Ca²⁺ regulation in detrusor smooth muscle from ovine fetal bladder after in utero bladder outflow obstruction

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Abstract:

Purpose: To characterize intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) regulation in fetal bladders following outflow obstruction by examining Ca^{2+} response to agonists in smooth muscle cells.

Materials and Methods: Severe bladder outflow obstruction (BOO) was induced in male fetal sheep by placing a urethral ring and urachal ligation midway through gestation at 75 days. Fetuses were examined 30 days after surgery. $[\text{Ca}^{2+}]_i$ in single smooth muscle cells isolated from bladder wall was measured with epifluorescence microscopy using Fura-2 AM during exposure to agonists, such as carbachol and ATP, and other activators, such as caffeine and KCl.

Results: Detrusor smooth muscle cells from obstructed bladders had similar resting $[\text{Ca}^{2+}]_i$ to those of sham-operated controls. The maximal response to carbachol was reduced following obstruction ($p < 0.05$), and construction of dose-response curves also demonstrated a higher EC_{50} values ($p < 0.05$). These changes were however not mirrored by caffeine-evoked Ca^{2+} release, which was not significantly different between the obstruction group and sham-operated controls. Kinetic analysis of carbachol transients further revealed an attenuated maximal rate of rise in obstructed bladders ($p < 0.01$). The magnitude of $[\text{Ca}^{2+}]_i$ to purinergic neurotransmitter ATP was also found to be smaller in cells from obstructed bladders ($p < 0.05$), although trans-membrane influx by high K depolarization was not significantly affected.

Conclusions: Muscarinic and purinergic pathways were down-regulated in fetal detrusor muscle following outflow obstruction. These major functional receptors appeared to be more susceptible to obstruction than other Ca^{2+} regulators and their impairment may contribute to compromised contractile function seen with in utero BOO.

Introduction

Human fetal bladder outflow obstruction (BOO) is an exclusively male disease, usually caused by posterior urethral valves (PUV). This condition affects 1/5000 male births and is the commonest cause of end-stage renal failure in children.¹ In addition to renal impairment, two-thirds of boys with PUV also suffer significant bladder dysfunction,² with common urodynamic findings of bladder instability, low capacity and poor bladder emptying.³

Our current understanding on the consequences of fetal bladder outflow obstruction in humans derives from postnatal studies of urinary tract function in boys with posterior urethral valves. In infancy these bladders generally have small capacity and thick walls,⁴ correlating with detrusor muscle overactivity on urodynamics.³ Later the bladder develops large capacity and becomes hypocontractile, often with progression to complete detrusor failure.³ Less is known about the effects of bladder outflow obstruction on human fetal urinary tract structure and function. In several studies of human fetuses with bladder outflow obstruction some histological changes have been documented. Freedman et al described thickened bladder walls with increased smooth muscle and collagen, but no change to their relative content.⁵ Kim et al reported increased muscle thickness but with decreased collagen relative to muscle.⁶ Workman and Kogan also found increased muscle thickness in human fetal bladders with posterior urethral valves, although in contrast to previous findings, there was a disproportionate increase in connective tissue.⁷ However, few studies have examined the pathophysiology of the fetal detrusor muscle exposed to bladder outflow obstruction.

The need to understand functional abnormalities as a consequence of PUV necessitates the use of experimental animal models to study the normally developing and obstructed fetal bladders. For these purposes, the ovine fetus has proved to be a useful model, as it is convenient for experimental intra-uterine surgical manipulation, and successful induction of bladder outflow obstruction produces dysregulation of normal growth and development.⁸ In two recent studies, severe obstruction of the fetal sheep bladder, by urachal and urethral obstruction, resulted in a large hypocontractile organ, accompanied by functional denervation and contractile abnormalities^{9,10} and bladder remodelling from enhanced apoptosis in detrusor muscle.¹¹ An altered collagen content in the detrusor wall has been shown to be a significant contributor to contractile abnormalities. However whether other cell components, smooth muscle in particular, are also involved, cannot be resolved in the multicellular preparations used in the study.¹⁰ A crucial question yet to be answered is whether intracellular Ca^{2+} regulation, a determinant of smooth muscle contraction, is altered due to obstruction. Of particular interest is whether alterations to cholinergic and purinergic pathways in detrusor muscle occur, such

