Ca²⁺ regulation in detrusor smooth muscle from developing fetal sheep bladders

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Abstract:

Aim: To characterize intracellular Ca^{2+} $[\text{Ca}^{2+}]_i$ regulation in fetal sheep bladders undergoing normal development by examining $[\text{Ca}^{2+}]_i$ responses to agonists and other physiological interventions in smooth muscle cells from different gestational ages. **Methods:** Three groups of fetal sheep bladders were investigated: 1) 65-70 days gestation (2nd trimester); 2) 105-110days (3rd trimester); 3) 135-140 days (term). Data from young adult sheep, aged 10-18months, were also included for comparison. $[\text{Ca}^{2+}]_i$ in single smooth muscle cells isolated from bladder wall was measured with epifluorescence microscopy using Fura-2 AM during either exposure to agonists: carbachol, caffeine, ATP and KCl or superfusion with low-Na solution. Specific inhibitors were applied to define the mechanisms of action. **Results:** Fetal detrusor smooth muscle cells had a similar resting to adult cells and exhibited transient $[\text{Ca}^{2+}]_i$ increases in response to carbachol, ATP, high K, caffeine and low-Na. The carbachol transients were abolished by atropine and prior application of caffeine; the ATP response was mimicked by α,β -methylene ATP (ABMA) and abolished by pretreatment with ABMA; high K-evoked $[\text{Ca}^{2+}]_i$ rises were antagonized by verapamil. The maximal responses to carbachol, high K, caffeine and low Na in fetal cells were similar to those of adult counterparts, whilst the ATP response was smaller ($p < 0.05$). These variables were largely similar between the three gestational groups and, with the exception of ATP-induced response, between myocytes from fetal and early adult bladders. Dose-response curves to carbachol demonstrated an increase of potency between mid-gestation and early adulthood ($p < 0.05$). **Conclusions:** Muscarinic receptors coupled to intracellular Ca^{2+} release, P2X receptor-linked Ca^{2+} entry, depolarization-induced Ca^{2+} rise via L-type Ca^{2+} channels, $\text{Na}^+/\text{Ca}^{2+}$ exchange and functional intracellular Ca^{2+} stores are all operational in fetal bladder myocytes. Whilst most of Ca^{2+} regulators are substantially developed and occur at an early fetal age, a further functional maturation for cholinergic sensitivity and purinergic efficacy continues throughout to the adult stage.

Introduction:

The urinary bladder undergoes several important changes during fetal development. In the human bladder a reservoir capacity is formed at the end of the 1st trimester -16 weeks gestation. By this time discrete inner and outer longitudinal layers, as well as a circular central layer have formed and the trigone and external urethral sphincter are also visible (Cuckow & Nyirady, 2001; Ludwikowski et al, 2001). Further progress to term is accompanied by increased muscularisation of the detrusor, trigone and urinary sphincter. During the same period there is a substantial increase in bladder capacity, which at intervals decreases rapidly with micturition (Nicolaidis et al, 1988; Rabinowitz et al, 1989).

In contrast to accumulating information on the morphology of bladder development, there are few functional studies. The fetal bovine bladder is relatively stiff at late first trimester, attributed to a high smooth muscle tone (Baskin et al 1994; Coplen et al 1994; Dean et al, 1997). Thereafter, compliance increases, accompanied by an increase of the collagen type-I:type-III ratio (Baskin et al., 1994; Koo et al, 1995). A similar pattern of collagen changes may also occur in human bladders (Kim et al, 1991). The progressive increase of compliance parallels the onset of urine production, and subsequent volume-dependent work that the bladder performs *in utero*. Furthermore by the end of the first trimester there is a significant cholinergic innervation to human fetal bladders (Jen et al., 1995), and in bovine fetal bladders there is an increasing contractile response to cholinergic agonists with gestational age (Koo et al 1995). Both the enhanced muscle activity and the increased bladder capacity are essential attributes in bladder development. Thus, in the 2nd trimester only half emptying occurred, which progressed to complete emptying later in gestation (Koo et al, 1995).

