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Inhibition of the contractile response of the rat detrusor muscle by the β_2-adrenoceptor agonist clenbuterol

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Abstract

The action of clenbuterol, β_2-adrenoceptor agonist, on the contractile response of isolated rat detrusor muscle strips was investigated in vitro. Clenbuterol (10^{-5} M) inhibited the detrusor muscle frequency response (1–40 Hz, $p < 0.02$) with a more pronounced effect at 1 Hz than 40 Hz. Clenbuterol (10^{-6} M) significantly inhibited the contractile response to exogenous ATP (10^{-4} to 10^{-2} M, $p < 0.05$) but not to carbachol (10^{-9} to 10^{-4} M). The presence of 10^{-5} M ICI 118, 551, β_2-adrenoceptor antagonist, shifted significantly the clenbuterol dose–response to 1 Hz electrical field stimulation (EC_{50} 3.4×10^{-6} M ($\pm 2.2 \times 10^{-6}$ M) for clenbuterol alone, to 4.1×10^{-4} M ($\pm 8.8 \times 10^{-5}$ M), $P < 0.05$). In conclusion, clenbuterol inhibits electrical field and ATP-stimulated contractions of detrusor muscle. Reversal of the clenbuterol inhibition of detrusor muscle contraction by ICI 118, 551 shows that clenbuterol is probably acting through postsynaptic β_2-adrenoceptors, which modulate the response to ATP released from purinergic nerves.

Keywords: Clenbuterol; Agonist; Contractile response

1. Introduction

Urinary continence is maintained by a combination of relaxation of the detrusor muscle and contraction of the urethral sphincter, whereas incontinence, due to detrusor instability, is caused by powerful detrusor muscle contractions, which overcome the urethral sphincter mechanism resulting in urinary leakage. Current treatment of detrusor instability involves the use of different pharmacological agents (Castleden and Robinson, 1998). Unfortunately, all of the drugs used to date lack bladder specificity and selectivity (Brading and Turner, 1994) and some, like oxybutynin, one of the most common treatments, have unacceptable side effects making treatment unsatisfactory (Yaker et al., 1995). The contractile activity of bladder smooth muscle has been shown to be inhibited by β-adrenoceptor agonists (Nording and Gosling, 1980; Levin et al., 1988; Morita et al., 1993, 1995). In a study of different β-adrenoceptor subtypes in the rabbit, canine, and human bladder, only β_2-adrenoceptors were found to be functionally active in the detrusor muscle of the human (Restorick and Mundy, 1989). In human tissue, the β_2-adrenoceptor agonist isoproterenol (isoproterenol bitarate) caused a concentration-dependent decrease in the pre-contraction of the detrusor muscle stimulated by 10^{-5} M carbachol. In addition, β-adrenoceptor agonists, like isoproterenol, have been shown to relax contraction of the detrusor muscle caused by depolarization of the membrane with high KCl concentrations (Nishimoto et al., 1995). This inhibition of the contractile force in detrusor muscle following stimulation of the β_2-adrenoceptor has been linked to increases in cyclic AMP (Rodbell, 1980; Levin et al., 1988; Morita et al., 1993; Nishimoto et al., 1995). In a recent study, Morita et al. (1995) compared the effectiveness of isoproterenol and the selective β_2-adrenoceptor agonist clenbuterol (4-amino-alpha-[(tert-butylamino) methyl]-3,5-dichlorobenzylalchol hydrochloride) on the contractile response of the rabbit vesicourethral muscle. Both caused relaxation of the urinary bladder dome and base, although clenbuterol had a

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more pronounced effect. In addition, clenbuterol potentiated the electrical field stimulation induced contraction of the external urethral sphincter. These data suggested that clenbuterol may have a role to play in the treatment of urinary incontinence by inhibiting detrusor muscle contraction and facilitating external urethral sphincter selectivity (Morita et al., 1995).

To contribute to the assessment of its potential use in the treatment of detrusor instability, we have investigated further the action of clenbuterol on rat detrusor muscle to dissect its effect on the contractile response of this tissue.

