The Effect of the $P2X_1$ Receptor Antagonist MRS2159 on ATP- and EFS-Evoked Contractions of Isolated Ovine Detrusor Muscle Strips

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Abstract

Background: Overactive bladder (OAB) is a storage symptom complex often associated with detrusor over activity (DO). Antimuscarinic drugs still the mainstay treatment for OAB but the adverse effects limit their effectiveness, hence, alternatives are needed. Recently, β3 agonists have been introduced in clinical use for the treatment of OAB. At present, experimental search focusing on the potential use of $P2X_1$ antagonist in DO.
Objectives: This study aims to investigate the possible existence of $P2X_1$ purinoceptors and their interplay with β-Adrenoceptors in the ovine detrusor smooth muscle (DSM), and to examine the interaction of selected purinoceptors agonists and antagonists on models of voiding and non-voiding detrusor contractions.
Methods: *In vitro* experiments were performed on ovine detrusor muscle preparations in an organ bath containing Krebs solution. The contractile response of intact and denuded ovine DSM strips evoked by either 500μM adenosine 5'-triphosphate (ATP) or 10μM α,β-methylene ATP (α,β-meATP) or EFS was measured before and after the addition of 100μM MRS2159 (MRS) and 10μM isoprenaline (ISO) separately.
Results: ATP (500μM) and α,β-meATP (10 μM) effectively contracted the isolated ovine DSM producing a response consisting of two phases an initial transient rapid phasic contraction and a later slowly developing tonic contraction. MRS inhibited the phasic contractions evoked by 500μM ATP and 10Hz EFS in intact and denuded tissues, but it is completely abolished the contractions evoked by 10μM α,β-meATP. The cumulative administration of 50μM α,β-meATP abolished the phasic and tonic contractions evoked by ATP.
Conclusions: The contractile activity evoked by 10Hz EFS in the ovine DSM is largely mediated by the activation of $P2X_1$ purinoceptors which are present abundantly in the ovine DSM and there is an intracellular inter talk between $P2X_1$ purinoceptors and β-AR in the ovine DSM.
Key Words: $P2X_1$ receptors, Ovine detrusor, Non-voiding model.

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Introduction

The parasympathetic nerves represent the principal excitatory pathway in the urinary bladder through releasing of ACh and ATP as main co-transmitters[1,2]. The purinergic mechanisms have been suggested to play a role in regulating both urine storage and voiding[3]. ATP is the transmitter responsible for the non-adrenergic non-cholinergic (NANC) component of bladder contraction, through the activation of $P2X_1$ purinoceptors[4,5], which are the most abundant P2X receptor subtype in the adult human bladder[6].

The urothelium releases ATP in response to bladder stretch and noxious stimuli to activate suburothelial afferents $P2X_3$ receptors[7] and transmitting signals about bladder extension or nociception[8]. The interaction of ATP with $P2X_1$ receptors located in the detrusor resulting in a rapid phasic contraction has been described by Yoshimura & Chancellor[9]. The Purinergic impact to bladder contraction varies with species[10] from being dominant in cat, mouse, and rabbit, to moderate in guinea pig, rat, and dog, to less prominent in pig and human[11].

Purinergic mechanisms in the detrusor muscle are up-regulated in pathological states[12], as in detrusor over activity (DO)[13], which is involuntary contractions of the detrusor muscle during bladder filling and often results in Overactive bladder (OAB)[14]. It has been found that DO is associated with either excess release of ATP or ATP is less efficiently broken down due to a reduction in ectonucleotidase activity[15]. Antimuscarinic drugs are still the mainstay in the treatment of OAB but the adverse effects limit their use[16]. Recently, several studies have been made aiming at the potential use of $P2X_1$ antagonist in ameliorating the DO[17].

This study aims to investigate the possible existence of $P2X_1$ purinoceptors and its interplay with β-Adrenoceptors in the ovine detrusor smooth muscle (DSM), and to examine the effect of MRS2159 on models of voiding and non-voiding detrusor **contractions.**

Materials and Methods

Specimens of ovine (male, < 1 year old) urinary bladder were taken in the early morning from a local abattoir and immediately transported to the laboratory in a pre-oxygenated chilled (4°C) Krebs-Henseleit solution containing the following (in mM): 118.4 NaCl, 4.7 KCl, 1.2 $MgSO_4.7H_2O$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 2.5 $CaCl_2.2H_2O$ and 11.7 glucose. The bladder body was cut from a level above the ureters and the tissue cleared of external fat and connective tissue and a four longitudinal strips measuring (10mm x 5mm) consisting of smooth muscle and urothelium were prepared from the anterior part. In some experiments, the urothelium was carefully removed from these strips using fine dissecting scissor. The bladder strips were mounted vertically for isometric recording of tension in 50 ml organ baths containing a Krebs-Henseleit solution which was kept at 37°C and gassed with 95% O_2–5% CO_2. The isometric tension was recorded through a force-displacement transducer. These strips were subjected to a resting tension of 1.0g and allowed to equilibrate for 60 min, during which time they were washed every 10–15 min and the resting tension was adjusted. Intramural nerves were stimulated by electrical field stimulation (EFS) which delivered with a Grass 88 stimulator. The bladder strips were mounted in an organ bath between two parallel platinum wire electrodes (diameter 0.25 mm, distance between them is 8mm) with sufficient gaps between

electrodes and strip to allow for unimpeded muscular contraction[18].

