Direct Inhibition of Rat Detrusor Muscle Contraction by Erythromycin

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Key words: Erythromycin, inhibition, detrusor, smooth muscle, contraction

ABSTRACT

Purpose

Detrusor instability is a common problem in the elderly, which is usually treated with anti-cholinergic medication. This study investigates the effect of erythromycin on rat detrusor muscle contractile response to characterise its potential as an alternative inhibitor of bladder muscle contraction**.**

Materials and Methods

Strips of rat detrusor muscle were suspended in a perfusion organ bath. The contractile response to direct muscle stimulation, electrical field stimulation (EFS, 0.5 to 60 Hz), carbachol (10^{-5} M), and potassium (10-80 × 10^{-3} M) were determined before and after the addition of erythromycin (10^{-4} M – 10^{-3} M). The contractile response to carbachol (10^{-5}M) in the presence of nifedipine (10^{-8} M or 10^{-6} M) or in calcium-free Kreb's solution was also determined in the absence and presence of erythromycin.

Results

Erythromycin 5 × 10^{-4} M inhibited the maximum contractile response to EFS, carbachol and potassium by 38% ($p<0.01$), 62% ($p<0.001$) and 17% ($p<0.05$) respectively, but did not significantly reduce the response to direct muscle stimulation. The atropine-resistant component of EFS-evoked contraction was inhibited by 19.5% ($p<0.01$) in the presence of erythromycin. In calcium-free Krebs solution, the maximum contractile response to carbachol was reduced by 42% of control ($p<0.0001$) and nifedipine 10^{-8} M had no additional effect. When erythromycin 5 × 10^{-4} M was added together with nifedipine 10^{-8} M, the response to carbachol was inhibited by a further 25% ($p<0.005$).

Conclusions

Erythromycin inhibits rat detrusor muscle contraction through the inhibition of calcium influx and the modulation of intracellular calcium movement.

INTRODUCTION

Estimates for the prevalence of overactive bladder (OAB) range between 3 and 43% depending on the study method used and the age of the patient [Milsom *et al.* 2000]. Urge incontinence may occur if intravesical pressure exceeds urethral closure pressure and is present in 30-50% of people with OAB [Appell *et al.* 2001, Latifpour *et al.* 1992, Liberman *et al.* 2001]. Health care estimates for the cost of urinary incontinence in the USA exceed 26.3 billion dollars in 1995 [Wagner *et al.* 1998]. OAB is frequently treated pharmacologically, but the commonly used agents (such as the anti-muscarinics) suffer from poor efficacy and tolerability, even in extended release formulations [Van Kerrebroeck *et al.*2001, Appell *et al.* 2001]. There is, therefore, interest in developing alternative avenues of treatment for this condition.

In the mammalian urinary bladder, there are two important excitatory neurotransmitters that stimulate the bladder to contract [Sibley 1984], acetylcholine and adenosine 5'-triphosphate (ATP) [Brading *et al.* 1990]. In humans, voiding is initiated predominantly by acetylcholine that activates muscarinic receptors in the normal human bladder [Messelink *et al.*1999]. In some patients with unstable bladders, an ATP-stimulated component of contraction becomes more evident [Wu *et al.* 1999]. In rats, ATP accounts for approximately 30% of the contractile force generated in the normal bladder, a situation that may resemble some of the changes found in unstable human detrusor, making this a useful model for the study of detrusor muscle contractile responses to stimuli.

The physiological effects of erythromycin, a macrolide antibiotic, on smooth muscle contraction have been investigated extensively in a variety of tissues and species and present a complex picture [Armstrong *et al.* 1992, Minocha *et al.* 1991, Nissan *et al.*

1997, Tamaoki *et al.* 1995]. Effects of erythromycin include actions on motilin receptors [Nissan *et al.* 1999, Peeters *et al.* 1989], Ca^{2+} channels [Armstrong *et al.* 1992] and nerves [Minocha *et al.* 1991, Nissan *et al.* 1997]. Nissan et al (1999) demonstrated that erythromycin inhibited the contractile response of rat detrusor muscle to carbachol and electrical field stimulation (EFS) but not to bradykinin, phenylephrine or substance P. This inhibitory effect of erythromycin on EFS-evoked responses was not altered in the presence of tetrodotoxin (TTX), which blocks presynaptic activation and neurotransmitter release. From this, it was concluded that the mode of action of erythromycin on rat detrusor smooth muscle contraction was due to a direct effect on the detrusor muscle. The present study was conducted to further investigate the mechanisms of action of erythromycin on the contractile response of isolated rat detrusor muscle and to investigate the hypothesis that an effect on calcium signalling is responsible.