as observed in both adult animal models of BOO and human neurogenic bladders¹²⁻¹⁵ when purinergic mechanisms assume a greater importance. We therefore tested the hypothesis that changes to intracellular Ca^{2+} regulation contribute to impaired contractility of smooth muscle from obstructed fetal bladders. We used single smooth muscle cells isolated from the obstructed sheep fetal bladder.

Materials and Methods

Experimental model. All work was conducted in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act 1986. Date-mated Romney Marsh ewes were purchased from the Royal Veterinary College, Potters Bar, UK. Partial BOO was induced in male fetuses by placing an omega-shaped silver ring around the urethra and completely ligating the urachus. The sham-operated fetuses experienced urethral and urachal exposure only. The experiments used seven fetuses for BOO and eleven age-matched fetuses served as control. Surgery was performed at mid gestation (75 days). At 30 days after surgery (105 days), the ewes were sacrificed by intravenous injection of Euthatal pentobarbitone sodium (Rhone Merieux, Athens, Georgia). Detailed protocols to induce *in utero* BOO in fetal sheep were described previously.⁹

Single cell preparation. The bladder dome was dissected and placed in Ca^{2+} -free HEPES-Tyrode's solution. Following removal of urothelium and adventitia, small strips of muscle (1 mm diameter, 5 mm length) were cut from the detrusor layer and placed in a collagenase-based enzyme mixture dissolved in the same solution. The muscle preparation was soaked in the enzyme at 25°C for 1 hour and then incubated at 37°C for 30 min. The enzymatic reaction was terminated by replacing the enzyme mixture with Ca^{2+} -free HEPES Tyrode's. The strips were gently triturated with a fire-polished glass pipette to release single cells. Dissociated cells were stored in Ca^{2+} -free HEPES Tyrode's at 4°C and used within 6 hours.

Measurement of intracellular free Ca^{2+} concentration, $[\text{Ca}^{2+}]_i$. $[\text{Ca}^{2+}]_i$ was measured with epifluorescence microscopy described previously. Cells were loaded with the Ca^{2+} -sensitive fluorochrome Fura-2 AM (Calbiochem, Germany) at 25°C for 30 min and de-esterified for 20 min before the experiment. An aliquot of cells was placed onto a perfusion chamber fitted on the stage of an inverted epifluorescence microscope (Nikon Diaphot, Japan) and 10 min were allowed for cells to settle and adhere to the glass bottom. Cells were continuously superfused in normal Tyrode's solution at 37°C, with a flow rate of $2 \text{ ml} \cdot \text{min}^{-1}$. The cell of interest was focused and an adjustable diaphragm was used to eliminate background interference. The excitation light at 340 nm and 380 nm was generated from a xenon light source and alternately directed to the cell via a dichroic mirror centered at 410 nm. The emitted fluorescent light was filtered at 510 nm and fluorescence from 410 nm-510 nm collected. The signals were amplified with a PMT (photonmultiplier) based spectrometer (Cairn UK). The analogue output was converted to digital data via an A-to-D converter (sampling interval 20ms) and acquired online to a PC for storage and analysis.

The intensity ratios at 340 nm and 380 nm excitation (R , 340/380) were calibrated using an in vitro method and converted into Ca^{2+} concentration according to the following equation:

$$[\text{Ca}^{2+}]_i = K_d ((R - R_{\min}) / (R_{\max} - R)) \beta$$

where K_d is the dissociation constant with a value of 224 nM, $R_{\min}$ and $R_{\max}$ are intensity ratios in 0 Ca^{2+} (no added Ca^{2+} and 5 mM EGTA) and saturating Ca^{2+} (2 mM CaCl_2) respectively, and β is the ratio of fluorescence intensities at 380 nm alone recorded in 0 Ca^{2+} and Ca^{2+} saturating solution.