Further, interest in fetal bladder development comes from the need to understand its pathophysiology as a consequence of congenital anomalies; for example bladder outflow obstruction due to posterior urethral valves. The sheep fetus is a convenient experimental model for this condition, and fetal bladder outflow obstruction produces dysregulation of normal growth and development (Peters et al,

1992). The functional characteristics of detrusor from 120-day fetal sheep (term is 145 days) using normal bladders, or after 30 days severe obstruction, have been measured (Nyirady et al, 2002; Thiruchelvam et al, 2003a,b), and are in harmony with similar studies (Levin et al, 2001; Matsumoto et al., 2003). In brief, obstruction generates a hypocontractile, flaccid organ, accompanied by functional denervation. However, to understand the basis of these functional changes, and if these abnormalities could be reversed by ablation of the obstruction, it is necessary to document when during gestational development functional characteristics appear

Recently, the time course of development of contractile characteristics in the fetal sheep bladder has been documented by measuring nerve-mediated and neurotransmitter-elicited responses (Nyirady et al 2005). This study showed that fetal development between 2nd trimester (70 days) and term in the sheep is associated with increased contractile activation: an increase of muscle responses at earlier stages (70-110 days) and continued development of innervation until term. However, these studies left a number of questions, namely; are changes to muscle contractility mirrored in changes to cellular Ca^{2+} regulation; do the responses to different agonists develop along similar time courses; does the sensitivity to excitatory transmitters alter during development. The aim of the current study was to answer these questions using detrusor myocytes isolated from fetal bladders, at the same time points as those used to make contractile recordings.

Results and Discussion

Resting $[Ca^{2+}]_i$. At rest, $[Ca^{2+}]_i$ in fetal detrusor cells was 120 ± 8 nM, (n=24), comparable to those of preparations from adult sheep – 124 ± 15 nM (n=9, $p>0.05$). Fetal cells from different gestational ages had similar resting $[Ca^{2+}]_i$: 119 ± 16 nM (n=7, 70 days,) 117 ± 10 nM (n=11, 110 days) 118 ± 18 nM (n=5, 140 days), with no statistical difference between any two groups. Spontaneous small, discrete intracellular resting Ca^{2+} -transients were observed in 10-20 % of cells from both fetal and adult preparations (figure 1A), whereas at other times the resting $[Ca^{2+}]_i$ remained entirely stable (figure 1B), similar to other species (Fry et al 2004). The exception was in myocytes from three of the seven 70-day fetal bladders, where long-lasting Ca^{2+} waves were also observed (Figure 1C).

Intracellular Ca^{2+} transients from intracellular stores.

Ca^{2+} transients were elicited by brief exposures to the muscarinic receptor antagonist carbachol, or caffeine. Caffeine at millimolar concentrations releases Ca^{2+} from the sarcoplasmic reticulum (SR) through activation of ryanodine receptors (Pessah et al 1987). Figure 2A shows the response to carbachol, a transient increase of $[Ca^{2+}]_i$ followed by an undershoot before return to normal. This waveform has been observed in adult detrusor cells from other species (Wu and Fry 1998; Wu et al 1999), and as well as other smooth muscle cell types (Baro et al 1993) and has been suggested to be a feature of Ca^{2+} release from intracellular stores. Application of 1 μ M atropine completely abolished the carbachol response (figure 2B, n=5), suggesting the action is mediated via muscarinic receptors.

Caffeine (20 mM) generated a large $[Ca^{2+}]_i$ transient, comparable to those by carbachol and with a similar morphology (Figure 2C). Immediate application of a second dose of caffeine failed to evoke a further Ca^{2+} -transient, an indication of store emptying; however the response was fully recovered after 4-5 minutes. In addition, application of carbachol immediately after a caffeine-induced Ca^{2+} -transient failed to elicit a second response, indicating a common source of Ca^{2+} for both transients. (Figure 2D).

Intracellular Ca^{2+} transients from transmembrane Ca^{2+} influx.

Fig 3A shows a Ca^{2+} -transient after application of 10 μM ATP, but in this case an undershoot was absent, and consistent with the hypothesis that the transient did not originate from intracellular Ca -stores (Baro et al., 1993; Wu et al., 1999). Furthermore application of the non-hydrolysable analogue α,β -methylene ATP (ABMA), produced a similar Ca^{2+} transient, suggesting that the receptor involved is P2X_1 or P2X_3 (North 2002) and abolished the response to concomitant addition of ATP (Figure 3B). The ATP response did however recover when the ABMA was removed. The magnitude of the Ca^{2+} -transients diminished when successive ATP exposures were administered every 15 minutes, but this did not occur with similar carbachol exposures and suggests that the former decline may be due to purinergic receptor desensitization.