Experimental Design

After equilibration, the contractile response (initial phasic contraction) of intact strips was elicited by direct addition to the bath of one of the agonists: ATP (500µM), α,β-meATP (10µM) or CCh (5µM) as a control. After that the tissues were washed three times every 15 min for about 45 min, until resting level of tension was attained and then incubated separately with either MRS (for 45min) or ISO (for 20min), then the agonist was applied again. Atropine (1µM) and prazosin (100nM) were present throughout the experiments except with EFS and CCh. Similar experiments were done using the denuded (detrusor muscle without urothelium) strips instead of the intact one, with potassium chloride (KCl, 10mM, in addition to the 4.7mM included in the preparation of Krebs-Henseleit solution) added during the equilibration period for induction of the spontaneous rhythmic activity in the isolated denuded strips.

In one series of experiments, the desensitizing effect of cumulative administration of α,β-meATP was used instead of antagonism with MRS, where the tissue was stimulated with ATP (500µM) as a control, and then washed for 45min. After that, a first dose of α,β-meATP (50µM) was added for 10min and then a same second dose of α,β-meATP was added cumulatively for additional 10min; subsequently, ATP (500µM) was added also cumulatively after 20min of the first application of α,β-meATP and the phasic contraction was measured before and after desensitization with α,β-meATP (50µM).

In another series, the contractile responses of DSM strips to EFS (10 and 40Hz, 100V, pulse width 0.5milliseconds in 20sec trains at 10min intervals) were measured as a control and then after incubation for 45min with MRS (100µM) or after incubation for 20min with ISO (10µM).

Drugs

The following pharmacological agents were used: adenosine 5'- triphosphate disodium salt (ATP, non-selective P2 agonist), α,β-methylene adenosine 5'-triphosphate lithium salt (α,β-meATP, selective $P2X_1$ agonist), MRS2159 (MRS, selective $P2X_1$ antagonist), carbachol (CCh, non-selective muscarinic agonist), atropine sulphate (non-selective muscarinic antagonist), prazosin hydrochloride (α_1-agonist), isoprenaline hydrochloride (ISO, non-selective β-adrenoceptor agonist), potassium chloride (KCl) and platinum wire (0.25mm diameter). All these agents were purchased from Sigma Aldrich Co. and were prepared daily by dissolving in distilled water.

Statistical Analysis

The responses to MRS and ISO were expressed as a percentage of the contraction evoked previously by either ATP, α,β-meATP or EFS. The results were presented as mean values ± standard error of the mean (SEM) from ''n'' bladder strips. Statistical analyses and graphics were performed using Graph Pad Prism 6.03 software. Comparisons between responses before and after incubation with MRS and ISO or between intact and denuded strips response were done by paired and unpaired Student's two-tailed *t*-test, respectively. The term n, used in the presentation of statistical analyses throughout, refers to the number of strips used. In all cases, a P value < 0.05 was regarded as statistically significant.

Results

The ovine DSM exhibited spontaneous rhythmic activity. Atropine (1µM) and prazosin (100nM) on their own did not appear to influence the basal tone or the spontaneous rhythmic activity of the

ovine DSM. Atropine slightly reduced contractions evoked by exogenous ATP (500µM) but not significantly (P=0.077; n= 5; Table 1).

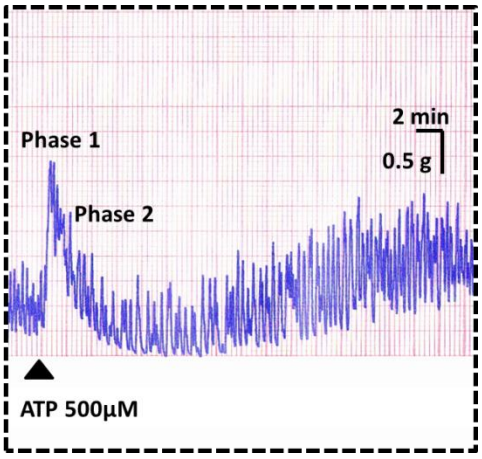
Atropine completely abolished contractions evoked by exogenous CCh (10µM) (mean 3.15±0.13 gm tension; n= 4).

I. Sensitivity of contractions to MRS

1. ATP with MRS

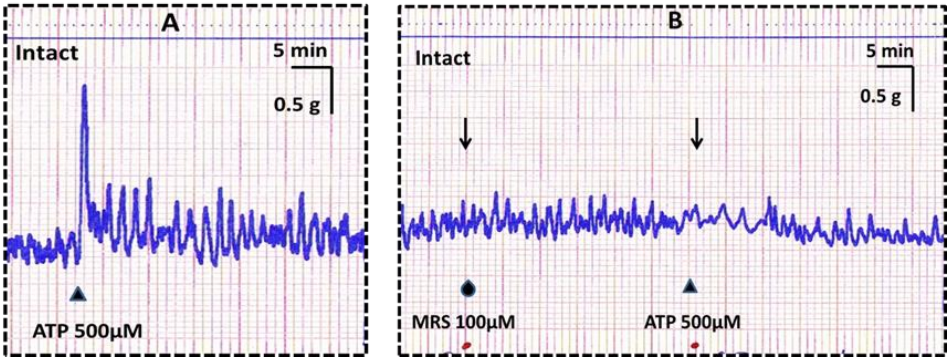
ATP (500µM) effectively contracted the isolated ovine DSM strips producing a response which consists of two phases an initial transient rapid phasic contraction (mean 0.57±0.04 gm tension; n=30) and a later slowly developing tonic contraction sometimes superimposed on the spontaneous rhythmic activity (Fig. 1).