2. Methods

Male and female Wistar rats (250–400 g) were killed with a blow to the head followed by dislocation of the neck. For each experiment a minimum of five rats were used, the results are a mean ± S.E.M. of two bladder strips from each rat. The bladders were removed and placed into cold Kreb's solution which was made up fresh and consisted of: NaCl 119 mM, KCl 4.4 mM, $NaHCO_3$ 20 mM, NaH_2PO_4 1.2 mM, $MgCl_2$ 1.2 mM, $CaCl_2$ 2.5 mM and Glucose 11 mM made up in distilled water (pH 7.2). Bladders were dissected free of any adhering fat or serosa and then cut into longitudinal strips of muscle ($4 \times 1 \times 0.5$ mm). These were suspended in a perspex organ bath of 0.2 ml volume (Brading and Sibley, 1983). The bladder strips were constantly perfused at the rate of 1 ml min^{-1} with Kreb's solution aerated with 95% oxygen and 5% carbon dioxide and the temperature was maintained at 37°C throughout the experiments.

The base of the bladder muscle strip was attached to the bottom of the organ bath chamber using a fine silk suture and the top to an isometric force transducer connected to a four-channel oscillograph (Harvard). Before experimentation, the bladder muscle strips were allowed to equilibrate for 1 h under a tension of 10 mN.

2.1. Electrical field stimulation with tetrodotoxin

The muscle strips were stimulated by electrical field stimulation using recessed platinum electrodes in the wall of the organ bath which were connected to a stimulator (Digitimer). Stimulation occurred at a frequency of 1–80 Hz, 50 V, with a pulse width of 1, 0.5, and 0.05 ms in 10-s trains at 3-min intervals. The bladders were then incubated with 1.6×10^{-6} M tetrodotoxin for 15 min and then re-stimulated.

2.2. Electrical field stimulation

Curves of frequency response were obtained by stimulating the samples with 1, 5, 10, 20, and 40 Hz at 0.5 ms and 50 V. Strips of bladder were then incubated with 10^{-5} M clenbuterol (Tocris Cookson, UK) alone, a β_2-adrenoceptor agonist, or ICI 118, 551 ((±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl) amino]-2-butanol) (Tocris Cookson), a β_2-adrenoceptor antagonist, alone and with clenbuterol 10^{-5} M, for 15 min before being re-stimulated. Additions were made to the Kreb's solution before warming or perfusion passed the tissue. For this reason, preparations were left to equilibrate for 15 min before any experiments began, we found that this was the optimum time for exposure under our experimental conditions. Response curves were presented as a percentage of the maximum response.

2.3. Effect of clenbuterol at 1 and 40 Hz

Bladder muscle strips were suspended in the organ bath as described previously and allowed to equilibrate for 1 h. Each strip was stimulated at 1 Hz and, following a 3-min recovery, stimulated again at 40 Hz. Increasing concentrations of clenbuterol 10^{-9} to 10^{-4} M were added to the bath to produce cumulative dose–response curves at both frequencies. Cumulative dose–response curves were expressed as a percentage of the contraction achieved at 1 and 40 Hz, respectively prior to the addition of clenbuterol. Experiments were repeated in the presence of atropine (10^{-6} M).

2.4. Inhibition of agonist-induced contractile response

Bladder muscle strips were stimulated at 1 and 40 Hz as a control. The antagonist ICI 118, 551 at a concentration of 10^{-5} M was added to the bath for 15 min before the strips were exposed to increasing concentrations of clenbuterol (10^{-9} to 10^{-4} M) to produce a cumulative dose–response curve in the presence of the antagonist. Again, each clenbuterol concentration was equilibrated for 15 min before stimulation at 1 and 40 Hz. This data was then compared with the cumulative dose–response data produced at 1 and 40 Hz for clenbuterol alone.

2.5. Adenosine 5′-triphosphate (ATP) stimulation

Bladder strips were stimulated with increasing concentrations of ATP between 10^{-6} and 10^{-2} M. ATP was made up as a stock in distilled water (10^{-2} M) and then was diluted with Kreb's solution (ATP–Kreb's) to produce the required concentrations. The bladder strips were perfused for 10 s with known concentrations of ATP–Kreb's solution to produce a cumulative dose–response curve. Either clenbuterol (2×10^{-5} M) or clenbuterol and ICI 118, 551 (2×10^{-5} M) was then added to the bath and the preparation equilibrated for 15 min before being re-stimulated with ATP as before (10^{-6} to 10^{-2} M).