It has been shown previously that ATP-induced Ca^{2+} transients can be abolished rapidly and reversibly by treatment with L-type Ca^{2+} channel antagonists suggesting transmembrane Ca^{2+} influx. Evidence for such Ca^{2+} entry was sought by exposing myocytes to a raised extracellular $[\text{K}]$ to depolarize the cells, as this has been observed in adult human and guinea-pig detrusor myocytes (Wu et al, 1999). Figure 4A shows a Ca^{2+} transient upon raising the extracellular KCl from 4 to 120 mM, with a distinct feature of a sustained phase following the initial peak of $[\text{Ca}^{2+}]_i$, as long as the high-K solution is maintained. The response was abolished by the L-type Ca^{2+} channel antagonist verapamil (20 μM , n=5).

Figure 4B shows also the existence of a functional $\text{Na}^+/\text{Ca}^{2+}$ exchanger, also demonstrated in adult guinea-pig detrusor myocytes (Wu and Fry 2001). A reduction of extracellular $[\text{Na}]$ from 147.4 mM to 29.4 mM produced a rise of intracellular Ca^{2+} , which remained elevated in the presence of the low-Na solution. The magnitude of the $[\text{Ca}^{2+}]_i$ rise was $198 \pm 96\mu\text{M}$, equivalent to $26.0 \pm 10.7\%$ when paired with corresponding maximum 30 μM carbachol transients in the same cell (n=6).

Comparison of agonist-mediated responses between fetal and adult bladders. The maximal response to carbachol (10-20 μ M) in fetal myocytes was 629 ± 108 nM (n=23), compared to a value of 373 ± 41 nM in cells from adult bladders (n=8). Although the mean values were not significantly different (Wilcoxon sign-rank test, $p>0.05$), the variability of the responses in fetal myocytes was significantly greater than in adult cells (F-test, $p<0.01$). When 20 mM caffeine was used to elicit Ca^{2+} -transients the maximum values were 540 ± 100 nM (n=23) and 471 ± 128 nM (n=8) respectively, in cells from fetal and adult bladders (figure 5B); neither the mean values nor the variability were significantly different. The ratios of the magnitude of caffeine:carbachol Ca^{2+} -transients were also similar in cells from fetal and adult bladders: $87\pm6\%$ (n=23) and $111\pm18\%$ (n=8) for respectively.

However, comparison of the membrane-dependent routes for increasing the $[\text{Ca}^{2+}]_i$ revealed some interesting differences. For the ATP-dependent Ca^{2+} -transients, the mean magnitude obtained from fetal myocytes was significantly smaller than their counterpart carbachol and caffeine-induced transients. Moreover the value was significantly smaller than corresponding Ca^{2+} -transients from adult bladder myocytes (191 ± 67 nM vs 587 ± 269 nM, $p<0.05$, fetal vs adult respectively). The magnitude of the high-K and low-Na induced Ca^{2+} transients in myocytes from fetal bladders were also significantly lower than their carbachol and caffeine-induced counterparts. In these cases however, the values were not significantly different in cells from adult bladders (high-K; 94 ± 22 nM (n=22) vs 125 ± 38 nM (n=8), fetal vs adult: low-Na; 198 ± 96 nM (n=6) vs 172 ± 69 nM (n=8).

To determine if there were changes to the agonist response during the fetal development period the data from fetal bladders were subdivided into the three groups and are displayed in figure 5, along with data from adult bladders. With respect to the carbachol and caffeine-induced Ca^{2+} transients their magnitudes were not significantly different in all four groups. The high-K Ca^{2+} -transient magnitudes were also not significantly different between the four groups. With respect to the ATP-

generated Ca^{2+} -transients the data was more equivocal. The mean Ca^{2+} -transient magnitude in the 70-day group (Group 1) was significantly smaller than the adult mean value, but there were no other significant differences. However, as indicated above when all the fetal bladder data was combined into a larger group the Ca^{2+} -transient magnitude was smaller than the adult data set.