Solutions: Normal Tyrode's solution contained (mM): NaCl, 118; NaHCO_3 , 24; KCl, 4.0; CaCl_2 , 1.8; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1.0; $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 0.4; glucose, 6.1; Na pyruvate, 5.0. Solutions were gassed with 95% O_2 : 5% CO_2 , pH 7.35. Ca^{2+} -free HEPES-Tyrode's contained (mM): NaCl, 105; HEPES, 19.5; KCl, 3.6; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.9; $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 0.4; NaHCO_3 , 22.3; glucose, 5.4; Na pyruvate, 4.5, pH 7.1 adjusted with 1 M NaOH. All chemicals were from Sigma-Aldrich Co Ltd, Poole, Dorset, UK.

Aliquots of 10 mM aqueous stocks of carbachol, ATP and ABMA (α , β methylene-ATP) were added to Tyrode's to achieve the desired concentrations. Powder forms of caffeine and KCl were added to Tyrode's solution to make 20 mM caffeine and high-K (120 mM) solutions; no osmotic corrections were made.

Data analysis and statistical methods: Results are expressed as means $\pm$ SE, n is the number of preparations unless otherwise indicated. Student's t -test was used to test the difference between two means. The null hypothesis was rejected for $p < 0.05$.

EC_{50} values (concentrations producing 50% of maximal effect) were obtained by the least-squares fit to the data points with the Hill equation:

$$y = (y_{\max} \cdot x^n) / (x^n + k^n),$$

Where y is the proportional response, $y_{\max}$ the maximum effect, x the concentration of an agonist, k the EC_{50} value, n the Hill coefficient. KaleidaGraph and Clampfit programs were used for curve fitting and kinetic analysis.

Results

Resting $[Ca^{2+}]_i$. The levels of resting $[Ca^{2+}]_i$ are a result of equilibrium of Ca^{2+} movement exerted by all Ca^{2+} regulators and represent homeostasis of Ca^{2+} metabolism. At rest, $[Ca^{2+}]_i$ in sham-operated normal fetal detrusor cells were 147 ± 11 nM, (n=35) and the value is similar in cells from BOO bladders: 141 ± 15 nM, (n=24, p=0.77). These data show that the steady-state Ca^{2+} equilibrium remained unchanged following outlet obstruction.

Evoked $[Ca^{2+}]_i$ rise via intracellular release. A major experimental objective was to investigate the effect of BOO on muscarinic receptor activation, the main functional receptor in bladder in all species using the agonist carbachol. Control experiments showed that carbachol-induced $[Ca^{2+}]_i$ transients in fetal detrusor myocytes were specific to muscarinic receptors, as they were blocked by 1 μ M atropine. In addition the source of Ca^{2+} was from intracellular stores, as they were abolished by prior application of high concentrations of caffeine. A maximal rise of $[Ca^{2+}]_i$ could be elicited by 10 – 100 μ M carbachol in these fetal detrusor myocytes. Figure 1 shows that in cells from obstructed bladders, the $[Ca^{2+}]_i$ transient in response to 30 μ M carbachol was attenuated compared with cells from sham-operated fetal bladders. In 22 cells from obstructed bladders, the maximal carbachol-induced $[Ca^{2+}]_i$ rise was 382 ± 55 nM, in contrast to 654 ± 74 nM from control cells (n=27, p<0.05), showing reduced efficacy of the receptors following obstruction (see also Figure 3).

Figure 1 near here

To ascertain whether an altered potency of carbachol also occurred, the dose-response relation was determined by application of carbachol over a range between 0.03 to 100 μ M. The minimal effective concentration was around 0.1 μ M and the maximal effect around 10 μ M. Figure 2A presents tracings of $[Ca^{2+}]_i$ transients at different concentrations of carbachol, showing a reduced relative magnitude at submaximal concentrations in cells from obstructed bladders. An EC_{50} of 1.0 ± 0.2 μ M was obtained from the dose-response relationship for cells from 11 control bladders, whilst the relationship was shifted to the right in cells from seven obstructed bladders (EC_{50} : 2.9 ± 0.8 μ M; p<0.05, Figure 2B). This observation suggests that in addition to attenuated efficacy, the sensitivity of muscarinic receptors to their neurotransmitter was also compromised. The shape of the two curves was similar, however, with a Hill coefficient near to unity in both groups (1.45 ± 0.22 and 1.13 ± 0.16 for control and obstruction respectively; p=0.32).