In further experiments, bladder strips were mounted in a 50-ml fixed volume organ bath at 37°C filled with Kreb's solution and tensioned and aerated as before. This method was used to allow drugs to be added directly to the bath

without having to perfuse the solutions past the tissue. The bladder strips were pre-contracted with ATP (10^{-5} and 10^{-4} M) as a control. After the contraction at each concentration, the preparation was washed with fresh Kreb's solution and left for 15 min to recover. The pre-contraction was then repeated and during the rising phase of the contraction clenbuterol (10^{-6} M) was added directly to the organ bath.

2.6. Carbachol stimulation

Mounted bladder strips were stimulated with carbachol at increasing concentrations (10^{-8} to 10^{-3} M) for 10 s. Carbachol, dissolved in distilled water (10^{-2} M) was diluted with Kreb's solution to make Carbachol–Kreb's and used in the same way as ATP–Kreb's, to produce a cumulative dose–response curve. Clenbuterol (2×10^{-5} M) or ICI 118, 551 (2×10^{-5} M) was then added to the bath and the preparation equilibrated for 15 min before being re-stimulated with carbachol at the same concentrations.

Additionally, bladder strips mounted in a 50-ml fixed volume organ bath, as described above, were pre-contracted with 10^{-6} and 10^{-5} M carbachol as a control. After washing with fresh Kreb's solution and 15 min recovery between each concentration, the contraction was repeated and clenbuterol (10^{-6} M) was added during the rising phase of the contraction.

2.7. Statistical analysis

Data were expressed as mean ± S.E.M. Statistical analysis of drug effect and difference between treatment groups was determined using: (1) analysis of variance (ANOVA), where Dunnett's test was used to compare groups to a single control. In all other circumstances a multiple comparison was performed using Tukey's HSD. (2) Paired or unpaired *t*-tests were performed as appropriate. A p value of < 0.05 was regarded as significant in all cases.

3. Results

3.1. Electrical field stimulation with tetrodotoxin

At the different pulse widths tested, tetrodotoxin decreased or abolished the contractile response of the detrusor muscle strips (Fig. 1). At 1 ms pulse width the contractile response was reduced at all frequencies and the remaining contractile response of the bladder strips was due to direct muscle stimulation (Brading, 1987). There was no significant difference between the contractile response obtained at 0.05 and 0.5 ms and both were abolished by tetrodotoxin indicating the neurogenic origin of the contractile response at these pulse widths.

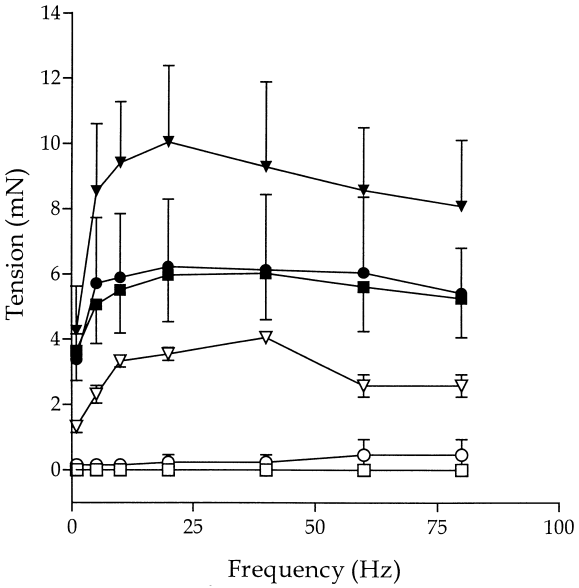

Fig. 1. Contractile response of rat detrusor muscle to electrical field stimulation before and after exposure to 1.6×10^{-6} M tetrodotoxin at different pulse widths. Responses to pulse widths 0.05 ms (■, □), 0.5 ms (●, ○) and 1 ms (▼, ▽). Control responses are shown by closed symbols. Responses obtained after exposure to tetrodotoxin are shown by open symbols ($n = 5$). The curves for electrical field stimulation at 0.5 and 0.05 ms are not significantly different. Nor are the responses at these frequencies significantly different following the addition of tetrodotoxin. However, all other curves are significantly different from each other. $P < 0.001$ in all cases.