Figure 5 near here

Although the magnitude of the Ca^{2+} -transient induced by a large carbachol concentration was not different in the various fetal groups and the adult group, it is possible that the potency of the agonist may vary during development. This was tested by constructing dose-response curves to carbachol and estimating the EC_{50} value. Figure 6 shows dose-response curves to carbachol for the three fetal and the adult group. The potency to carbachol was greatest in myocytes from adult bladders, least in the group 1 bladders and intermediate in the group II/III bladders. The values for the four groups were 2.75 ± 1.13 , 1.03 ± 0.17 , 1.05 ± 0.12 and 0.42 ± 0.05 μM for Group I, II, III fetal bladders and adult bladders respectively. Between Group I and Group II/III bladders the increase of potency was significant, and the same was true between Groups II/III and adult bladders.

Discussion, Cellular Ca^{2+} regulation in fetal detrusor smooth muscle. The present study has demonstrated that fetal detrusor smooth muscle cells maintain a similar low resting $[\text{Ca}^{2+}]_i$ to that in adult cells, and that the major Ca^{2+} entry pathways and intracellular Ca^{2+} regulators are operational as early as mid-gestation. To date, there is only limited work on intracellular Ca^{2+} regulation in fetal smooth muscle cells and no study has followed changes during development. In cerebral arteries from near-term fetal sheep, noradrenaline-induced Ca^{2+} -transients have been described (Long et al 2000), and in full-term fetal rabbits pulmonary artery smooth muscle cells respond to caffeine with Ca^{2+} -transients via ryanodine-sensitive release from the sarcoplasmic reticulum (SR) (Porter et al 2000). These observations together with those from the present study, support the notion that

receptor-coupled intracellular Ca^{2+} rise as well as functional intracellular Ca^{2+} stores are sufficiently developed in some smooth muscles before birth.

Activation of the main functional receptors to muscarinic and purinergic agonists generated Ca^{2+} -transients with profiles similar to those observed in adult detrusor cells. The variability of the carbachol-induced responses in fetal cells may suggest that the distribution of muscarinic receptors may be more heterogeneous in different fetal cells, but it is not known if this represents regional variation in the fetal bladder. Carbachol-induced Ca^{2+} -transients are probably generated by Ca^{2+} release from intracellular stores, as exposure to caffeine immediately prior to carbachol addition abolished the carbachol response. Caffeine releases completely Ca^{2+} from the SR via its effect on ryanodine receptors in many cell types (Pessah et al 1987; Poulsen et al 1995), and a similar complete release was observed in fetal detrusor cells. The similarity of the caffeine- and carbachol-evoked Ca^{2+} -transients implies that a large concentration of carbachol (10 μM) is able to release completely intracellular Ca^{2+} stores.

Transmembrane Ca^{2+} fluxes were also recorded in fetal detrusor cells. Exogenous ATP evoked a large intracellular Ca^{2+} transient that was mirrored by the non-hydrolysable analogue α,β -methylene ATP (ABMA). ABMA binds preferentially to ionotropic P2X_1 or P2X_3 purinoceptors (North 2002), that depolarise the membrane and permit Ca^{2+} entry through Ca^{2+} channels. ABMA also desensitises these purinoceptors, as demonstrated by the ineffectiveness of ATP to evoke a Ca^{2+} -transient when applied immediately after ABMA exposure. Consistent with depolarising-induced Ca^{2+} entry, raised extracellular $[\text{K}]$ also generated Ca^{2+} -transients that were abolished by the L-type Ca^{2+} channel antagonist verapamil.

Of interest is that the magnitude of the Ca^{2+} transients evoked by ATP and high-K were similar in myocytes from fetal bladders, and significantly smaller than the corresponding carbachol transients.

This observation was mirrored by larger carbachol-induced contractures in fetal detrusor, compared to ABMA or high-K contractures (Nyirady et al., 2005), and implies that the intracellular release of Ca^{2+} via muscarinic receptor activation is more important than Ca^{2+} influx in fetal myocytes to initiate contraction. Another transmembrane route for Ca^{2+} is via $\text{Na}^+/\text{Ca}^{2+}$ exchange, as in adult cells: the increase of $[\text{Ca}^{2+}]_i$ was similar to that generated by membrane depolarisation. The importance of the exchanger may be to maintain intracellular Ca^{2+} and store refilling rather than initiate contraction (Wu et al 2001).

