (Fig. 3A). Contraction frequency and amplitude were not significantly altered. In five of the seven biopsy specimens, contractions were completely halted by addition of 100 μmol/L of MDC, and in four of these five specimens, contractions were resumed following flushing with PSS and restimulation with 100 pmol/L of oxytocin (Fig. 3B). However, contractile activity was significantly attenuated (49.0% ± 6.2% of initial contractile activity, n = 4) compared to that of control strips (84.2% ± 7.9% of initial contractile activity; $P < 0.05$, n = 6) (Fig. 3C).

Cumulative additions of MDC also resulted in a significant attenuation of the contractile activity of spontaneous contractions, with significant inhibition achieved at 10 μmol/L ($P < 0.05$, n = 7) and data fitting to a sigmoidal dose-response curve giving a log IC_{50} of −5.4 ± 0.5 mol/L (Fig. 3A). Neither contractile frequency nor amplitude was significantly reduced. For four of nine tissue strips, contractions were completely halted by 100 μmol/L of MDC.

Effect of Cystamine and MDC on Phenylephrine-Stimulated Myometrial Contractions

Cumulative additions of both polyamine transglutaminase inhibitors, cystamine and MDC, produced a dose-dependent attenuation of phenylephrine-stimulated myometrial contractions. Contractile activity was significantly reduced at 100 μmol/L of cystamine ($P < 0.01$, n = 9) and at 100 μmol/L of MDC ($P < 0.001$, n = 9). Derived log IC_{50} values were greater than −3 and −4.9 ± 0.3 mol/L for cystamine and MDC, respectively (Fig. 4A). Except in the case of complete cessation of contraction, no significant difference in contraction frequency or amplitude was found with either inhibitor. In five of nine tissue strips for cystamine and three of nine tissue strips for MDC, inhibition of transglutaminase caused total