Figure 2 near here

To explain the observed reduction of carbachol-induced Ca^{2+} release, the effect of bladder obstruction on the magnitude of the intracellular Ca^{2+} store was investigated by examining the $[\text{Ca}^{2+}]_i$ rise after brief application of 20mM caffeine. Figure 3 shows that the average rise of $[\text{Ca}^{2+}]_i$ in myocytes from obstructed bladders was not statistically different from that in control cells (415 ± 104 nM, $n=10$ vs 568 ± 134 nM, $n=15$ respectively, $p=0.57$). This suggests that total releasable Ca^{2+} from intracellular stores was not significantly reduced by fetal BOO.

Figure 3 near here

The similar caffeine-evoked Ca^{2+} rise suggests that a reduced Ca^{2+} storage capacity was unlikely to underlie the suppressed carbachol-response seen in BOO myocytes, and the reason for a compromised ability to release Ca^{2+} was sought. The maximum rate of $[\text{Ca}^{2+}]_i$ rise following carbachol application ($d[\text{Ca}^{2+}]_i/dt$) correlates with Ca^{2+} release from IP_3 -mediated release from intracellular stores. Carbachol-induced Ca^{2+} -transients in myocytes from an obstructed and control bladders are superimposed in figure 4A, each expressed as 100% of their own maximum value. The upstroke phase is shown on an expanded time-base in figure 4B. Analysis of the upstrokes showed that with cells from obstructed bladders this rate was reduced significantly compared to control (0.66 ± 0.09 $\mu\text{moles.l}^{-1}.\text{s}^{-1}$, $n=22$ vs 1.47 ± 0.23 $\mu\text{moles.l}^{-1}.\text{s}^{-1}$, $n=27$, $p<0.01$).

Figure 4 near here

Evidence for impaired Ca^{2+} extrusion/re-uptake was also sought by measurement of maximal rate of relaxation following Ca^{2+} release by carbachol. A slower rate is apparent in Figure 4A following the peak of the $[\text{Ca}^{2+}]_i$ transient. The maximum rates of relaxation were 0.59 ± 0.10 $\mu\text{moles.l}^{-1}.\text{s}^{-1}$ ($n=22$) and 1.33 ± 0.27 $\mu\text{moles.l}^{-1}.\text{s}^{-1}$ ($n=27$) in cells from obstructed and control bladders, respectively ($p<0.01$).

Evoked $[\text{Ca}^{2+}]_i$ transients by transmembrane influx. Further experiments examined purinergic pathways as purinergic neurotransmission is a functional component in many non-primate species and also becomes operational in abnormal human bladders. The maximal Ca^{2+} rise by 30 μM ATP was 60 ± 20 nM ($n=10$) in myocytes from obstructed bladders, statistically different from 175 ± 52 nM ($n=18$) in control cells ($p<0.05$ Figure 3).

Ca^{2+} influx via Ca^{2+} channels by membrane depolarization is an important contributor to Ca^{2+} regulation in many smooth muscle cell types and has been shown to be sufficient to generate contraction and participate in purinergic activation in detrusor smooth muscle. As ATP-induced $[\text{Ca}^{2+}]_i$ transients were decreased following bladder obstruction, the involvement of Ca^{2+} channels was examined. In this study, a depolarization-induced Ca^{2+} rise was evoked by raising extracellular KCl from 4 mM to 120 mM. Such transients were abolished by the Ca^{2+} channel antagonist verapamil, indicating specific involvement of L-type Ca^{2+} channels. Our measurement showed a net rise of $[\text{Ca}^{2+}]_i$ by 108 ± 42 nM (n=8) and 96 ± 15 nM (n=14) for obstructed bladder and control myocytes, respectively and no statistical difference was found between these two means (p=0.98, Figure 3). Thus an attenuation of ATP transient is unlikely to be mediated by an effect on Ca^{2+} influx via Ca^{2+} channels.