Comparison of Ca^{2+} regulation in myocytes from adult different fetal age bladders. The similarity of the values of the resting $[\text{Ca}^{2+}]$ in all three fetal groups and adult myocytes suggests that mechanisms which regulate intracellular $[\text{Ca}^{2+}]$ develop at an early gestational age. However, spontaneous rises of intracellular Ca^{2+} were more pronounced in 70-day (group I) myocytes, possibly due to the fact that these processes have not yet attained full capacity. These spontaneous Ca^{2+} transients may contribute to greater active muscle tone and contribute to the reduced compliance of bladders in earlier fetal age (Dean et al 1997). This spontaneous activity may also be necessary to promote voiding when the neuronal control of the bladder is incomplete (Nyirady et al, 2005).

The Ca^{2+} -transients in myocytes from bladders of different fetal age may be compared to the corresponding contractile responses from our previous study. The magnitude of Ca^{2+} -transients were similar throughout the gestation period from 70 days to near term (groups I-III). This contrasts with the contractile data (Nyirady et al., 2005) that showed a significant increase of tension between groups I and II. It is unlikely therefore that the increase of force is due to maturation of intracellular signalling pathways, which seem to be fully developed by mid-gestation. The simplest explanation is that there is an increase of relative muscle mass, development of contractile proteins (Arens et al 2000), and/or innervation. However, because the carbachol contracture increased more than the nerve-mediated contraction then the muscarinic system itself must also have been upregulated. One

explanation is the increased sensitivity of the carbachol-induced Ca^{2+} -transient over this period, as a fixed concentration of carbachol (10 μM in the previous study) may not have generated a maximum contracture.

ATP-generated Ca^{2+} -transients appeared to demonstrate a difference between fetal and adult bladder myocytes, although a progression throughout development was not observed, due in part to the relatively small numbers of available bladders. However, because a similar trend was not seen with the high-K Ca^{2+} transients, this is consistent with the hypothesis that the number of purinergic receptors, rather than the number of Ca^{2+} channels increases in adult versus fetal bladder. This is consistent with a previous study which showed a higher expression P2X receptor mRNA in adult human bladders than in fetal tissue (O'Reilly et al 2001): there is no corresponding data for L-type Ca^{2+} channels. These overall observations are contrary to the general belief that the intracellular Ca^{2+} pool in fetal bladder is under-developed, as inferred from indirect variables (Longhurst 2004).

In summary, major receptors coupled to intracellular Ca^{2+} are substantially developed in fetal bladder and occur at an early fetal age, although a small further refinement of function takes place later. Ca^{2+} entry via membrane ion channels and exchangers is well developed, and is mirrored by an equal efficacy of Ca^{2+} release from the intracellular stores. Whilst these Ca^{2+} regulators contribute dynamic changes to intracellular Ca^{2+} in response to physiological stimuli, a tightly controlled resting Ca^{2+} level below the threshold for many cellular activations provides a stable environment to maintain normal cellular function. Altered Ca^{2+} regulation is likely to contribute to abnormal contractile function during fetal development.

Experimental procedures

Animals and experimental groups. All work was conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986. Date-mated Romney Marsh ewes and young adult sheep were purchased from the Royal Veterinary College, Potters Bar, UK. Detrusor smooth muscle samples from the bladders of 22 fetuses between 70 and 140 days of gestation (term is 145 days) were used. Fetuses were obtained from three gestation groups: I. 2nd trimester, 65-70 days gestation (n=7); II. 3rd trimester, 105-110 days gestation (n=10); III pre-term, 135-140 days gestation (n=5). Fetuses from groups I and III were controls from a non-urological study investigating the application of gene therapy to fetal sheep using ultrasound-guided injection of replication-deficient adenoviral vectors. Bladders were obtained at post mortem at least two days following the injection procedure. Group II fetuses were a control group for a study of infravesical obstruction (Nyirady et al, 2002). No sheep in any group underwent urogenital tract manipulation or instrumentation. The fetuses were of either sex, but it has been shown previously (Nyirady et al., 2002; Thiruchelvam et al., 2003a) that there are no functional sex differences in fetal sheep. Young adult sheep, of either sex (10-18 months old), were controls from a study of ageing on brain function; again no functional differences were found between sexes. Ewes or young adult sheep were sacrificed by intravenous injection of Na pentobarbitone (40 ml vet, diEuthatal, Rhone Merieux, Athens, USA).