Figure 1: Typical trace showing the contractions evoked by ATP (500µM) in ovine detrusor muscle (n=30).



MRS (100µM) significantly inhibited the phasic (79% ±4.8; P=0.003; Table 1) and the tonic contractions evoked by ATP (500µM), without interfering with the spontaneous rhythmic activity or the basal tone of the isolated intact DSM strips (Fig. 2B).

Figure 2: Typical traces showing the effect of 100µM MRS2159 on isolated intact ovine



detrusor muscle strips contractions evoked by 500µM ATP (n=5).

Table 1: The effect of MRS2159, isoprenaline and atropine on contractions evoked by ATP in intact ovine detrusor muscle strips.

Agonist Control (tension gm)	Agent	% of inhibition (tension$ gm)	Statistics
ATP 500µM 0.784±0.086	MRS2159 (100µM)	79±4.8 * (0.148±0.0168)	P= 0.003 (n=5)
ATP 500µM 0.484±0.048	isoprenaline (10µM)	85.8±3.04 * (0.064±0.0191)	P= 0.0028 (n=5)
ATP 500µM 0.498±0.0146	atropine (1µM)	3±1.341 ** (0.482±0.0177)	P= 0.077 (n=5)

Data presented as mean ± S.E.M, n represent the number of strips.* P < 0.05, ** P > 0.05 when compared with the respective control values using paired Student's *t*-test, $ tension stated here is the remaining observed tension.

The denuded strips failed to exhibit any spontaneous rhythmic activity and ATP (500µM) did not induce any response in these strips unless their spontaneous rhythmic activity had been stimulated with KCl (10mM). After induction with KCl, ATP effectively contracted the denuded strips as done in the intact one. MRS (50µM) significantly inhibited the phasic (92%±3.572; P<0.0001; Table 2) and tonic contractions evoked by ATP (500µM) in the denuded tissue without interfering with the basal tone or the rhythmic activity
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Table 2: The effect of MRS2159 and isoprenaline on contractions evoked by ATP in denuded ovine detrusor muscle strips.

Agonist Control (tension gm)	Agent	% of inhibition (tension$ gm)	Statistics
ATP 500µM 0.45±0.028	MRS2159 (50 µM)	92.83±3.572 * (0.033±0.0162)	P< 0.0001 (n=6)
ATP 500µM 0.492±0.043	isoprenaline (10 µM)	61±5.0299 * (0.196±0.033)	P= 0.0003 (n=5)

Data presented as mean ± S.E.M, n represent the number of strips.* P < 0.01 when compared with the respective control values using paired Student's *t*-test, $ tension stated here is the remaining observed tension.

2.α,β-meATP with MRS2159

The application of α,β-meATP (10µM) effectively contracted the intact isolated ovine detrusor muscle producing a response similar to that produced by ATP (500µM) and the mean phasic contraction was (0.712±0.073 gm tension, Fig. 3A).

MRS (100µM) completely abolished the phasic and tonic contractions evoked by 10µM α,β-meATP, but neither the spontaneous rhythmic activity nor the basal tone was influenced (Fig. 3B).

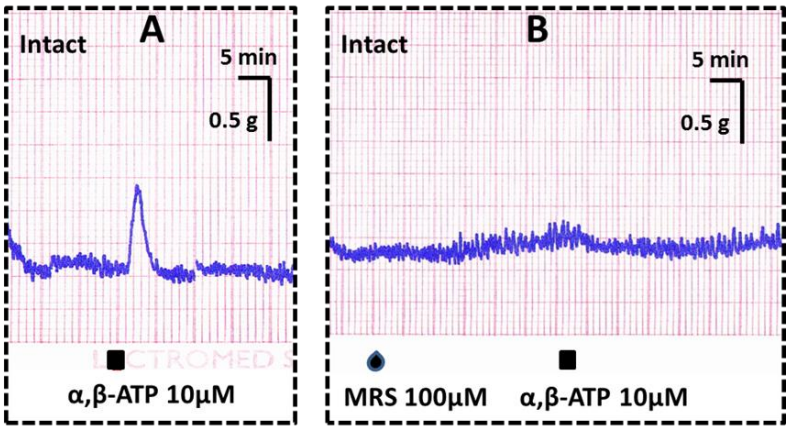

Figure 3: Typical traces showing the effect of 100μM MRS on isolated intact ovine detrusor muscle strips contractions evoked by 10μM α,β-meATP (n=5).

Like ATP, α,β-meATP did not induce any response in the denuded ovine strips unless their spontaneous rhythmic activity had been stimulated once during the equilibration period with KCl. After induction with KCl, α,β-meATP (10μM) effectively contracted the denuded ovine detrusor muscle strips in a similar manner to that observed in the intact ones. Only 50μM MRS was required to completely abolish the phasic and tonic contractions evoked by α,β-meATP (10μM) in the denuded tissue without interfering with the basal tone or the rhythmic activity induced by KCl.

3. CCh with MRS

CCh (5μM) induced contraction of the ovine DSM being composed of three phases: rapid phasic contraction (mean 1.782±0.14gm tension; n=5), then slowly declining tonic contraction and sustained (plateau) contraction. In this series of experiments, MRS (100μM) did not significantly influence the contractions evoked by CCh (5μM) (P=0.690) whether it is phasic, tonic or plateau. Also, MRS did not appear to influence the spontaneous rhythmic activity or the basal tone of ovine DSM.