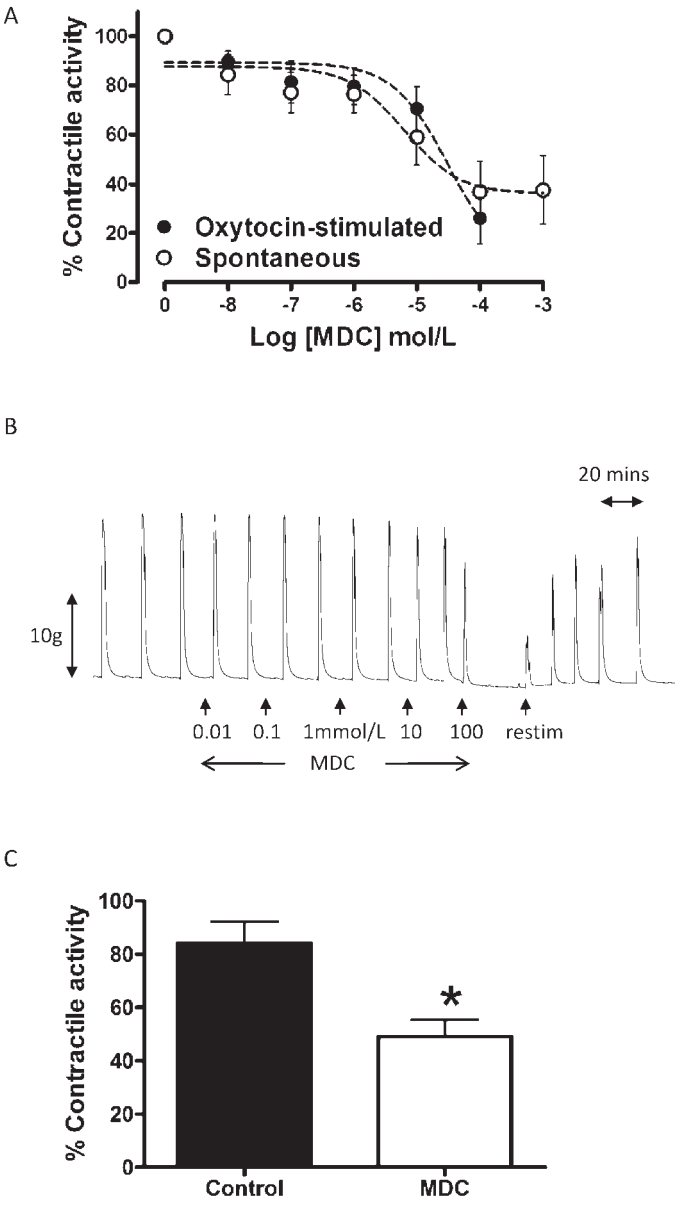

FIG. 3. MDC addition affects oxytocin-stimulated and spontaneously generated contractions. **A**) Cumulative additions of MDC attenuated oxytocin-stimulated contractions (closed circles; n = 7) and spontaneously generated contractions (open circles; n = 9). **B**) Representative trace recording of oxytocin-stimulated contractions in myometrial strips following cumulative MDC addition (range, 10 nmol/L to 100 μmol/L; units shown are in μmol/L), subsequent flushing with PSS, and restimulation (restim) with 100 pmol/L of oxytocin. **C**) Contractile activity, as a percentage of that observed initially, of resumed contractions following flushing with PSS and restimulation with 100 pmol/L of oxytocin is significantly reduced compared to control strips (*$P < 0.05$, n = 4).

abolition of contraction between 100 μmol/L and 1 mmol/L and between 10 μmol/L and 1 mmol/L, respectively.

Effect of Cystamine and MDC on Bradykinin-Stimulated Myometrial Contractions

Cumulative additions of both cystamine and MDC produced a dose-dependent attenuation of bradykinin-stimulated myometrial contractions. Contractile activity was significantly reduced at 10 μmol/L of cystamine ($P < 0.05$, n = 8) and at 10 μmol/L of MDC ($P < 0.01$, n = 6). Derived log IC_{50} values