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 muscle strips (1 mm diameter, 5 mm length) were cut from the detrusor layer and placed in an enzyme mixture dissolved in the same solution. The muscle strip 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, $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured with epifluorescence microscopy described previously (Wu et al 1998). 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 use for experiment. An aliquot of cells were 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 glass bottom. Cells were continuously superfused in normal Tyrode's solution at 37°C, with a flow rate of 2 ml.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 410nm. The emitted fluorescent light was filtered at 510nm and fluorescence from 410nm-510nm collected. The signals were amplified with a photomultiplier-based spectrometer (Cairn, Sittingborne, UK). The analogue output was converted to digital data via an A to D converter and acquired online to a PC for storage and analysis.

The intensity ratios at 340nm and 380nm excitation (R , 340/380) were calibrated using an in vitro method (Wu et al 1998) and converted into Ca^{2+} concentration according to the following equation (Grynkiewicz et al 1985):

$$[Ca^{2+}]_i = K_d ((R - R_{min}) / (R_{max} - R)) \beta \quad (1)$$

where K_d is the dissociation constant with a value of 224nM, R_{min} and R_{max} are intensity ratios in 0 Ca^{2+} (no added Ca^{2+} and 5mM EGTA) and saturating Ca^{2+} (2mM $CaCl_2$) respectively, and β is the ratio of fluorescence intensities at 380nm 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; $MgCl_2 \cdot 6H_2O$, 1.0; $NaH_2PO_4 \cdot 2H_2O$, 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; $MgCl_2 \cdot 6H_2O$, 0.9; $NaH_2PO_4 \cdot 2H_2O$, 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. Low Na solution was composed of (mM): Tris·HCl, 118; NaHCO₃, 24; KCl, 4.0; CaCl₂, 1.8; MgCl₂·6H₂O, 1.0; NaH₂PO₄·2H₂O, 0.4; glucose, 6.1; Na pyruvate, 5.0, thus containing 29.4mM Na⁺, compared to 147.4 mM Na⁺ in normal Tyrode's solution. NaCl was substituted for by Tris·HCl, while NaHCO₃ and Na pyruvate were not altered to in order to maintain a normal cellular pH regulation and metabolism.

Carbachol (10 mM), atropine (1 mM), ATP (10 mM) and verapamil (5mM) were stored as aqueous stocks, and diluted in Tyrode's to the desired concentrations. Powder forms of caffeine and KCl were added to Tyrode's solution to make 20mM caffeine and high-K (120 mM) solutions; no osmotic correction was made.

Data analysis and statistical methods: Results are expressed as means $\pm$ SE, n is the number of bladders unless otherwise indicated. Analysis of variance was applied to test the significance of the overall difference among the age groups and the Student-Newman-Keuls test was further performed to examine differences between the groups. For comparison of two samples, Student's t -test was used for data of normal distribution and equal variance, and non-parametric tests were used for data with unknown distribution or unequal variance. The null hypothesis was rejected for $p < 0.05$.

The EC₅₀ (concentrations producing 50% of maximal effect) were obtained by a least square fit to the data points with the Hill equation:

$$y = (y_{\max} \cdot x^n) / (x^n + k^n), \quad (2)$$

y is the proportional response of $y_{\max}$, the maximum effect; x the concentration of an agonist; k the EC₅₀ value; n the Hill coefficient.