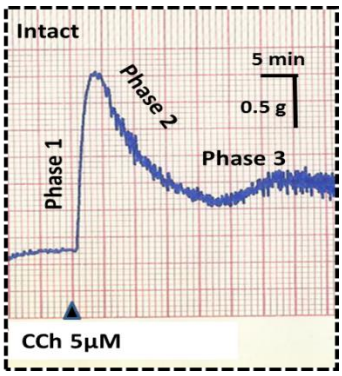

Figure 4: Typical trace showing the the contractions evoked by CCh (5μM) in isolated intact ovine detrusor muscle strips contractions (n=10).

4. EFS with MRS

EFS induced a frequency-dependent increase in the amplitude of contractions of the isolated intact ovine DSM strips. Stimulation of the intramural nerves of the ovine DSM by a low frequency EFS (10Hz) and a high frequency EFS (40Hz) elicited phasic contraction of (0.611±0.033gm tension, Fig. 5A) and (1.22±0.033gm tension, Fig. 6A) respectively.

MRS (100µM) significantly suppressed the phasic contractions evoked by low frequency EFS (10Hz) (75.16%±2.676; P=0.0003; Fig. 5B; Table 3) with a slight reduction in the contractions evoked by high frequency EFS (40Hz) (27.25%±0.853; P=0.0003; Fig. 6B; Table 3). Neither the spontaneous rhythmic activity nor the basal tone was influenced by the addition of MRS.

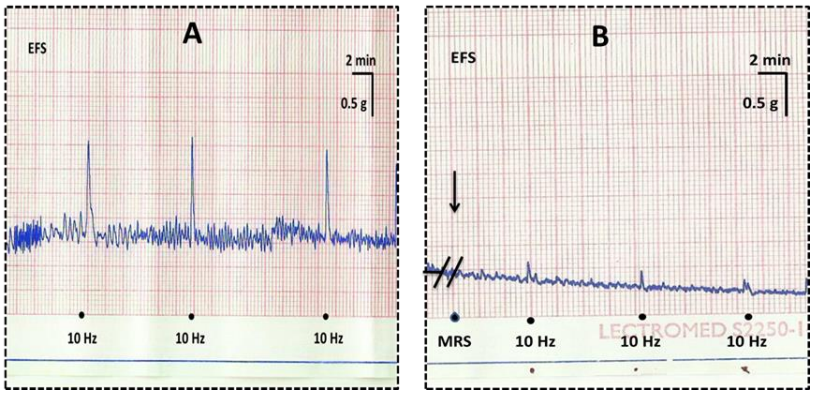

Figure 5: Typical traces showing the effects of 100µM MRS on the amplitude of intact ovine detrusor contraction induced by 10Hz EFS (n= 6).

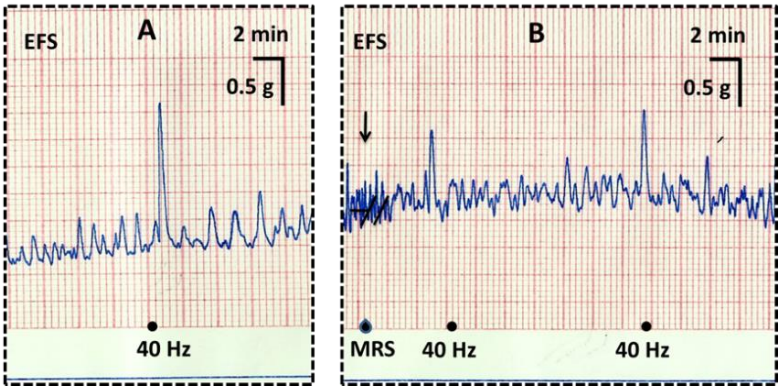

Figure 6: Typical traces showing the effects of 100µM MRS on the amplitude of intact ovine detrusor contraction induced by 40Hz EFS (n= 6).

Table 3: The effect of MRS2159 and isoprenaline on contractions evoked by low and high frequency electrical field stimulation of intramural nerves of intact ovine detrusor muscle.

Frequency	Control (tension gm)	Antagonist	% of inhibition (tension$ gm)	Statistics
10 Hz	0.65 ± 0.0534	MRS2159 (100 µM)	75.16 ± 2.676* (0.156 ± 0.0114)	P= 0.0003
	0.573 ± 0.037	isoprenaline (10 µM)	77.33 ± 4.601* (0.125 ± 0.0196)	P= 0.0002
40 Hz	1.225 ± 0.033	MRS2159 (100 µM)	27.25 ± 0.853* (0.887 ± 0.0165)	P= 0.0003

Data presented as mean ± S.E.M, n= 6.* P < 0.01 when compared with the respective control values using paired Student's *t*-test, $ tension stated here is the remaining observed tension

II. The effect of cumulative administration of α,β-meATP

Initially the intact strip was stimulated with 500µM ATP to produce its contractile effect (Fig. 7A). It has been found that the cumulative administration of high concentration (50µM) of α,β-meATP abolished the phasic and tonic contractions evoked by exogenous ATP (500µM), where the first dose of α,β-meATP (50µM) effectively contracted the ovine DSM strips and elicited a large transient phasic contraction while the second dose when added cumulatively failed to produce any response (i.e. tachyphylaxis). Subsequently, ATP (500µM) when added cumulatively also failed to produce any response (Fig. 7B).