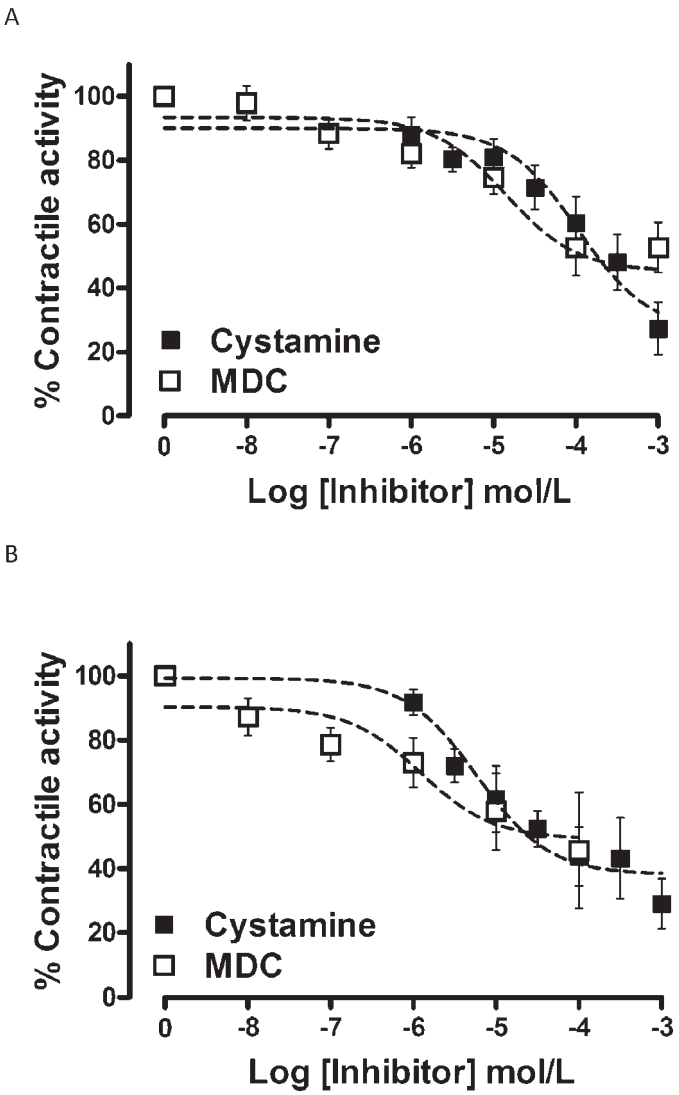

FIG. 4. Cystamine and MDC attenuate phenylephrine- and bradykinin-induced contractions. **A**) Phenylephrine-induced contractions were attenuated by cystamine (closed squares; n = 9) and by MDC (open squares; n = 10). **B**) Bradykinin-induced contractions were attenuated by cystamine (closed squares; n = 8) and by MDC (open squares; n = 6).

were −4.5 ± 0.4 mol/L for cystamine and −5.5 ± 0.7 mol/L for MDC (Fig. 4B). Except in the case of complete cessation of contraction, no significant difference in contraction frequency or amplitude was found with either inhibitor. In three of eight tissue strips for cystamine and three of six tissue strips for MDC, inhibition of transglutaminase caused total abolition of contraction at between 100 μmol/L and 1 mmol/L and at 100 μmol/L, respectively.

Effect of Potent, Site-Specific TGM2 Inhibitors on Spontaneously Generated and Oxytocin-Stimulated Myometrial Contractions

Both cystamine and MDC act to inhibit TGM2, but data in the literature suggest that at high concentrations, these compounds might inhibit other enzymes, including caspase 3 [21]. Therefore, to ensure that the effects seen with these compounds are related to an effect on transglutaminase activity, we used two irreversible, site-specific inhibitors, R281 and R283 [22, 23]. Contractility of all biopsy specimens

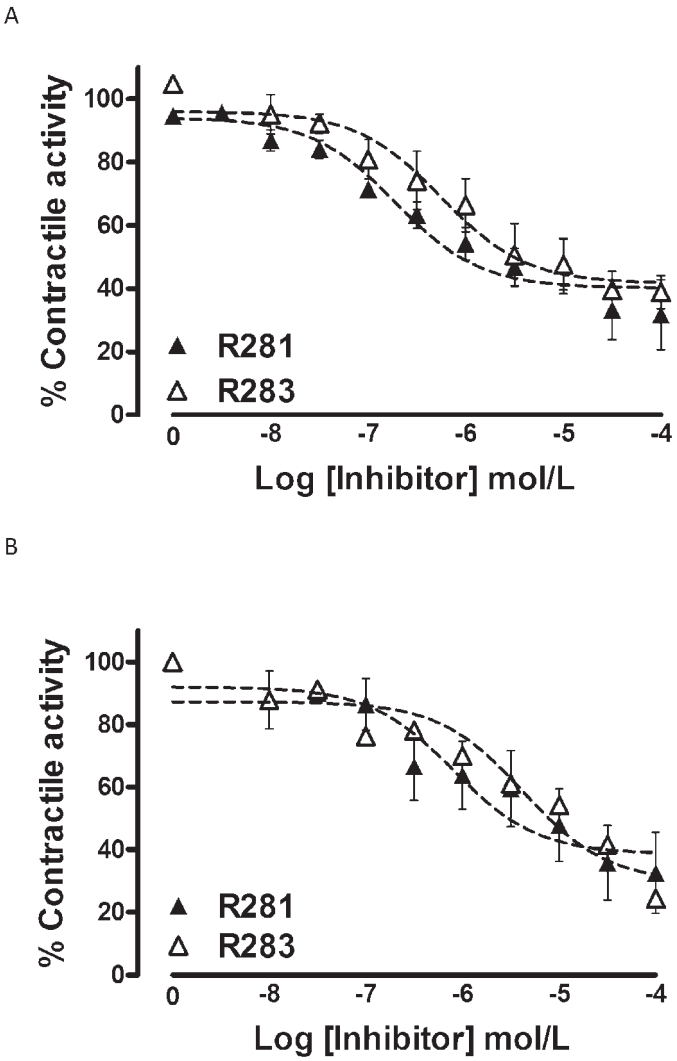

FIG. 5. Site-specific transglutaminase inhibitors, R281 and R283, attenuate spontaneously generated and oxytocin-induced contractions. **A**) Spontaneously generated contractions were attenuated by R281 (closed triangles; n = 3) and by R283 (open triangles; n = 4). **B**) Oxytocin-induced contractions were attenuated by R281 (closed triangles; n = 6) and by R283 (open triangles; n = 5).

was significantly attenuated following treatment with at least one of the site-specific inhibitors, although the two inhibitors acted differentially, with some biopsy specimens only responding to one.