3.2. Electrical field stimulation

After 15 min incubation with 10^{-5} M clenbuterol the electrical field stimulated response at all stimulation frequencies was significantly reduced when compared to the control, $p < 0.02$ in all cases (Fig. 2). The contractile response to electrical field stimulation of the detrusor muscle in bladder strips incubated for 15 min in the presence of the β_2-adrenoceptor antagonist ICI 118, 551 alone was not significantly affected (not shown). In addition, at a concentration of 10^{-5} M clenbuterol was observed to inhibit the spontaneous contractions normally recorded in the bladder muscle preparations in 50% of the samples examined (not shown).

3.3. Inhibition by clenbuterol of contractions stimulated by electrical field stimulation at 1 and 40 Hz

The contractile responses of bladder muscle strips stimulated at 1 and 40 Hz in the presence of clenbuterol at different concentrations (10^{-9} to 10^{-4} M) were inhibited in a dose-dependent manner (Fig. 3). When stimulated using a frequency of 1 Hz, clenbuterol had an EC_{50} of 3.4×10^{-6} M ($\pm 2.2 \times 10^{-6}$ M). A similar result (EC_{50} 3.0×10^{-6} M ($\pm 2.3 \times 10^{-6}$ M) was observed when experiments were performed in the presence of atropine

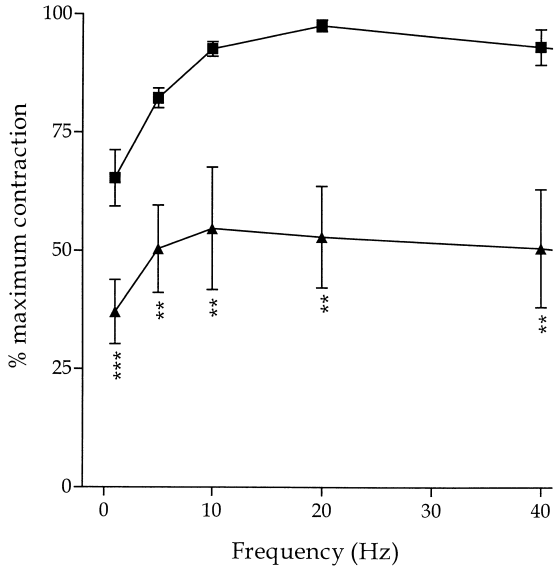

Fig. 2. Electrical field stimulation of rat detrusor muscle at 1–40 Hz ($n=5$) control stimulation (■) ($n=5$) and (▲) in the presence of clenbuterol 10^{-5} M ($n=5$). $^{**}p<0.01$ and $^{***}p<0.001$. The maximum contractile responses normalized to 100% for responses to 1 and 40 Hz had a mean tension and S.E.M of 40.0 ± 1.5 and 10.8 ± 2.5 mN, respectively.

10^{-6} M (not shown). However, when the same preparations were stimulated at 40 Hz, the EC_{50} was 7.3×10^{-6} M ($\pm1.8\times10^{-6}$ M). There was a statistically significant difference in the effect of clenbuterol at the two stimulation frequencies when compared with a Student's *t*-test ($p<0.05$) for all doses of clenbuterol except 10^{-4} M. In the presence of atropine, an inhibitory effect of clenbuterol was apparent on the small residual response (not shown).

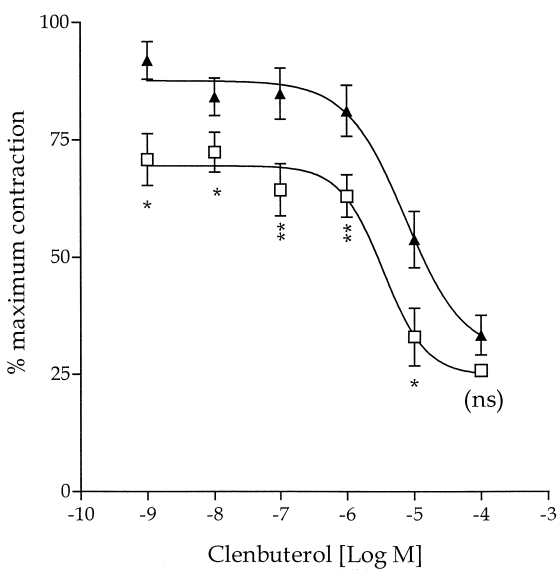

Fig. 3. The effect of cumulative addition of clenbuterol (10^{-9} to 10^{-4} M) to rat detrusor muscle stimulated at (□) 1 Hz and (▲) 40 Hz electrical field stimulation. $^{*}p<0.05$ and $^{**}p<0.01$.