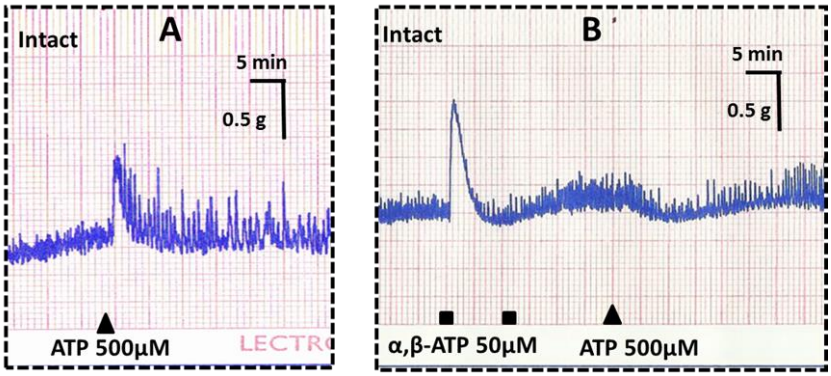

Figure 7: Typical traces showing the desensitizing effect of cumulative administration of 50µM α,β-meATP on intact ovine detrusor muscle strips evoked by 500µM ATP (n=5).

III. Sensitivity of contractions to isoprenaline

1. ATP with isoprenaline

ISO induced a concentration dependent relaxation for the isolated ovine DSM strips. ISO (10µM) significantly inhibited and almost abolished the phasic (85.8%±3.04; P=0.0028;

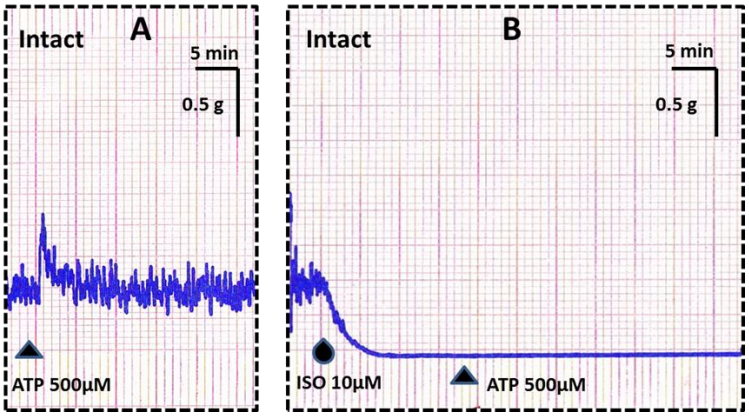

Figure 8: Typical traces showing the effect of 10µM isoprenaline on isolated intact ovine detrusor muscle strips contractions evoked by 500µM ATP (n=5).

Table 1) and tonic contractions of intact ovine DSM strips evoked by ATP (500µM) (Fig. 8B). Also, ISO completely abolished the spontaneous rhythmic activity and substantially reduced the basal tone of the ovine DSM. ISO in a concentration less than 10µM produced a lesser inhibitory effect on ATP- evoked phasic and tonic contractions.

For denuded strips, ISO (10µM) markedly inhibited the phasic (61%±5.029; P=0.0003; Table 2) and tonic contractions of isolated denuded ovine DSM strips evoked by ATP (500µM), while completely inhibited the spontaneous rhythmic activity of the ovine DSM and substantially reduced the basal tone as done with the intact strips.

2. EFS with isoprenaline

ISO (10µM) produced an approximately equivalent inhibitory effect to that of MRS (100µM) on phasic contractions evoked by the low frequency EFS (10Hz) (77.33%±4.601; P=0.0002; Table 3). ISO completely abolished the spontaneous rhythmic activity and reproducibly and substantially reducing the basal tone (Fig. 9B).

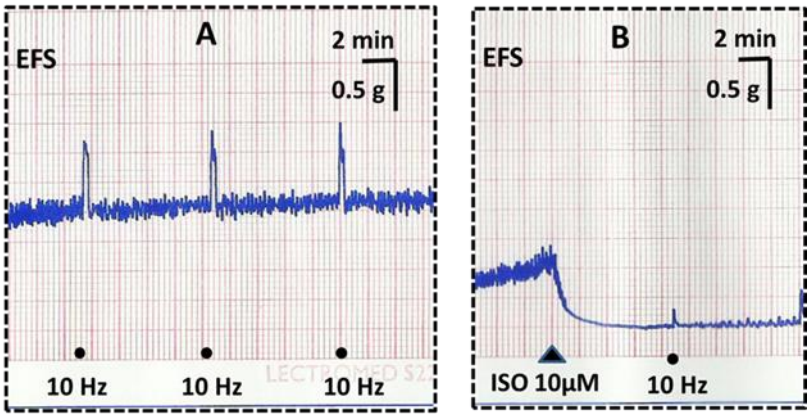

Figure 9: Typical traces showing the effects of 10µM isoprenaline on the amplitude of intact ovine detrusor contraction induced by 10Hz EFS (n= 6).