Cumulative additions of site-specific inhibitors on spontaneously generated contractions attenuated contractions between 40% and 80% (six of nine biopsy specimens for R281 and five of seven biopsy specimens for R283), with complete abolition of contractions in one tissue strip following addition of 100 μmol/L of R283. Contractile activity was significantly reduced at 10 nmol/L of R281 ($P < 0.01$, n = 6) and at 1 μmol/L of R283 ($P < 0.05$, n = 5). Derived log IC_{50} values were −6.9 ± 0.3 mol/L for R281 and −6.5 ± 0.5 mol/L for R283 (Fig. 5A).

Similarly, cumulative additions of site-specific inhibitors produced variable responses in oxytocin-stimulated myometrial contractions, with three of six and four of seven tissue strips responding to treatment with R281 and R283, respectively. Contractility was attenuated by between 44% and 80% of initial activity, with complete abolition of contractions in one tissue strip following the addition of 30 μmol/L of R281. Contractile activity was significantly reduced at 10 μmol/L of R281 ($P < 0.05$, n = 3) and 30 μmol/L of R283 ($P < 0.01$, n = 4). Derived log IC_{50} values were −6.4 ± 0.3 mol/L for R281 and −6.3 ± 0.9 mol/L for R283 (Fig. 5B).

Effect of Transglutaminase Inhibitors on Oxytocin-Induced Calcium Mobilization

To determine whether the transglutaminase inhibitors, cystamine and MDC, were able to affect oxytocin-mediated calcium signaling, we studied the effect of inhibitor preincubation on cultured primary myometrial smooth muscle cells.

Oxytocin stimulation produced a dose-dependent increase in calcium mobilization, as measured by fluorescence intensity, in cultured myometrial cells, with a log EC_{50} of −7.7 ± 0.2 mol/L (n = 18) (Fig. 6A). Response to oxytocin stimulation was immediate, with an increase in fluorescence upon addition at 15 sec and a peak at 30 sec. Whereas the maximal calcium response was dependent on oxytocin concentration, no statistical difference in time to peak and rate of decay was found (data not shown).

Preincubation with cystamine at increasing concentrations (100 nmol/L to 10 μmol/L) appeared to cause a rightward shift in the dose response to oxytocin, with the highest concentration of cystamine (10 μmol/L) displaying a reduced maximum calcium mobilization (E_{max}). However, no significant difference was found in either E_{max} or log EC_{50} (n = 9–12) (Fig. 6B). Following preincubation with 1 μmol/L of MDC, a significant decrease was observed in the maximal calcium mobilization compared to that seen without inhibitor (E_{max}, 79.6% ± 3.6% of control; $P < 0.01$, n = 6), but no significant difference was observed in log EC_{50} (Fig. 6, C and D). No delay in response to oxytocin stimulation or duration of calcium mobilization was observed for either inhibitor.

DISCUSSION

The data presented here demonstrate significant attenuation of spontaneous and agonist-stimulated contractions in isolated pregnant human myometrial strips by the action of the polyamine TGM2 inhibitors, cystamine and MDC, and the site-specific TGM2 inhibitors, R281 and R283, suggesting a potential role for TGM2 in regulating uterine contractility in pregnancy. These observations are supported by the work of Houlihan et al. [24], who showed the attenuation of spontaneously contracting and oxytocin-mediated contractions in human pregnant myometrium by spermine, another polyamine inhibitor of TGM2 activity [25]. The potential importance of TGM2 in regulating contractility of the pregnant myometrium is evidenced by the inability of cystamine, at concentrations showing a significant effect in our studies, and of spermine to inhibit spontaneous contractions of nonpregnant human myometrium [24, 26]. Together with our data, this suggests modified signaling pathways operating in pregnant and nonpregnant uteri.