MATERIALS AND METHODS

Adult Wistar rats were humanely culled by cervical dislocation using a schedule 1 procedure. The bladders were removed and placed in ice-cold Krebs solution. Bladder muscle strips, approximately 1 mm × 5 mm and dissected free of fat and serosa, were placed in an organ bath of 0.2 ml capacity and perfused at a rate of 1 ml min^{-1} with freshly prepared Kreb's solution containing NaCl 119 mM, KCl 4.4 mM, $NaHCO_3$ 20 mM, NaH_2PO_4 1.2 mM, $MgCl_2$ 2.5 mM, glucose 11 mM, $CaCl_2$ 2.5 mM, in distilled water pH 7.2, aerated with 95% O_2, 5% CO_2 and warmed to 37^oC. The apex of the muscle strip was attached to an isometric transducer (Pioden Control Ltd) connected to a four-channel Harvard Universal Oscillograph. The muscle strip was placed under tension of 10 mN for 1 hour to equilibrate. Final concentrations of erythromycin (Sigma UK) were prepared by first dissolving the solid in ethanol. Atropine (Sigma UK) and potassium chloride (Sigma UK) were made up in Kreb's solution on the day of the experiment. Nifedipine (Sigma UK) was dissolved in ethanol and kept in a darkened container. Carbachol (Sigma UK) was made up in either normal Kreb's solution or calcium-free Kreb's solution, being made without $CaCl_2$ and with the addition of EGTA 10^{-5} M. Repeated contractile responses of rat detrusor muscle strips over time showed no significant alteration.

Effect of ethanol and erythromycin on EFS-evoked responses

Control frequency response curves were obtained and then repeated in the presence of 0.25% ethanol, 0.5% ethanol and 0.5% ethanol plus erythromycin 5 x 10^{-4} M. These experiments were carried out to determine the effect of ethanol alone on the contractile response to of rat detrusor muscle.

Effect of erythromycin on EFS-evoked responses in the absence and presence of atropine

Electrical field stimulation (EFS) was performed using a Harvard dual impedance stimulator at 0.5, 1, 5, 10, 20, 40 and 60 Hz, 50V and 0.05 msec pulse width. Control frequency response curves were obtained and then repeated after incubation with erythromycin, 10^{-4} M, 5×10^{-4} M, and 10^{-3} M for 20 minutes each. Further responses were obtained after incubation with atropine 10^{-6} M alone and then with atropine 10^{-6} M plus erythromycin, 10^{-4} M, 5×10^{-4} M and 10^{-3} M, to determine the effect of erythromycin on the purinergic component of nerve stimulation.

The effect of erythromycin on ATP evoked responses

Control responses to adenosine 5-triphosphate (ATP) 10^{-2}M were obtained and then repeated in the presence of erythromycin 5 x 10^{-4} M.

Effect of erythromycin on carbachol-evoked responses

A control contractile response of rat detrusor muscle to a sub-maximal concentration of carbachol (10^{-5} M) was determined. Responses were then repeated in the presence of erythromycin, 10^{-4} M, 5×10^{-4} M and 10^{-3} M after equilibration for 20 minutes.

Effect of erythromycin on potassium-evoked responses

To determine the effect of erythromycin on contractile response to potassium, a control contractile response curve was obtained to potassium chloride, 10×10^{-3} M – 80×10^{-3} M and then repeated after the strips were perfused with erythromycin 5×10^{-4} M in Krebs solution for 20 minutes.

Effect of erythromycin and nifedipine on carbachol-evoked responses

The effect of erythromycin on the contractile response of detrusor muscle to carbachol was determined in the presence of erythromycin and nifedipine. Control contractile responses were obtained to carbachol 10^{-5} M and then repeated after incubation with Krebs and nifedipine 10^{-6} M alone or with nifedipine 10^{-6} M plus erythromycin 5×10^{-4} M.

Effect of erythromycin and nifedipine on carbachol-evoked responses in calcium-free Kreb's solution

To determine the effect of erythromycin on intracellular calcium release, carbachol-evoked contractile responses were determined in calcium-free Krebs solution. Two strips from each bladder used were studied in parallel. Control contractile responses to carbachol 10^{-5} M were determined in normal Kreb's solution and then in Ca^{2+} free Kreb's solution. The evoked response to carbachol in calcium-free Kreb's solution was obtained following the disappearance of spontaneous contractions after the Kreb's solution was changed from calcium containing to calcium-free. The bladder strips were then reincubated with normal Kreb's solution for 20 minutes to allow the intracellular calcium stores to be replenished. The normal Kreb's solution was then switched to calcium-free Kreb's solution and the samples incubated with either nifedipine 10^{-8} M alone or with nifedipine 10^{-8} M plus erythromycin 5×10^{-4} M before being stimulated with carbachol 10^{-5} M again, once the spontaneous contractions had ceased.