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References

- Arens YH, Rosenfeld CR and Kamm KE (2000). Maturation differences between vascular and bladder smooth muscle during ovine development. *Am J Physiol* 278: R1305-1313.
- Baro I, O'Neill SC and Eisner DA (1993). Changes of intracellular $[Ca^{2+}]$ during refilling of sarcoplasmic reticulum in rat ventricular and vascular smooth muscle. *J Physiol* 465:21-41.
- Baskin LS, Constantinescu S, Duckett JW, Snyder HM, Macarak E 1994. Type III collagen decreases in normal fetal bovine bladder development. *J Urol* 152: 688-691.
- Coplen DE, Macarak EJ and Levin RM (1994). Developmental changes in normal fetal bovine whole bladder physiology. *J Urol* 151: 1391-1395.
- Cuckow PM, Nyirady P. Embryology of the Urogenital Tract. In: Gearhart JP, Rink RC, Mouriquanad PDE, editors. *Pediatric Urology*. Philadelphia: W.B. Saunders, 2001: 3-13.
- Dean GE, Cargill RS, III, Macarak E, Snyder HM, Duckett JW, Levin R 1997. Active and passive compliance of the fetal bovine bladder. *J Urol* 158: 1094-1099.
- Fry CH, Sui GP, Severs NJ and Wu C (2004). Spontaneous activity and electrical coupling in human detrusor smooth muscle: implications for detrusor overactivity? *Urology* 63 (3 Suppl 1):3-10.
- Grynkiewicz G, Poenie M and Tsien RY (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 260: 3440-3450.
- Jen PY, Dixon JS, Gosling JA 1995. Immunohistochemical localization of neuromarkers and neuropeptides in human fetal and neonatal urinary bladder. *Br J Urol* 75: 230-235.
- Kim KM, Kogan BA, Massad CA, Huang YC 1991. Collagen and elastin in the normal fetal bladder. *J Urol* 146: 524-527.
- Koo HP, Macarak EJ, Zderic SA, Duckett JW, Snyder HM, III, Levin RM 1995. The ontogeny of bladder function in the fetal calf. *J Urol* 154: 283-287.
- Levin RM, Macarak E, Howard P, Horan P, Kogan BA 2001. The response of fetal sheep bladder tissue to partial outlet obstruction. *J Urol* 166: 1156-1160.

- Long W, Zhang L and Longo LD (2000). Cerebral artery K(ATP)- and K(Ca)-channel activity and contractility: changes with development. *Am J Physiol* 279: R2004-2014.
- Longhurst PA (2004). Developmental aspects of bladder function. *Scand J Urol Nephrol Suppl* 215: 11–19.
- Ludwikowski B, Oesch H, I, Brenner E, Fritsch H 2001. The development of the external urethral sphincter in humans. *BJU Int* 87: 565-568.
- Matsumoto S, Kogan BA, Levin RM, Howard PS, Macarak EJ 2003. Response of the fetal sheep bladder to urinary diversion. *J Urol* 169: 735-739.
- Nicolaides KH, Rosen D, Rabinowitz R, Campbell S 1988. Urine production and bladder function in fetuses with open spina bifida. *Fetal Ther* 3: 135-140.
- North RA (2002). Molecular physiology of P2X receptors. *Physiol Rev* 82:1013-1067.
- Nyirady P, Thiruchelvam N, Fry CH, Godley ML, Winyard PJD, Peebles DM, Woolf AS, Cuckow PM 2002. Effects of in utero bladder outflow obstruction on fetal sheep detrusor contractility, compliance and innervation. *J Urol* 168: 1615-1620.
- Nyirady P, Thiruchelvam N, Godley ML, David A, Cuckow PM and Fry CH (2005). Contractile properties of the developing fetal sheep bladder. *Neurourol Urodyn* 24:276-281.
- O'Reilly BA, Kosaka AH, Chang TK, Ford AP, Popert R, Rymer JM and McMahon SB (2001). A quantitative analysis of purinoceptor expression in human fetal and adult bladders. *J Urol* 165: 1730-1734.
- Pessah IN, Stambuk RA and Casida JE (1987). Ca^{2+} -activated ryanodine binding: mechanisms of sensitivity and intensity modulation by Mg^{2+} , caffeine, and adenine nucleotides. *Mol Pharmacol* 31:232-238.
- Peters CA, Vasavada S, Dator D, Carr M, Shapiro E, Lepor H 1992. The effect of obstruction on the developing bladder. *J Urol* 148: 491-496
- Porter VA, Reeve HL and Cornfield DN (2000). Fetal rabbit pulmonary artery smooth muscle cell response to ryanodine is developmentally regulated. *Am J Physiol* 279: L751-757.