Discussion

The ovine DSM exhibited spontaneous rhythmic activities observed in our work similar to that reported by Anderson[10] on humans and that by Hashitani & Suzuki[19] on guinea pig. Atropine (1µM) and prazosin (100nM) on their own did not appear to influence the basal tone or the spontaneous rhythmic activity of the sheep DSM. Atropine (1µM) only slightly reduced contractions evoked by exogenous ATP (500µM) but not significantly (P=0.077) while it is completely abolished contractions evoked by 10µM exogenous Carbachol (CCh; 3.15±0.13 gm tension; n=4). Hence, atropine (1µM) and prazosin (100nM) were present throughout the remainder of the experiments to exclude the involvement of muscarinic effects due to possible ATP-induced non-neuronal release of Ach from urothelial cells Hanna-Mitchell *and coworkers*[20] and to make sure that α_1-adrenoceptors (if possibly present) do not interact with the test agonists like ATP and α,β-meATP. Similar approach was adopted by Kennedy and coworkers[21].

ATP (500µM) effectively contracted the isolated ovine DSM producing biphasic contractions and this is consistent with that reported elsewhere on isolated rabbit urinary bladder[18] and on isolated guinea pig urinary bladder[21]. α,β-meATP was more potent than ATP, where approximately similar responses were attained by ATP (500µM) and α,β-meATP (10µM) in the ovine DSM. This is in agreement with that reported by Harvey and coworkers[15] in guinea pig DSM and this high potency of α,β-meATP is probably due to being relatively resistant to degradation by ectoenzymes; similar suggestions were made by others[22,23].

Earlier, α,β-meATP was used for desensitization of $P2X_1$ receptors due to the absence of P2X receptor antagonists at that time[21]. In our work, it was found that the cumulative administration of α,β-meATP (50µM) abolished the phasic and tonic contractions evoked by ATP. Further, as it is generally accepted that α,β-meATP is a selective agonist for $P2X_1$ purinoceptors[1,24] and also antagonist by virtue of causing desensitization of these receptors when used in a higher dose and cumulatively[23,25]. Similar approaches had been used in marmoset by McMurray *and coworkers*[26] and in guinea pig by Kennedy *and coworkers*[21]. Thus, it is suggested that this contractile action of ATP is mediated by $P2X_1$ subtype of purinoceptors.

In intact DSM preparation, MRS (100µM) did not influence the spontaneous rhythmic activity of ovine DSM and thus it is postulated that $P2X_1$ purinoceptor is not involved in the mediation of this spontaneous rhythmic activity; however, other subtypes of purinoceptors cannot be excluded. MRS (100µM) significantly inhibited the phasic contractions induced by ATP (500µM) (79% ±4.8; P=0.003) while it is completely abolished the phasic contractions evoked by α,β-meATP (10µM). Therefore, due to the high selectivity of MRS to $P2X_1$ receptor[27,28], these ATP- and α,β-meATP-induced responses are suggested to be mediated by $P2X_1$ receptor. Similar lines of evidence for characterization of $P2X_1$ in the urinary bladder of small animals have been presented in mouse by Vial and Evans[29] and in guinea pig by Kennedy *and coworkers*[21]. Kennedy and coworkers[21] reported that MRS (100µM) inhibited around 38% of contractions evoked by exogenous ATP (300µM), while this concentration completely blocked responses to α,β-meATP (10µM) in the guinea pig bladder. They concluded that ATP-mediated its contractile action via $P2X_1$ plus other subtypes of purinoceptors while α,β-meATP produced its action via activation of only $P2X_1$ receptors, i.e. $P2X_1$ receptors coexist with variant purinoceptors

which are collectively responsible for mediating contractile responses. Therefore, it is suggested that the presence of $P2X_1$ receptor in the ovine DSM is more predominant than that described in the guinea pig bladder. Further, as $P2X_1$ receptor have been described to be the most abundant P2X receptor subtype in the adult human bladder[6]; hence, it is prposed that the ovine DSM could be a good model to study experimentally the potential use of $P2X_1$ antagonist in detrusor overactivity (DO).

In denuded DSM preparation, ATP and α,β-meATP consistently failed to produce any response in the absence of spontaneous rhythmic activity, therefore, KCl (10mM) was added only once during the equilibration period to initiate rhythmic activity which was found to persist even after repeated washings throughout the experiment. It appears that this spontaneous activity of the ovine DSM is greatly influenced by the presence of the urothelium, a similar suggestion was made by Buckner *and coworkers*[30]. It is worth noting that MRS appear to be more potent in the denuded preparation than the intact one, only 50µM MRS was required to inhibit (92%±3.572; P<0.0001; Table 2) and complete inhibition of the phasic contractions evoked by ATP (500µM) and α,β-meATP (10µM) respectively, whereas in intact preparation 100 µM MRS inhibited 79% and complete inhibition of contractions evoked by the respective agents. It follows the presence of the urothelium is a prerequisite for spontaneous rhythmic activity and the latter is a prerequisite for ATP and α,β-meATP to produce their contractile actions. Hence, it is hypothesized that some elements are released from the urothelium, and such elements may play modulatory roles in the mediation of spontaneous rhythmic activity and also may be essential for ATP to produce its contractile activity. Further, high KCl concentration produces contractile responses by virtue of causing depolarization and in turn influx of extracellular calcium via voltage-gated Ca^{2+} channels to the intracellular space of the DSM[31]; hence, it is postulated that KCl may compensate for the urothelial-missing elements and thus reverts the spontaneous activity and in turn restores the ATP-induced responses. Therefore, a higher concentration of MRS appears to be required to inhibit the action of ATP and α,β-meATP in the intact tissue compared to that required in the denuded tissue, this is probably due to the absence of the urothelial-derived elements that modulate the contractile activity of ATP and α,β-meATP.