Effect of erythromycin on direct muscle stimulation

The contractile response to EFS at an increased pulse-width of 0.5 ms [Brading & Williams 1990] was determined at 1, 5, 10, 20, 30, 40 and 60 Hz, alone and in the presence of TTX 1.6×10^{-6} M (to eliminate neurogenic stimulation), and in the presence of TTX and erythromycin 5×10^{-4} M to investigate the effect of erythromycin on direct muscle stimulation alone.

Statistics

Results are presented as mean ± SEM. Statistical analyses were carried out using Student's t test and one-way analysis of variance followed by either Dunnett's or Bonferroni's correction for multiple comparisons. A *P*-value of <0.05 was considered significant. All analyses were performed using GraphPad Prism software.

RESULTS

Effect of ethanol on EFS-evoked responses

Ethanol 0.25% had no effect on the maximum contractile response of rat detrusor muscle to EFS (Fig 1). The maximum contractile response was, however, slightly inhibited by 0.5% ethanol by 14% (N.S) compared to the control response. The addition of erythromycin 5 x 10^{-4} M to 0.5% ethanol significantly reduced the contractile response by 46% ($p<0.01$) compared to the control response. The difference in the inhibition of the maximum response by 0.5% ethanol alone compared to 0.5% ethanol plus erythromycin 5 x 10^{-4} M was 32% ($p<0.01$).

Effect of erythromycin on EFS-evoked responses in the absence and presence of atropine

When stimulated with EFS the rat bladder detrusor strips contracted with a maximal force of 54 ± 11.4mN. Incubation with erythromycin 10^{-4} M, 5×10^{-4} M, and 10^{-3} M reduced the maximum contractile response to EFS compared to control by 3% (N.S), 39% ($p<0.01$), and 65% ($p<0.01$), respectively. When frequency response curves were compared using Dunnett's multiple comparison, the curves obtained after the addition of erythromycin 5×10^{-4} M and 10^{-3} M were significantly different ($p<0.01$) compared to the control (Fig. 2A).

Incubation of the bladder strips with atropine 10^{-6} M reduced the contractile response of detrusor muscle and inhibited the maximal response to EFS (10 Hz) by 23% ($p<0.001$) to 77% of the control response at that frequency. The further addition of erythromycin 10^{-4} M, 5×10^{-4} M, and 10^{-3} M inhibited the atropine-resistant response by 3% (NS), 27% ($p<0.01$) and 69% ($p<0.0001$), respectively at 10 Hz (Fig.2 B).

The effect of erythromycin on ATP evoked responses

Erythromycin 5 x 10^{-4} M significantly inhibited the contractile response to ATP 10^{-2} M by 27% $p<0.05$ (Fig 3). The magnitude of this inhibition was the same as the inhibitory effect of erythromycin 5 x 10^{-4} M on the atropine resistant response to EFS.

Effect of erythromycin on carbachol-evoked responses

A sub-maximal concentration of carbachol (10^{-5} M) elicited a contraction with a force of 13.7 ± 8.4 mN. The contractile response to carbachol was inhibited by erythromycin in a concentration-dependent manner. Erythromycin 10^{-4} M, 5×10^{-4} M, and 10^{-3} M inhibited the carbachol-evoked contraction by 30% ($p<0.05$), 62% ($p<0.001$) and 84% ($p<0.0001$), respectively (Fig. 4).

Effect of erythromycin on potassium-evoked responses

Potassium induces detrusor muscle contraction by opening L-type Ca^{2+} channels in the sarcolemma [Latifpour *et al.* 1992]. To investigate whether erythromycin affects calcium influx an experiment was carried out to determine the effect of erythromycin on contractile responses to depolarisation-evoked calcium influx. The addition of erythromycin 5 x 10^{-4} M reduced the contractile response to potassium significantly at all potassium concentrations tested except 50×10^{-3} M (Fig. 5). The maximal contractile response, at 60 x10^{-3} M KCl, was reduced by 17% ($p<0.05$) by erythromycin 5×10^{-4} M These results suggest that erythromycin has an inhibitory effect on excitation-coupling mechanisms and may inhibit calcium influx through voltage-sensitive calcium channels.