- Poulsen JC, Caspersen C, Mathiasen D, East JM, Tunwell RE, Lai FA, Maeda N, Mikoshiba K and Treiman M (1995). Thapsigargin-sensitive Ca^{2+} -ATPases account for Ca^{2+} uptake to inositol 1,4,5-trisphosphate-sensitive and caffeine-sensitive Ca^{2+} stores in adrenal chromaffin cells. *Biochem J* 307: 749-758.
- Rabinowitz R, Peters MT, Vyas S, Campbell S, Nicolaides KH 1989. Measurement of fetal urine production in normal pregnancy by real-time ultrasonography. *Am J Obstet Gynecol* 161: 1264-1266.
- Sibley GN (1984). A comparison of spontaneous and nerve-mediated activity in bladder muscle from man, pig and rabbit. *J Physiol* 354:431-443.
- Thiruchelvam N, Wu C, David A, Woolf AS, Cuckow PM, Fry CH 2003a. Neurotransmission and viscoelasticity in the ovine fetal bladder after in utero bladder outflow obstruction. *Am J Physiol*; 284: R1296-R1305
- Thiruchelvam N, Nyirady P, Peebles DM, Fry CH, Cuckow PM, Woolf AS 2003b. Urinary outflow obstruction increases apoptosis and deregulates Bcl-2 and Bax expression in the fetal ovine bladder. *Am J Pathol* 162: 1271-1282.
- Wu C and Fry CH (1998). The effects of extracellular and intracellular pH on intracellular Ca^{2+} regulation in guinea-pig detrusor smooth muscle. *J Physiol* 508:131-143.
- Wu C and Fry CH (2001). $\text{Na}^+/\text{Ca}^{2+}$ exchange and its role in intracellular Ca^{2+} regulation in guinea pig detrusor smooth muscle. *Am J Physiol* 280:C1090-1096.
- Wu C, Bayliss M, Newgreen D, Mundy AR and Fry CH (1999). A comparison of the mode of action of ATP and carbachol on isolated human detrusor smooth muscle. *J Urol* 162:1840-1847.

Legends

Figure 1. Record of intracellular Ca^{2+} in unstimulated fetal detrusor smooth muscle cells.

A, small, fast spontaneous transients – cell from 100-day bladder: B, constant level of $[\text{Ca}^{2+}]_i$ - cell from 110-day bladder: C, large, spontaneous Ca^{2+} waves - cell from 70-day bladder.

Figure 2. Carbachol and caffeine-evoked Ca^{2+} -transients in fetal detrusor myocytes.

A. Ca^{2+} -transient on brief application of 10 μM carbachol (carb); the thick bar in all parts represents duration of exposure to the agonist and the dotted line the base level $[\text{Ca}^{2+}]_i$: B, the effect of 10 μM atropine on carbachol-evoked Ca^{2+} -transients: C, Ca^{2+} -transients evoked by 20 mM caffeine (caff): D, the effect of carbachol exposure immediately after a second caffeine-evoked Ca^{2+} transient.

Figure 3. ATP-induced Ca^{2+} -transients in fetal detrusor cells.

A, Ca^{2+} -transient on application of 10 μM ATP - the dotted line represents the base line $[\text{Ca}^{2+}]_i$ value: B, effect of pre-treatment with 10 μM α,β methylene-ATP (ABMA) on ATP-evoked Ca^{2+} -transient.

Figure 4. High-K and low-Na-induced Ca^{2+} -transients in fetal detrusor myocytes.

A, the extracellular $[\text{KCl}]$ was raised from 4 to 120 mM. Before the second exposure the cell was superfused with 20 μM verapamil for the period indicated: B, the effect of lowering the extracellular $[\text{Na}]$ from 147.4 to 29.4 mM Na. Before this exposure a 10 μM carbachol Ca^{2+} -transient was generated for comparison.

Figure 5. Magnitude of Ca^{2+} transients in fetal and adult detrusor myocytes.

The mean ($\pm$ S.E.) increase of intracellular $[\text{Ca}^{2+}]_i$ on exposure on detrusor myocytes to different interventions: 10 μM carbachol (carb); 20 mM caffeine (caff); 10 μM ATP; 120 mM KCl. The four bars in each group, labeled a-d in the first group) are the data from Group 1 (n=7), Group II (n=11) and Group III (n=5) fetal bladders, and adult (n=9) bladders respectively. * $p < 0.05$.

Figure 6. Carbachol dose-response curves for generation of Ca^{2+} -transients.

The dose-responsiveness of Ca^{2+} -transient magnitude in myocytes from Group 1 (n=7), Group II (n=11) and Group III (n=5) fetal bladders, and adult (n=9) bladders. All curves are fitted to the maximum transient height as 100%. Mean data $\pm$ S.E.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6