It is well established that the physiological voiding contractions of the human DSM are mainly muscarinic, while the pathological non-voiding contractions involve non-cholinergic mechanisms to a much larger extent[32,33] and that ATP may be one of the most important non-cholinergic pathophysiological stimuli to elicit bladder contraction[34]. CCh (5µM) induced contraction of the ovine DSM being composed of three phases and this has been adopted as a voiding model in this study. A similar approach using more than 3µM CCh has been adopted on isolated guinea pig bladder by Gillespie *and coworkers*[35] and on isolated ovine bladder by Dawood and Lafi[36] to produce global contractions of the bladder.

Further, MRS (100µM) failed to produce any significant (P=0.69) inhibition on CCh (5µM)-evoked contractions; similar findings were reported in the guinea pig bladder by Kasakov & Burnstock[37] and by Fujii[22]. As it is suggested above the effect of MRS may be related to its high selectivity to $P2X_1$ subtype of purinoceptors; hence, it is proposed that there is no direct intertalk between $P2X_1$ receptors and muscarinic receptors expressed in the ovine DSM. Therefore, it is suggested that a drug which can acts

specifically on the human bladder $P2X_1$ receptor, may be considered as a promising drug candidate for the treatment of DO as it is theoretically does not interact with physiological voiding.

ISO (10µM) did not inhibit the ovine DSM contractions evoked by CCh (5µM), similar observations were reported by Dawood & Lafi[36]; while it substantially and significantly inhibited the phasic contraction evoked by ATP (500µM). Similar findings were reported in the rat bladder by Michel & Sand[38] where β-AR agonists caused a weaker relaxation against contractions evoked by CCh than against other non-cholinergic stimuli. The possible explanation of this differential effect of β-AR agonists on CCh-evoked contractions may be related to a specific intertalk between postsynaptic M_2 muscarinic receptors and β_3-ARs at the cyclic adenosine-3,5-monophoshate (cAMP) level where the stimulation of postsynaptic M_2 receptors by high levels (5µM) of CCh antagonizes the relaxation response of ISO by inhibiting adenylate cyclase enzyme; similar explanations were put forward by Hegde *and coworkers*[39] in rats and by Ehlert *and coworkers*[40] in mice. Further, the differential effect of ISO (10µM) on ATP-induced responses may suggest that ATP shares a common pathway with that responsible for spontaneous and basal tone activities but differs from that responsible for mediating responses to CCh. This common pathway is not likely to be on the level of $P2X_1$ receptors as neither the spontaneous activity nor the basal tone were influenced by the selective $P2X_1$ antagonist.

Moreover, this inhibitory effect of ISO on CCh- and ATP-evoked contractions is proposed to be related to its ability to stimulate β-ARs, especially β_3-receptors, which have been suggested to be expressed predominantly in the ovine bladder[36], and in human bladder[41,42]. It is generally accepted that the stimulation of β-AR increases intracellular cAMP which activates protein kinase A (PKA) that, in turn, phosphorylates specific proteins resulting in DSM relaxation[43]. However, another mechanism is suggested that K^+ channels, particularly large conductance voltage activated and Ca^{2+} activated K^+ (BK_{Ca}) channels, may be involved in the ISO-mediated relaxation of the ovine DSM. The involvement of K^+ channels were also suggested by others, in guinea pig DSM by Petkov & Nelson[44], in rat DSM by Hristov *and coworkers*[45] and in ovine DSM by Dawood & Lafi[36].

The inhibitory effect of ISO on contractions evoked by ATP in the intact tissues is more significant (P=0.0096; unpaired Student's *t*-test) than that in the denuded tissues; a similar finding was reported in the rat urinary bladder by Birder *and coworkers*[46]. Further, other workers reported that the urothelium contains functional β-ARs[42,47]. Hence, it is postulated that stimulation of β-ARs by ISO results in the release of nitric oxide (NO) due to an increase in intracellular Ca^{2+} following activation of the adenylate cyclase pathway in the urothelial cells and the released NO augments the inhibitory effect of ISO. This suggestion is in line with that presented by Andersson *and coworkers*[48] in the rat urinary bladder where the removal of the urothelium abolished the NO effects, suggesting that the urothelium is being the origin of NO.

However, Otsuka *and coworkers*[47] provided evidence argues against the involvement of NO in this setting, they showed that the presence of the urothelium reduced the relaxant effects of ISO on human DSM, therefore, they suggested that the stimulation of β-ARs in the human urothelium induces the release of a urothelium-derived factor (UDF) which inhibits the relaxation response of the human DSM to ISO, and that this inhibitory mechanism might not involve NO. Moreover, in earlier work, Murakami *and*

coworkers[49] suggested that ISO stimulates the release of an unidentified UDF which in turn inhibits the contractions evoked by CCh in intact porcine DSM preparation. This discrepancy between our results and that reported by Otsuka *and coworkers*[47] and Murakami *and coworkers*[49] regarding the effect of ISO on denuded and intact strips may be related to species differences (ovine *vs.* human and porcine, respectively) and also to the experimental conditions for DSM preparations related to the contractile activity (e.g. basal tone) being spontaneous, or contracted by CCh or ATP.