Of the two polyamine TGM2 inhibitors used in the present study, MDC was more potent than cystamine in all experiments, although both inhibitors demonstrated the same rank order of potency in response to the different contractile stimuli (bradykinin > spontaneous = phenylephrine > oxytocin). The lack of significant difference in IC_{50} values between either the inhibitors or the methods of contraction stimulus suggests a common method of inhibitory action, in keeping with an effect at the level of a subcellular enzyme. The concentration of cystamine required in the present study to inhibit myometrial contractility is comparable to that used to

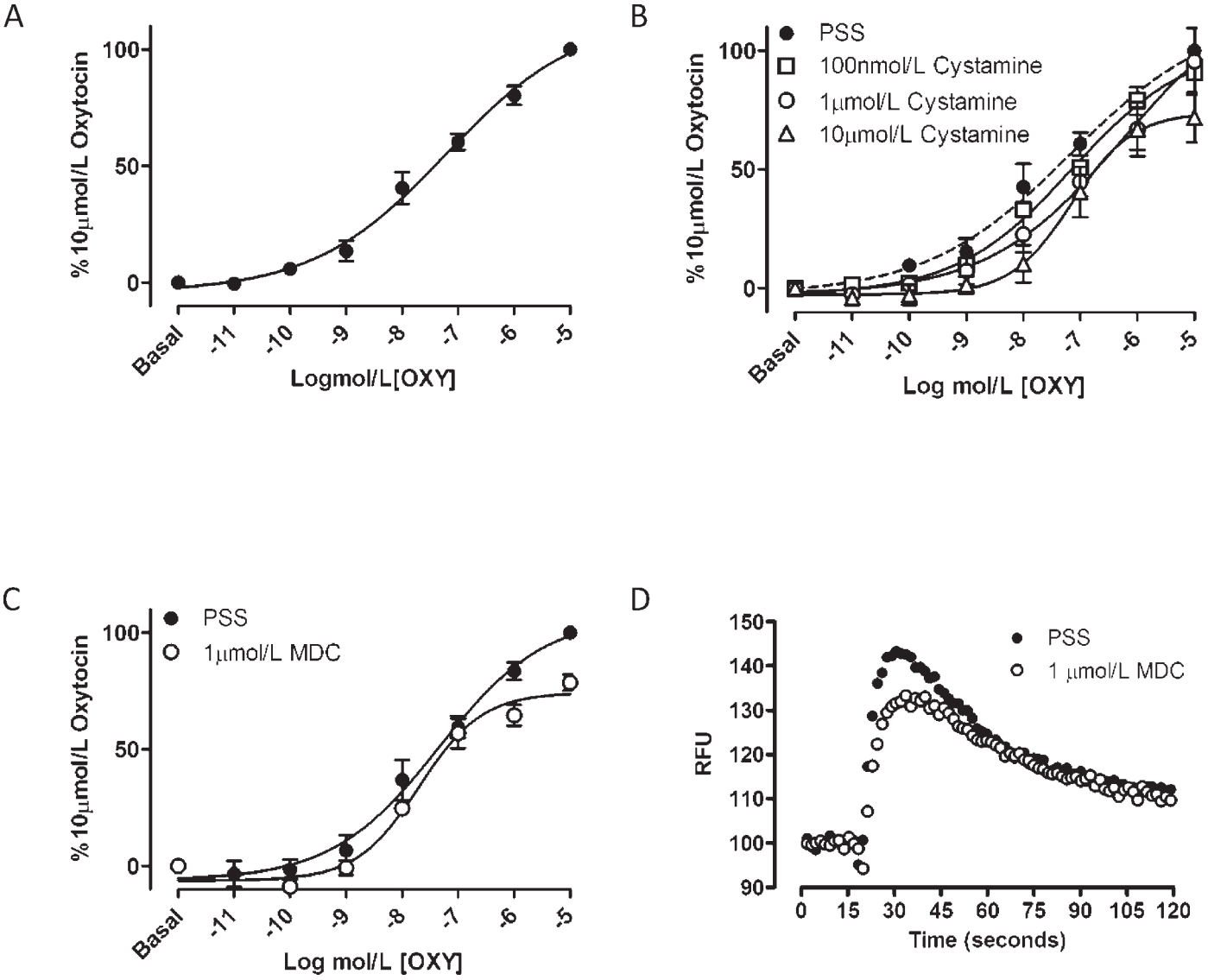

FIG. 6. Cystamine and MDC affect calcium mobilization in cultured myometrial cells. **A**) Oxytocin induced a concentration-dependent response on calcium mobilization in cultured, passage 1–2 myometrial cells. Fluorescent intensity is plotted as a percentage of that seen with the highest oxytocin addition (10 μmol/L). **B**) Preincubation for 30 min with cystamine (100 nmol/L to 10 μmol/L) produced a progressive rightward shift in the response to oxytocin (10 pmol/L to 10 μmol/L); however, no significant change was observed in log EC_{50}. **C**) Preincubation for 30 min with MDC (1 μmol/L) produced a depressed maximal response to oxytocin stimulation (10 pmol/L to 10 μmol/L) but no significant change in log EC_{50}. **D**) Representative trace of relative fluorescence intensity (RFU) over time seen following preincubation with MDC (open circles) or with PSS (closed circles). No delay in response or duration of peak was observed.

inhibit transglutaminase activity in an in vitro assay [27], demonstrating that despite the polar nature of these compounds, adequate penetration of tissue was achieved. The increased variability in response to cystamine, in terms of the concentration required to completely inhibit contractions, compared to MDC may potentially be a result of the conversion of cystamine to cysteamine within the tissue environment. Reduction of cystamine to cysteamine can occur in the cytoplasmic environment [28], and whereas cysteamine can still inhibit TGM2 activity, it has been shown to be the least effective transglutaminase inhibitor in comparison to cystamine, spermine, spermidine, and