3.4. Inhibition of contractile response to electrical field stimulation

When bladder strips were stimulated at 1 Hz in the presence of the β_2-adrenoceptor antagonist ICI 118, 551 at a concentration of 10^{-5} M (Fig. 4A), the dose-dependent effect of clenbuterol was significantly different to that obtained in the absence of ICI 118, 551 at all concentrations ($P<0.05$ in all cases). There was also a significant shift in the EC_{50} values of 3.4×10^{-6} M ($\pm2.2\times10^{-6}$ M) for clenbuterol alone 4.1×10^{-4} M ($\pm8.8\times10^{-5}$ M) for clenbuterol in the presence of ICI 118, 551 ($p<0.05$) (analyzed using Graph pad prism sigmoid dose–response curves best fit.).

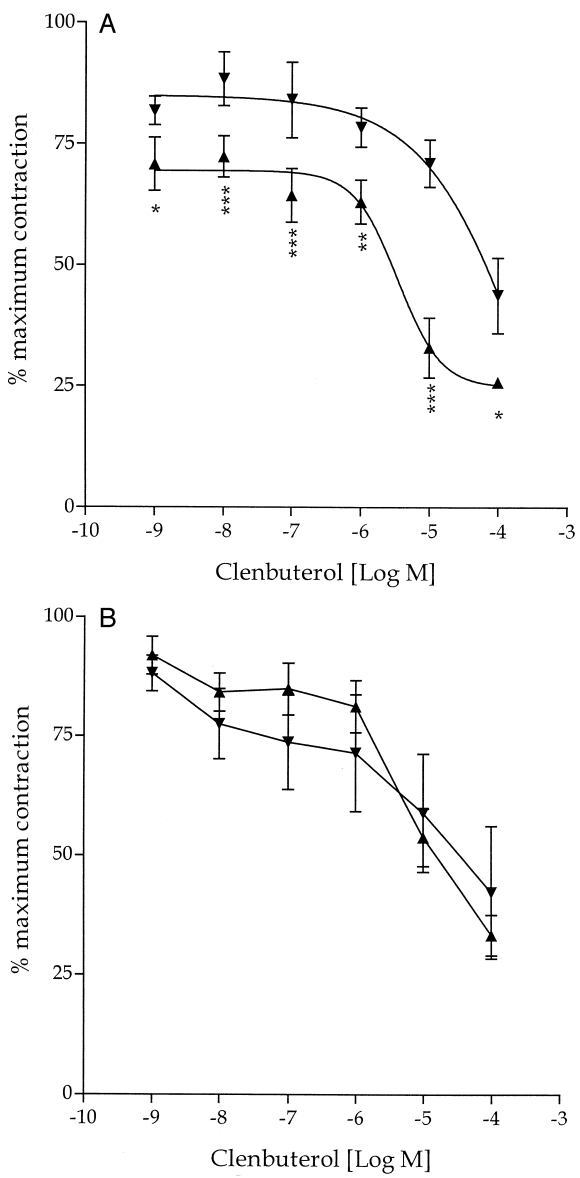

Fig. 4. The cumulative response curve of clenbuterol (10^{-9} to 10^{-4} M) stimulated at (A) 1 Hz and (B) 40 Hz electrical field stimulation ($n=5$) in the absence (▲) and in the presence (▼) of ICI 118, 551 (10^{-5} M) ($n=5$). $^{*}p<0.05$, $^{**}P<0.01$ and $^{***}p<0.001$.

When the bladder muscle strips were stimulated at 40 Hz in the presence of the antagonist at 10^{-5} M, the dose–response curve was not significantly different at any of the concentrations examined (Fig. 4B).