Dawood[50] found that ISO had the ability to relax the non-voiding contractions evoked by many contractile stimuli like KCl, histamine, serotonin and prostaglandin E_2 at a concentration ten times less than that required to induce relaxation of CCh (0.3µM) pre-contracted strip; hence, he suggested a potential usefulness of the β-AR agonist in the treatment of DO syndrome induced by pathological non-voiding non-cholinergic stimuli with a little effect against physiological voiding contractions. This provides further support to our finding and suggestion that β-AR agonists have a good pharmacological profile in which they can suppress DO without impairing physiological voiding and this proposition has also been presented by Yamaguchi[51] in the human DSM.

Transmural stimulation of the parasympathetic nerves of the ovine DSM by a low frequency EFS (10Hz) and high frequency EFS (40Hz) elicited phasic contraction of (0.611±0.033gm tension) and (1.22±0.033gm tension) respectively. The low frequency EFS has been adopted to be equivalent to abnormal involuntary non-voiding contractions while the high frequency EFS has been adopted to be equivalent to voiding contraction. Similar approaches had been used in previous studies on isolated rat DSM strips by Aronsson[52] and on ovine DSM strips by Dawood & Lafi[36]. MRS (100µM) significantly suppressed (75%) contractions induced by EFS (10Hz), while a significantly smaller ($P<0.0001$; unpaired Student's *t*-test) reduction (27%) in the contractions evoked by 40Hz was observed. This substantial differential effect may justify the proposition that ATP can be released by EFS of the parasympathetic nerves of the ovine DSM to act via $P2X_1$ receptors to elicit phasic contraction; this is in line with that proposed by Andersson and Wein[53], and Fry *and coworkers*[54]. Several workers have suggested that ATP release was frequency dependent where high level of ATP released at low frequency and low level released at high frequency, this is consistent with that reported in rats and guinea pigs by Brading and Williams[55] where they showed that α,β-meATP reduced EFS-induced contractions at frequencies <10 Hz, whereas atropine had its maximum effects at frequencies >20 Hz; also they showed that a combination of α,β-meATP and atropine abolished the EFS-evoked contractions.

Maggi *and coworkers*[56] stated that the Frequency-dependent contractions can be sub-divided into fast transient phase (phasic) and longer lasting phase (tonic). It has been demonstrated that the purinergic system is of a greatest importance in the rapid phasic contraction, whereas the cholinergic system is mainly responsible for the tonic contraction[57]. Chancellor *and coworkers*[58] has been postulated that the purinergic component is needed for the initiation of micturition. However, Tong *and coworkers*[23] suggested that the purinergic component is relatively unimportant for bladder emptying while the cholinergic-mediated tonic contraction is primarily responsible for urine expulsion.

MRS (100µM) inhibited 75% of EFS (10Hz)-induced contractions, in the ovine DSM while the same concentration inhibited only 45% of EFS (4Hz)-induced contractions in the guinea pig DSM as

reported by Kennedy and coworkers[21]. This finding further supports our above suggestions that the $P2X_1$ receptors in the ovine bladder are more abundant than that reported in the guinea pig bladder. A similar line of argument supporting the presence of $P2X_1$ in the urinary bladder was put forward by Vial and Evans[29] where they found that genetic deletion of the $P2X_1$ receptor abolished non-cholinergic neurogenic (EFS-evoked) contractions in the mouse urinary bladder. It is worth noting that P2X receptors have been described in the marmoset urinary bladder by McMurray *and coworkers*[26], they reported that both neuronally released and exogenous ATP mediate their actions through the activation of P2X receptors.

ISO (10µM) produced an approximately equivalent inhibitory effect to that of MRS (100µM) on the phasic contraction evoked by EFS (10Hz) (77.33%±4.601; $P<0.01$). Similar findings about the effect of ISO (10µM) on low frequency EFS-induce contractions were reported in the human bladder by Tyagi *and coworkers*[41] and in the ovine bladder by Dawood & Lafi[36]. Hence, some sort of an antagonistic interplay between $P2X_1$ and β-AR is postulated in the ovine DSM. Further, Dawood & Lafi[36] found that ISO (100µM) was required to attenuate the high frequency EFS-induced contractions. This dose-dependent pharmacological difference of β-agonists may offer a therapeutic advantage over antimuscarinic agents, with a lesser possibility of interfering with physiological voiding, in the treatment of DO.

It is tempting to hypothesize that the initial phasic contraction induced by low frequency EFS in the ovine DSM is mainly purinergic and mediated by $P2X_1$ receptors since it is largely inhibited by the selective $P2X_1$ receptor antagonist (MRS), while ISO inhibited to a similar extent the phasic contraction evoked by either exogenous ATP or low frequency EFS. Several workers have concluded that purinergic mechanisms are responsible for the mediation of phasic contractions evoked by low frequency EFS, in rats and guinea pigs by Brading and Williams[55], in guinea pigs by Kennedy and coworkers[21] and in rats by Aronsson[52]. Thus, this may lend a support to hypothesize a possible existence of intracellular intertalk between cascades of events initiated by activation of $P2X_1$ receptor and β-adrenoceptors by the respective agonists resulting in pharmacological antagonism.

Conclusion

It is concluded that the contractile activity evoked by low frequency EFS (10Hz) in the ovine DSM is largely mediated by the activation of $P2X_1$ purinergic receptors which are appeared to be present abundantly in the ovine DSM. An intracellular intertalk between the cascades of events mediated by $P2X_1$ purinergic receptors and β-adrenoceptors exists in the ovine DSM.

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