Discussion

The present study has demonstrated that while steady-state cytosolic Ca^{2+} levels remain unaltered, the major receptor functions coupled to a rise of intracellular Ca^{2+} are down-regulated in fetal detrusor smooth muscle following severe BOO. These changes appear to be specific as Ca^{2+} entry by high K-induced depolarization was not significantly affected. These cellular lesions are likely to affect the contractile response to neurotransmitters and thus bladder function *in utero*. Both muscarinic and purinergic receptors were implicated. Of particular importance is the muscarinic system, as it is the predominant mechanism to provide Ca^{2+} for contractile proteins in bladder, evidenced by the large magnitude of carbachol-induced Ca^{2+} transients compared with other pathways (Figure 3). The compromised receptor function manifests as decreased receptor efficacy, indicated by the attenuated response to maximal dose of the agonist. Consistent with reduced receptor sensitivity to the ligand, the potency of the agonist was also decreased.

To date, no study has examined changes to intracellular Ca^{2+} regulation in fetal detrusor myocytes due to BOO. However, reduced receptor responses to neurotransmitters have been documented in hypertrophic muscle in adult hollow organs following obstruction or increased afterload. Impaired muscarinic as well as purinergic responses have been observed in hypertrophied human bladders and in adult models of severe bladder hypertrophy¹²⁻¹⁵. A reduction in β -adrenergic receptor responsiveness and subsequent altered myocardial contractile function has also been observed with pressure overload-induced cardiac hypertrophy.¹⁶ This implies that compromised receptor function may be part of the pathogenesis of muscular hypertrophy. Interestingly hypertrophic changes to smooth muscle cells have been observed from the present model of BOO,¹¹ suggesting that changes to receptor function are part of hypertrophic remodeling of fetal detrusor muscle following obstruction. The underlying mechanisms for altered receptor function are not fully understood at present but are likely to be multi-factorial. A primary cause for the impaired receptor function may be due to reduced receptor density, as has been observed with muscarinic receptors in detrusor muscle.¹⁷ However, a reduction of the total number of receptors cannot explain attenuated agonist potency, which further indicates reduced receptor sensitivity. The latter can at least in part be attributable to decreased receptor affinity to its transmitter. Indeed, lower receptor affinity has been observed in hypertrophic detrusor muscle, consistent with a shift in the [ratio of M2/M3 subtypes](#).¹⁶ A switch from subtype M3 to M2 would have an effect on the receptor affinity and hence the sensitivity to cholinergic neurotransmitters. Apart from alterations to receptors *per se*, abnormalities “downstream” from the receptors may also be responsible, which has been suggested for impaired

responsiveness to adrenergic stimulation.¹³ As such, impaired downstream coupling to the SR Ca^{2+} release (see later) has been shown in the present study, which would additionally contribute to the reduced efficacy and sensitivity of muscarinic receptors.

Altered Ca^{2+} responses to neurotransmitters may be compared with contractile abnormalities in obstructed fetal bladders. In the same model of *in utero* BOO, reduced contractile responses to nerve-mediated stimulation, as well as to agonists, have been observed.^{9,10} Denervation and a reduced muscle response to neurotransmitters/agonists were both implicated, but it was not possible to determine if the latter observation was due to a cellular defect or to a greater deposition of extracellular matrix in the form of collagen and elastin. The present study suggests that compromised receptor function in smooth muscle is at least a partial contributor to reduced contractility. Both the reduced maximal Ca^{2+} rise and potency may explain the reduced contractile response to cholinergic and purinergic agonists such as carbachol and ABMA (α , β methylene-ATP). In addition, a slower Ca^{2+} removal following contractile activation (see later discussion) would render myofilaments exposed to prolonged above-threshold levels of Ca^{2+} and compromise their ability to generate phasic contractions. This could be another contributing factor to reduced contractility in BOO. The change in EC_{50} of carbachol-induced Ca^{2+} transients was in contrast to the previous study, which did not show a change to EC_{50} to carbachol, when tension was recorded.¹⁰ However, the highest carbachol concentration in the previous study was 10 μM , and may not have produced a maximal response, in particular in obstructed bladders. An earlier work by Levin et al.¹⁸ found no significant differences between contractile responses to ATP, carbachol and KCl in fetal sheep bladders following a shorter-term obstruction. Together with our observation this suggests that these changes to excitatory stimuli only occur following longer-term obstruction, which represent cellular lesions in the de-compensated phase of BOO.