3.5. ATP stimulation

As previously reported (Burnstock, 1986; Brading, 1987), ATP (10^{-2} to 10^{-7} M) caused a dose-dependent contraction of the detrusor muscle. This contraction was inhibited by 2×10^{-5} M clenbuterol (Fig. 5) at all concentrations of ATP. The inhibition was not significant at 10^{-6} and 10^{-2} M but was significantly different at all other concentrations tested ($p < 0.05$). Inhibition of ATP-induced contractile response by clenbuterol was prevented in the presence of β_2 antagonist ICI 118,551 (10^{-6} M) (Fig. 5). Pre-contraction of the bladder with ATP was also inhibited by 10^{-6} M clenbuterol: control contractions to 10^{-3} M ATP of 11.9 ± 7.4 mN normalized to 100% of maximum contraction, were reduced to 65.7 ± 4.4% with the addition of clenbuterol ($p < 0.006$) (not shown).

3.6. Carbachol stimulation

As previously reported (Brading, 1987) carbachol (10^{-8} to 10^{-3} M) caused a dose-dependent contraction of the detrusor muscle. This dose-dependent contraction was not significantly affected by the β_2-adrenoceptor agonist clenbuterol (2×10^{-5} M) (Fig. 6) or by the β_2-adrenoceptor antagonist ICI 118, 551 (2×10^{-5} M) (not shown). Additionally, pre-contraction of the bladder with 10^{-5} M carbachol was not significantly affected by the addition of 10^{-6} M clenbuterol (not shown).

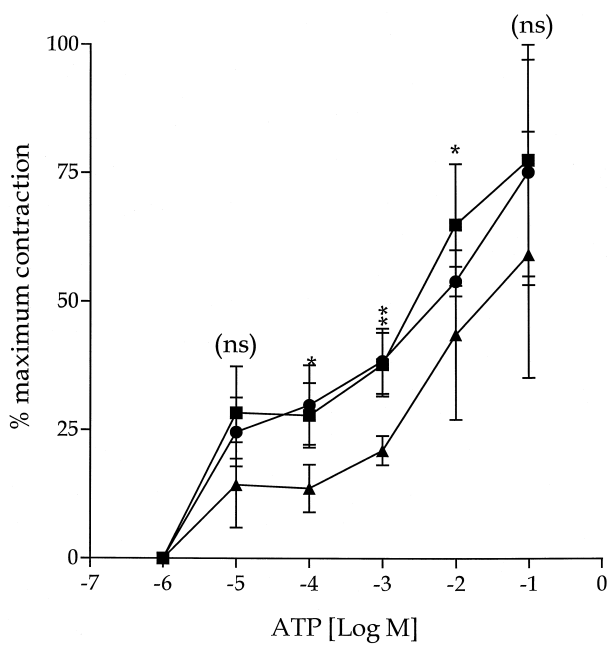

Fig. 5. Inhibition by clenbuterol of ATP stimulated of rat detrusor muscle contraction ($n = 5$). The percentage of maximum contraction caused by ATP (10^{-7} to 10^{-2} M) is shown before (■) and after (▲, ●) the addition of clenbuterol (10^{-5} M) in the absence (■, ▲) and presence (●) of ICI 118, 551 (10^{-6} M) ($n = 5$). Clenbuterol significantly inhibited the contractile response to ATP (10^{-4}–10^{-2} M) in the absence but not in the presence of ICI 118, 551. Ns = not significant, $^{*}p < 0.05$ and $^{**}P < 0.01$. The control tensions at maximum contraction in response to ATP had a mean of 18.3 ± 2.1 mN.