Effect of erythromycin and nifedipine on carbachol-evoked responses

To investigate the effect of erythromycin on calcium release from intracellular stores, responses to carbachol were obtained in the presence of nifedipine, a potent inhibitor of Ca^{2+} influx through L-type [Zar *et al.* 1990]. Addition of nifedipine (10^{-6} M) to the organ bath perfusate markedly inhibited carbachol evoked contractions by 81%, ($p<0.0001$). The addition of erythromycin 5×10^{-4} M and nifedipine 10^{-6} M to the perfusing solution reduced the contractile response by a further 13% ($p<0.05$) to 6% of the control contractile response (Fig. 6A), suggesting an effect of erythromycin on intracellular calcium mobilization as well as calcium entry mechanisms.

Effect of erythromycin and nifedipine on carbachol-evoked responses in Ca^{2+} free Kreb's solution

The experiment above was repeated in calcium-free Kreb's solution to further investigate the effect of erythromycin on responses involving intracellular calcium stores only. A lower concentration of nifedipine (10^{-8} M) was used to reduce the possibility of non-specific effects, and a shorter incubation period (until spontaneous contractions ceased) was used to minimise loss of calcium from intracellular stores. The contractile response to carbachol in calcium-free Kreb's solution was inhibited by 42% ($p<0.0001$) compared to control response in normal Kreb's solution. The addition of nifedipine (10^{-8} M) to calcium-free Kreb's solution had no additional inhibitory effect on the contractile response to carbachol (Fig. 6B). When erythromycin 5×10^{-4} M was added together with nifedipine 10^{-8} M, the contractile response to carbachol was reduced by a further 25% ($p<0.005$) to 33% of the control contractile response. Similarly erythromycin 5 x 10^{-4} M alone inhibited the response

to carbachol in Ca^{2+} free Kreb's solution to 34% of the control contractile response (not shown).

Effect of erythromycin on direct muscle stimulation

Erythromycin (5×10^{-4} M) did not significantly inhibit the EFS-evoked (0.5 ms pulse width, 1-50 Hz) contraction in the presence or absence of TTX (result not shown) suggesting that erythromycin does not inhibit detrusor smooth muscle contraction by an action on presynaptic mechanisms.

DISCUSSION

The results of this study demonstrate that erythromycin has an inhibitory action on the contractile response of rat detrusor muscle strips to EFS, carbachol, ATP and potassium. The effect of erythromycin on EFS was concentration dependent, with no significant effect at 10^{-4} M, but significant inhibition at 5×10^{-4} M and 10^{-3} M. Erythromycin also inhibited the atropine-resistant response to EFS, indicating that this inhibitory effect of this drug was not due solely to an effect on cholinergic transmission, as previously suggested by the findings of Nissan *et al* [1999]. Confirmation of the inhibition of non cholinergic transmission by erythromycin was further demonstrated by the inhibitory action of this drug on direct purinergic activation with ATP.

Stimulation by potassium results in depolarisation of the sarcolemma and activation of L-type calcium channels, permitting calcium entry and contraction to be initiated, independent of muscarinic or purinergic receptor activity. The inhibition of potassium-stimulated contractile responses by erythromycin suggested that the mode of action of this drug was not centred on the receptors themselves but occurred via downstream signalling or contractile mechanisms. Inhibition by a sub-maximal dose of erythromycin (5×10^{-4} M) of the total contractile response to EFS was greater (39%) than the inhibition of the peak contractile responses to either membrane depolarizing potassium or purinergic (atropine-resistant) stimuli (17% and 27%, respectively) that are dependent solely on extracellular calcium entry, suggesting that erythromycin was active on additional mechanisms independent of sarcolemmal calcium entry. At the same concentration of erythromycin, the contractile responses to

carbachol were inhibited to a greater extent (62%). In detrusor muscle, contractile responses to muscarinic receptor activation are mediated by the release of intracellular calcium stores, in addition to the entry of extracellular calcium [Maggi *et al.* 1998]. The greater susceptibility of carbachol-stimulated contractile response to erythromycin compared to stimuli resulting in depolarisation-evoked contractile responses suggested that there may be an inhibitory action of erythromycin on intracellular calcium release, in addition to an inhibition of extracellular calcium entry. To investigate this possibility further the inhibition by erythromycin of carbachol-evoked contractile responses of detrusor strips perfused with calcium-free Krebs's solution containing nifedipine, to remove the calcium entry component, was investigated. Contractile responses were reduced by 42% in the absence of extracellular calcium. These responses were not inhibited further by the calcium entry blocker, nifedipine, indicating that the residual contractile activity in these experiments was due to mobilisation of intracellular calcium stores. The addition of erythromycin (5×10^{-4} M) to calcium-free, nifedipine-containing Kreb's solution inhibited the contractile response to carbachol further suggesting an inhibitory action on the release of intracellular calcium from the sarcoplasmic reticulum or alteration of its availability to the contractile machinery within the cell. Together, the data are suggestive of actions of erythromycin to inhibit both calcium entry and release of internal calcium stores, possibly by the modulation of the Ca^{2+} -induced Ca^{2+} release (CICR) initiation of muscle contraction. However, effects on the contractile mechanism itself cannot be ruled out.