At the cellular level, the reduced receptor efficacy and sensitivity to muscarinic stimulation in BOO preparations may be associated with cellular signaling/coupling dysfunction. The decreased maximal rate of $[\text{Ca}^{2+}]_i$ rise of the carbachol-induced Ca^{2+} transient and the unaltered caffeine-induced Ca^{2+} transient following BOO means that reduced IP_3 receptor-coupled Ca^{2+} release may underlie the attenuated carbachol response, while the total releasable Ca^{2+} in the store remains the same. This observation also indicates that ryanodine receptors in the SR are not affected by BOO. Apart from the rate of Ca^{2+} rise, the relaxation was also slower, which may explain slower contractile relaxation (P Nyirady et al, unpublished data). Detrusor muscle from BOO rabbits exhibit a more tonic contraction¹⁹, and the slower kinetics of the Ca^{2+} transient as observed here may in addition to the

altered myosin subtypes contribute to this phenomenon. In hypertrophied heart, impaired removal of Ca^{2+} following excitation has also been noticed and found to be associated with reduced SR Ca^{2+} ATPase (SERCA2) activity.²⁰ The observed changes to the Ca^{2+} removal in the present study may be due to compromised SR re-uptake and/or sarcolemmal Ca^{2+} extrusion via Ca^{2+} -ATP pump, which are the major contributors to Ca^{2+} removal in detrusor muscle, although $\text{Na}^+/\text{Ca}^{2+}$ exchange cannot be excluded.

Conclusions:

Muscarinic and purinergic signaling in fetal bladder smooth muscle is compromised following BOO. This is accompanied by attenuated receptor-mediated Ca^{2+} release and Ca^{2+} removal. These changes in Ca^{2+} regulation may contribute to abnormalities in contractile function seen with *in utero* BOO.

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Figure legends**Figure 1**

Figure 1. Carbachol-induced (30 μ M) Ca^{2+}_i transients in fetal detrusor smooth muscle myocytes. Left: carbachol transient from a control myocyte; right: record from a myocyte of an obstructed bladder.

Figure 2

A

B

Figure 2. Comparison of the concentration-dependence of carbachol responses in myocytes from control and obstructed bladders. A. Representative record of carbachol-induced Ca^{2+} transients at different agonist concentrations; a supermaximal concentration was included as a reference: top, a control myocyte; bottom, a BOO counterpart. B. Dose-response relationships: the curves were fitted with the Hill equation to the average data points (mean $\pm$ SE, n=11 control, 7 BOO). Each curve is plotted with respect to its own maximum value at high carbachol concentrations.

Figure 3

Figure 3. Ca^{2+} transient magnitudes to maximum concentrations of several agonists in cells from control (sham) and obstructed (BOO) bladders: carbachol (10-100 μ M), caffeine (20mM), ATP (30 μ M) and high K (120mM), * $p < 0.05$ difference from control; mean $\pm$ SE, n=10-27.

Figure 4**A** **B**

Figure 4. Profile of carbachol-induced Ca^{2+} transients. A. Time course of transients from control and obstructed bladder myocytes; the onset of the events was aligned and the magnitude normalized for comparison. B. Initial phase of the Ca^{2+} transients on an expanded time-scale to reveal the difference in the rate of rise.