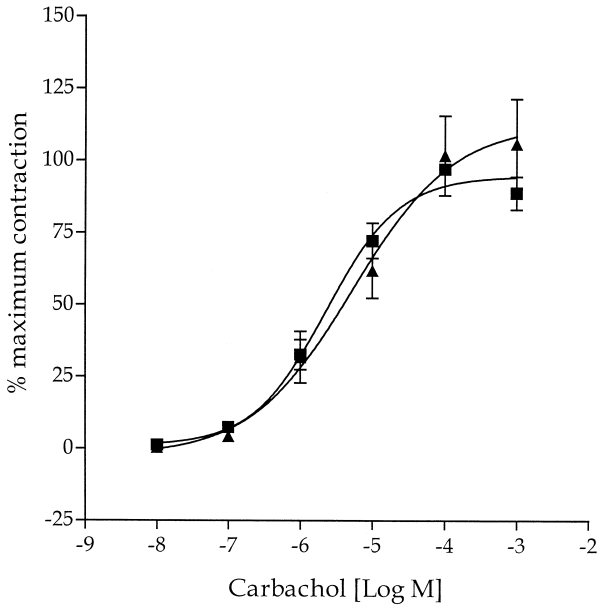

Fig. 6. Inhibition by clenbuterol of carbachol-stimulated rat detrusor muscle contraction ($n = 5$). The percentage of maximum contraction caused by carbachol (10^{-7} to 10^{-2} M) is shown before (■) and after (▲) the addition of clenbuterol (10^{-5} M) ($n = 5$). No significant differences, before or after the addition of clenbuterol, were observed. The maximum contractile response to carbachol in the control had a mean of 37.2 ± 11.3 mN.

4. Discussion

The present study was undertaken to evaluate the effects of a β_2-adrenoceptor agonist, clenbuterol, on the contractile response of the rat detrusor muscle to electrical field, ATP and carbachol stimulation. The three main observations were that clenbuterol inhibited the contractile response of the detrusor muscle to electrical field stimulation, that it inhibited ATP but not carbachol-stimulated contractions and that the inhibition caused by clenbuterol was reduced using a β_2-adrenoceptor antagonist at 10^{-5} M.

Electrical field stimulation of bladder muscle at different frequencies produces contraction of the bladder by stimulating both the cholinergic and purinergic components of the nervous innervation (Brading, 1987). The non-adrenergic non-cholinergic component was first described by Langley and Anderson (1895). Brading and Williams (1990) have since shown that responses to parasympathetic nerve stimulation cannot be abolished totally by muscarinic acetylcholine receptor blockers. At a

frequency of 1 Hz, ATP released from purinergic nerves is responsible for most of the observed contraction (Brading and Williams, 1990). As the frequency of electrical field stimulation increases, the percentage of the contraction due to neurotransmitters released from cholinergic nerves increases (Brading and Williams, 1990). In a review of bladder physiology, Brading (1987) detailed current theories on the non-cholinergic component of the response to electrical field stimulation, stating that either there is dual excitatory innervation or that cholinergic nerves release a co-transmitter (ATP) along with the acetylcholine; the ratio of release of these two neurotransmitters being dependent on the frequency of stimulation. Thus, when preparations were stimulated at 1 and 40 Hz in the present study, there was a significant difference in the proportions of neurotransmitters released responsible for the observed contractions.

The present experiments have shown that the inhibition of contraction caused by clenbuterol was greater when the bladder strips were stimulated at 1 Hz than at 40 Hz suggesting that this agonist was more effective on purinergic than cholinergic neurotransmission. In the presence of atropine, to block cholinergic transmission, the inhibitory effect of clenbuterol at 1 Hz was not significantly different from that observed with clenbuterol alone, consistent with a predominant effect on purinergic transmission. At 40 Hz, in the presence of atropine, purinergic transmission was similarly inhibited by clenbuterol. It was noted that clenbuterol inhibited the contractile response to 40 Hz electrical field stimulation in the absence of atropine with an EC_{50} significantly higher than its effect on isolated purinergic transmission at 1 Hz. This results suggests that in addition to its putative inhibitory effect on purinergic transmission, clenbuterol may have additional effects at high concentrations on high frequency electrical field-induced contraction.

ICI 118, 551 has been shown to antagonize β_2-adrenoceptor responses in other smooth muscle studies in rabbit and in man (Levin et al., 1988). When the bladder strips were stimulated at 1 Hz in the presence of both clenbuterol and the β_2-adrenoceptor antagonist ICI 118, 551, the dose–response curve for clenbuterol was shifted significantly to the right indicating that the inhibitory action of clenbuterol on detrusor muscle contraction is mediated through β_2-adrenoceptors in a competitive manner.