There are many studies demonstrating a wide variety of pharmacological effects attributable to erythromycin [Armstrong *et al.* 1992, Minocha *et al.* 1991, Tamaoki *et*

al. 1995, Nissan *et al.* 1999, Peeters *et al.* 1989, Zara *et al.* 1985]. Erythromycin inhibited nerve-mediated contractions in guinea pig ileum by actions on enteric nerves and on longitudinal muscle but not circular muscle [Nissan *et al.* 1999]. Human and guinea pig gallbladder smooth muscle contractions were also inhibited by erythromycin [Nissan *et al.*1997]. In human bronchus, erythromycin inhibited nerve-mediated contraction, probably by reducing exocytotic release of acetylcholine [Tamaoki *et al.* 1995]. Conversely, in rabbit terminal ileum, erythromycin stimulated motility by activation of dihydropyridine-sensitive calcium channels [Armstrong *et al.* 1992]. Similarly, in the human gastrointestinal tract at low concentration erythromycin had pro-motile effects, mainly through an action on motilin receptors and of opening calcium channels [Peeters *et al.* 1989, Nissan *et al.* 1999] but at higher concentrations, erythromycin had an inhibitory effect on gastrointestinal smooth muscle contraction [Depoortere *et al.* 1997]. Depoortere and Peeters reported that in rabbits, this inhibitory effect of erythromycin was mediated via block of dihydropyridine-sensitive calcium channels, thereby reducing calcium influx. Motilin receptors in rat detrusor muscle have not been reported in the literature**,** and the results presented herein indicate that the inhibitory action of this macrolide on rat bladder contraction is due to the inhibition of calcium movement into and within smooth muscle cells.

The concentrations of erythromycin required to inhibit rat detrusor muscle contraction in this study were higher than could reasonably be achieved by oral dosing in humans [Mannisto *et al.* 1975]. Erythromycin is known to have significant gastrointestinal effects (increased motility) [Peeters *et al.* 1989] making oral treatment with erythromycin an unattractive option for the treatment of unstable bladder. Intra-

vesical administration could remain an option but poor absorption through the urothelial barrier and the frequency of instillation that would be required make this unappealing. In addition, erythromycin is an antibiotic and its wide application for urinary incontinence would greatly increase the risk of development of resistant bacterial species. For these reasons, use of erythromycin itself for urinary incontinence cannot be considered but further screening of erythromycin-related compounds may reveal more potent and/or specific analogues that could be considered.

CONCLUSION

At high concentrations, erythromycin has a significant inhibitory effect on isolated detrusor muscle contraction. Our results suggest that erythromycin has inhibitory effects on both calcium entry through voltage-sensitive calcium channels and the release of calcium from intracellular stores. Further effects on the contractile machinery directly cannot be ruled out. Although erythromycin itself is unsuitable for the treatment of urinary incontinence, related compound may hold potential for future development.