putrescine [27]. The residual inhibitory effect of cystamine and MDC on the contractile activity of recovered contractions following washing out of compound and agonist restimulation may be indicative of a lasting or prolonged effect on contractile activity, but it is more likely a result of incomplete washing out of inhibitor from the tissue strip.

Although both polyamine TGM2 inhibitors, cystamine and MDC, could potentially have had additional nonspecific but functional effects within the myometrial environment [21], the use of the site-specific, irreversible TGM2 inhibitors, R281 and R283, also resulted in inhibition of oxytocin-stimulated and spontaneous contractility, and these compounds were more potent in their effect. The effect of these peptide inhibitors on specific biopsy specimens was more variable, but all specimens responded by attenuation of contractile activity to at least one inhibitor. The variability between biopsy specimen responses is likely to be a function of human variability, possibly in relation to the potential interaction between TGM2 and factor XIII. The catalytic α-subunit of factor XIII (F13A1) is also a member of the transglutaminase family, and although generally thought of as a blood coagulation factor, F13A1 has been shown to work

in conjunction with TGM2 extracellularly to promote chondrocyte maturation [29]. Moreover Bakker et al. [30] have postulated the role of F13A1 in small artery remodeling to compensate for TGM2 loss. F13A1 is found within the uterus, but it is produced from monocyte-derived tissue macrophages [31] and not the smooth muscle cells. Despite the lack of F13A1 in the smooth muscle cells of the uterus, we cannot exclude the possibility of an interaction between the TGM2 inhibitors, R281 and R283, and F13A1 in our isometric studies.

Data from the present study also suggest that TGM2 may play a role in oxytocin-stimulated calcium signaling. Both cystamine and MDC appear to cause a rightward shift in the dose-response curve to oxytocin stimulation, with a repression of the maximum response at the highest concentrations used (significant for 1 μmol/L of MDC). These results could indicate a noncompetitive inhibition of the oxytocin-mediated calcium response, as would be expected for a downstream effect on the receptor.