When the bladder strips were stimulated at 40 Hz, the effect of clenbuterol was not significantly modified by the additional presence of the β_2-adrenoceptor antagonist ICI 118, 551. This was probably due to insensitivity in the experimental system, as the purinergic component accounts for only 40% of the contraction at this frequency (Brading and Williams, 1990).

Direct stimulation of the bladder with exogenous ATP produced bladder muscle contraction over the range 10^{-6} to 10^{-2} M. ATP-stimulated contraction was significantly inhibited by clenbuterol and this inhibitory action was completely reversed in the presence of the β_2-adrenoceptor antagonist ICI 118,551. By contrast, cholinergic activation appeared to be unaffected by the β_2-adrenoceptor agonist clenbuterol as the contractile responses to exogenous stimulation of the bladder with carbachol were not inhibited by clenbuterol. These observations led to the conclusion that clenbuterol inhibits predominately the detrusor muscle contractile response to purinergic neurotransmission. The absence of a direct effect on carbachol-stimulated detrusor muscle contraction by clenbuterol also suggests that the inhibition at high clenbuterol concentration of the response to 40 Hz electrical field stimulation was due to a presynaptic effect. At a concentration of 10^{-5} M, the β_2-adrenoceptor antagonist ICI 118, 551 produced a competitive reduction in the clenbuterol-induced inhibition of the contractile response of the detrusor muscle to electrical field stimulation. From our combined results, we suggest that the inhibition caused by clenbuterol was mediated by β_2-adrenoceptors located postsynaptically in purinergic synapses.

Contraction of the detrusor muscle by acetylcholine depends predominantly on the release of intracellular calcium ions from calcium stores, whereas contraction initiated by ATP is dependent on membrane depolarization and entry of extracellular calcium (Fry and Wu, 1998). The results of our study suggest that it is the latter mechanism that is affected by activation of β_2-adrenoceptors. This inference is consistent with the results of another study, which has shown that isoproterenol, another β_2-adrenoceptor agonist, inhibits the contraction of the detrusor muscle in response to depolarization of the membrane with KCl (Nishimoto et al., 1995). In addition, the spontaneous contractions seen in rat detrusor muscle, thought to be caused by depolarization (Brading, 1992), were inhibited by clenbuterol (10^{-6} M). It appears that, unlike anticholinergics that are unable to affect spontaneous contractions (Brading and Turner, 1994; Turner and Brading, 1995), β_2-adrenoceptor activation has an effect on this activity.

As the inhibition of the detrusor muscle via activation of the β_2-adrenoceptor appears to be linked to changes in membrane potential, we anticipate that, as in other smooth muscles, the stimulation of β_2-adrenoceptors will be linked to the activation of ATP-sensitive potassium channels (Chang, 1997) resulting in hyperpolarization of the membrane. Further work has investigated this relationship (Hudman et al., submitted).

Rats, along with all non-human mammals with the exception of old world monkeys, possess both a cholinergic and purinergic component to their nervous innervation of the detrusor muscle (Sibley, 1984). The human stable bladder has no atropine-resistant component that is still tetrodotoxin-sensitive (Kinder and Mundy, 1985; Brading, 1987). However, unstable human bladders possess an atropine resistance (Sjögren et al., 1982; Nergardh and Kinn, 1983), which may be important in the functional changes that occur during instability (Fry and Wu, 1998). Clen-

buterol or related β_2-adrenoceptor agonists may, therefore, have an important role in controlling the unstable bladder. In a small double-blind clinical trial of clenbuterol in 39 women with detrusor instability, Gruneberger (1984) reported that clenbuterol treatment was very effective with few side effects when compared with the parasympathetic agent flavoxate (antispasmodic). Further work on human detrusor samples will investigate whether clenbuterol has the ability to inhibit the atropine-resistant component of muscle contraction in unstable human bladders in vitro.

In conclusion, this work significantly increases our understanding of the action of β_2-adrenoceptors in the detrusor muscle of the rat. It shows that clenbuterol inhibits detrusor muscle contraction in response to electrical field stimulation and ATP stimulation, but not to carbachol. The results show that clenbuterol is probably acting through β_2-adrenoceptors located postsynaptically which modulate the response to ATP released from purinergic nerves.

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