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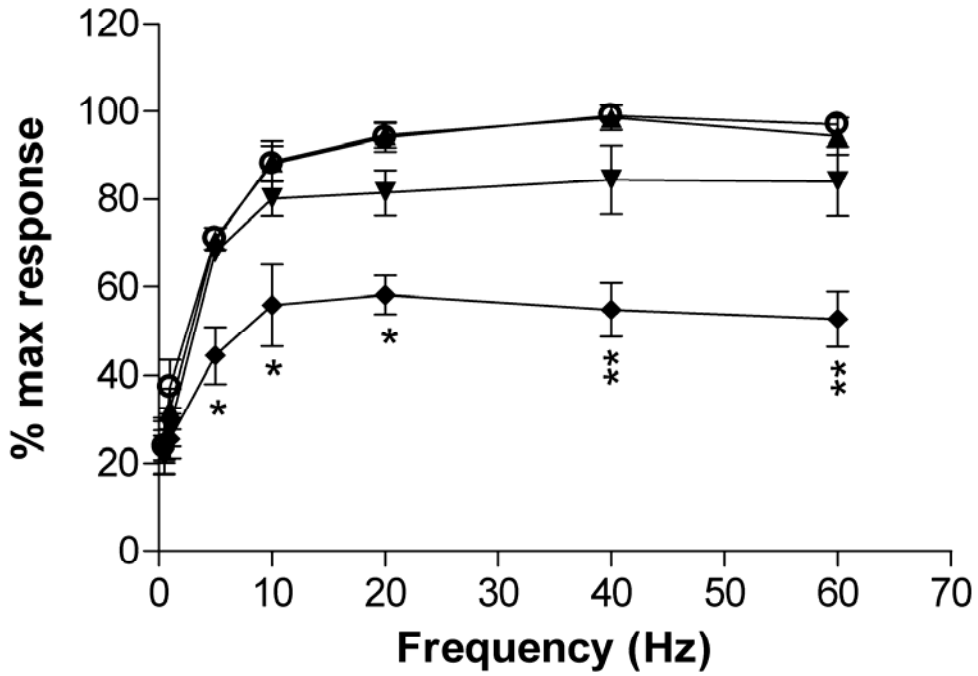
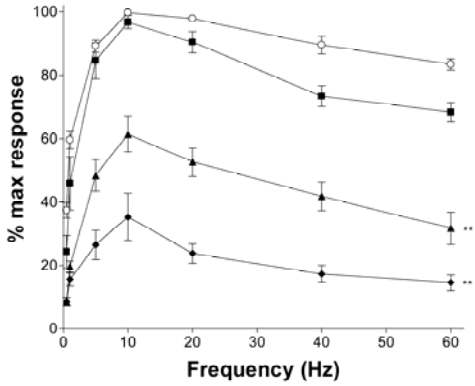


LEGENDS

Fig 1. The effect of ethanol alone and with erythromycin on the contractile response of rat detrusor muscle. O control response, ▲ ethanol 0.25%, ▼ ethanol 0.5%, ◆ ethanol 0.5% plus erythromycin 5 x 10^{-4} M. * $p<0.05$, **$p<0.01$ compared to 0.5% ethanol alone. (n=4).



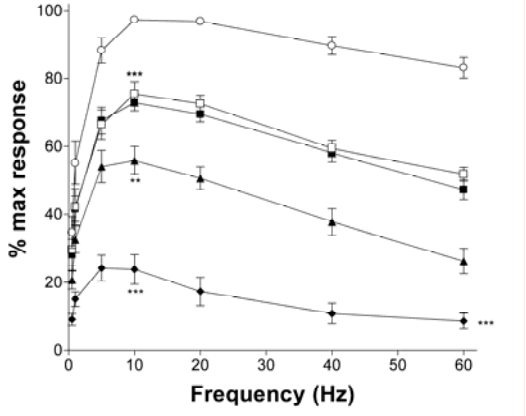


Figure 2 A. The effect of erythromycin 10^{-4} M to 10^{-3} M on the contractile response of rat detrusor to EFS, 0.5 Hz to 60 Hz. ○ Control response, ■ after the addition of erythromycin 10^{-4} M, ▲ erythromycin 5 x 10^{-4} M, ◆ erythromycin 10^{-3} M.

** $p < 0.01$, (n=5).

Figure 2 B. The contractile response of rat detrusor muscle to EFS in the presence of atropine 10^{-6} M, with and without the addition of erythromycin 10^{-4} to 10^{-3} M.

○ Control response, □ after the addition of atropine 10^{-6} M, ■ after the addition of atropine 10^{-6} M plus erythromycin 10^{-4} M, ▲ atropine 10^{-6} M plus erythromycin 5 x 10^{-4} M, ◆ atropine 10^{-6} M plus erythromycin 10^{-3} M. ** $p < 0.01$, *** $P < 0.001$, (n=9).