Although normally an inactive enzyme, *Tgm2* gene expression levels have been demonstrated to be high in murine uterus (unknown pregnancy state) and placenta [17], and both myometrial TGM2 protein and mRNA levels are increased in a rat model of pregnancy, with peak values observed at term [18]. Both the GTPase and transglutaminase activity of TGM2 could potentially play a role in regulating uterine contractility. Through action as a GTPase, TGM2 has been demonstrated to couple α-adrenoceptors and oxytocin receptors to phospholipase C-δ1 (PLCD1) in pregnant rat and nonpregnant human myometrial tissue, respectively [18, 32]. The role of G protein-coupled receptor (GPCR) and phospholipase C (PLC) signaling in oxytocin-mediated contractility is well documented (for review, see [33]); however, the precise role of the PLCD1 isoform in modulating calcium signaling remains elusive (for discussion, see [34]). It has previously been reported that TGM2 can activate PLCD1 [35–37] and that both phenylephrine and oxytocin can signal through PLCD1 in pregnant rat and nonpregnant human myometrial tissue [32, 38], suggesting a potential role of the GTPase activity of TGM2 in regulating contractility. However, the same cannot be said of bradykinin. Bradykinin is linked to PLC activation in nonpregnant rat uterine strips via G_q and $G_{i/o}$ [39, 40], and potential sensitivity to TGM2 inhibition may be a secondary effect following the calcium influx initiating a PLCD1-TGM2-mediated contractile signal. Even so, when bradykinin-stimulated PLC activation was analyzed in a rat adrenal cell line (PC12), TGM2 was not detectable [41]. In relation to its transglutaminase activity TGM2 has been shown to transamidate and activate the monomeric G-protein RhoA [42], which initiates a cascade resulting in phosphorylation of myosin light chains and, hence, smooth muscle contraction (for review, see [43]). The proportion of activated (GTP-bound) RhoA has been shown to increase in human myometrium upon pregnancy and to display a significant shift in spontaneous preterm labor [44].

The specific activity of TGM2, GTPase versus transglutaminase, is dependent on its conformation, with the two main activities being mutually exclusive. Both cystamine and MDC bind competitively to the active site of the normal TGM2 amine substrate and cause a conformational change resulting in an altered stereochemistry that prevents the enzyme from acting as a transglutaminase or a GTPase [45, 46]. Both R281 and R283, however, bind irreversibly to the calcium-activated TGM2 and, hence, are more likely to only inhibit the transglutaminase function [22, 23]. Contractile attenuation from R281 and R283 therefore could potentially indicate that it is the transamidating role of TGM2 that is crucial. Indeed, because both R281 and R283, which have differing membrane permeability, are able to affect contractility with a similar potency, it may be that an extracellular function of TGM2, not an intracellular GTPase role, is likely to be most important in maintaining myometrial contractility.

Whereas a number of drugs can be used as tocolytics, the time period over which they can be administered is limited to 72 h. To date, none has demonstrated improved neonatal outcomes [5, 47], and as such, the need remains for further research into the pathways behind uterine contractility to provide novel tocolytic targets. This time restriction lies in the severe cardiovascular side effects that can arise from prolonged administration of beta-mimetics, such as ritodrine [48, 49], or in the loss of effectiveness with oxytocin antagonists, such as atosiban, which also have to be delivered intravenously [50]. Unlicensed therapeutics include the calcium-channel blocker nifedipine and the cyclo-oxygenase inhibitor indomethacin. Usually, however, these are only employed following failure or contraindication with licensed tocolytics, because nifedipine can lead to serious maternal hypotension and concerns exist that indomethacin could carry significant neonatal complications [51, 52]. Therefore, a clear need exists to identify novel targets for the development of tocolytic drugs. The attenuation of both agonist-induced and spontaneous contractions in pregnant myometrium and effect on calcium signaling by TGM2 inhibitors shown here suggests that TGM2 may represent a novel target for tocolytic drugs [53], although these data can only be regarded as a first step in the process toward this end. Further delineation of the specific activity and role of TGM2 in pregnant human myometrial contractility is critical, as is the development of TGM2 inhibitors that have a more consistent effect in human myometrial tissue, before TGM2 can be regarded as a potential therapeutic target for tocolysis.

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