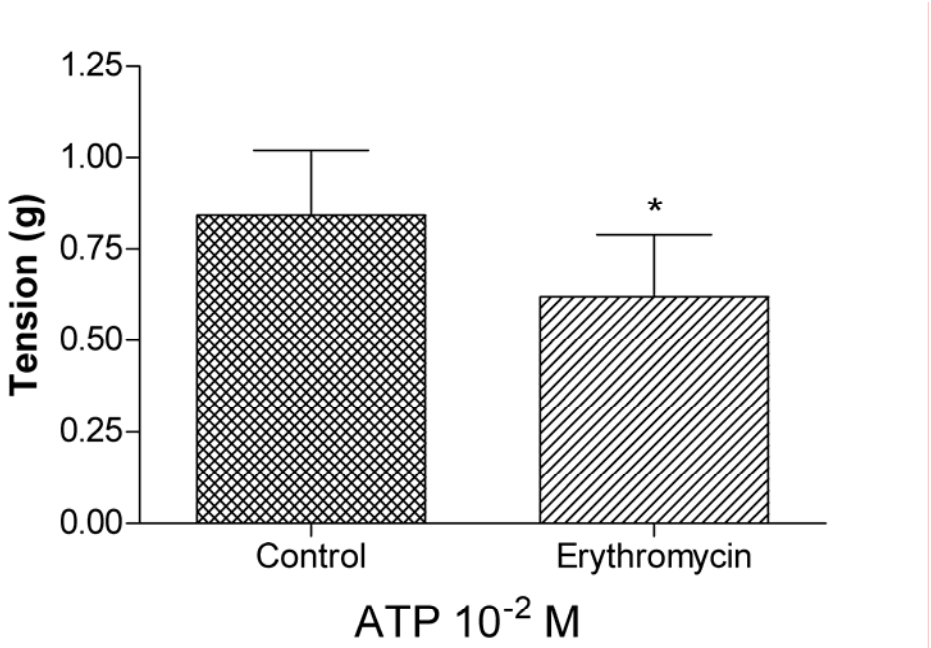


Fig 3. The effect of erythromycin on the contractile response of rat detrusor muscle to ATP 10^{-2} M. Crossed hatch = control, left hatch = erythromycin 5 x 10^{-4} M.

* $p<0.05$. (n=4)

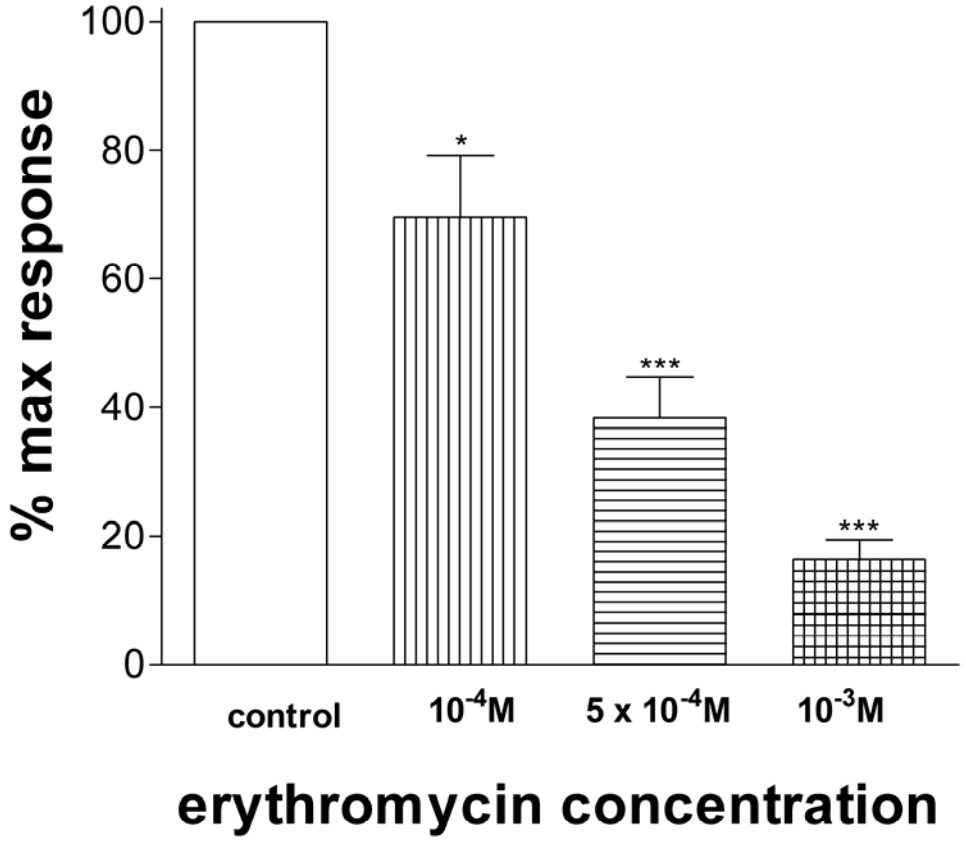


Figure 4. The effect of erythromycin 10^{-4} M - 10^{-3} M on the contractile response to carbachol 10^{-6} M. Open bar = control, vertical lines = erythromycin 10^{-4} M, horizontal lines = erythromycin 5×10^{-4} M, squares = erythromycin 10^{-3} M.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, (n=5).

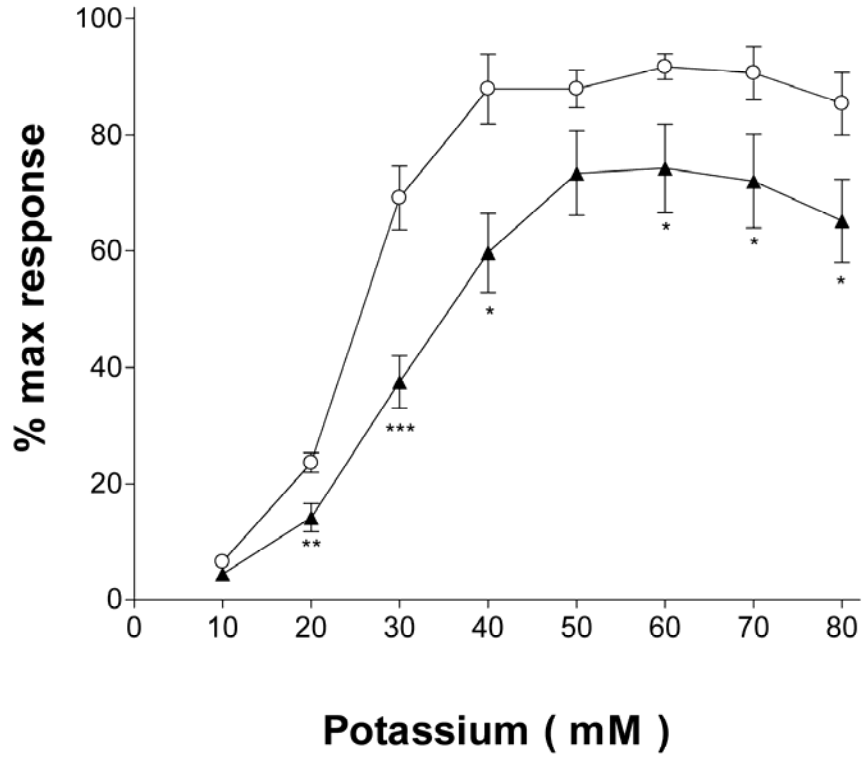
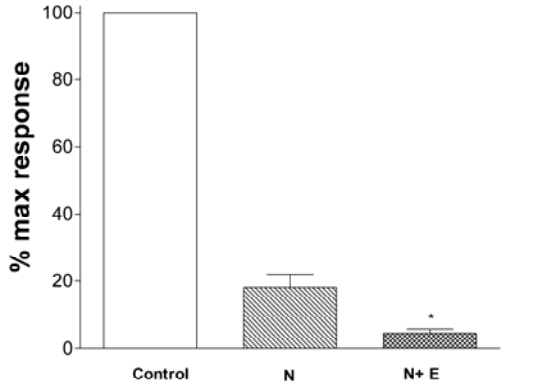


Figure 5. The effect of erythromycin 5×10^{-4} M on the potassium-evoked contractile response of rat detrusor muscle (10–80 mM). ○ Control, ▲ erythromycin 5×10^{-4} M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, (n=7).



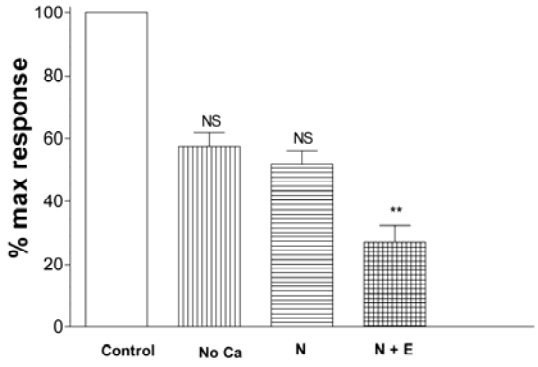


Figure 6 A. The effect of nifedipine 10^{-6} M alone with erythromycin 5×10^{-4} M on the contractile response of rat detrusor muscle to carbachol 10^{-6} M. Open bar = control, right hatch = nifedipine 10^{-6} M (N), crossed hatch = nifedipine 10^{-6} M and erythromycin 5×10^{-4} M (N+E). * $p < 0.05$, *** $p < 0.001$, (n=6).

Figure 6 B. The effect of nifedipine 10^{-8} M alone and nifedipine 10^{-8} M together with erythromycin 5×10^{-4} M on the contractile response of rat detrusor muscle to carbachol 10^{-6} M in Ca^{2+} free Kreb's solution. Open bar = control, vertical lines = calcium-free Kreb's solution, horizontal lines = calcium-free Krebs with nifedipine 10^{-8} M (N), squares = calcium-free Krebs with nifedipine 10^{-8} M and erythromycin 5×10^{-4} M (N+E),

NS. No statistical differences between the response to carbachol in calcium-free Kreb's solution alone and the response in calcium-free Kreb's with nifedipine 10^{-8} M.

** $p < 0.001$ compared to the response in calcium-free Kreb's solution alone. (n=6)