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Lack of effect of zaprinast on methacholine-induced contraction and inositol 1,4,5-trisphosphate accumulation in bovine tracheal smooth muscle

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1 The effects of zaprinast (M&B 22948), a selective guanosine 3′:5′-cyclic monophosphate (cyclic GMP) phosphodiesterase inhibitor, and sodium nitroprusside on cyclic GMP content, phosphoinositide hydrolysis and airway smooth muscle tone were examined in flurbiprofen pretreated bovine tracheal smooth muscle (BTSM).

2 Anion-exchange chromatography of the soluble fraction of BTSM homogenates resolved three peaks of Ca^{2+}/calmodulin-independent phosphodiesterase (PDE) activity that corresponded to type Ia (cyclic GMP-specific, zaprinast-inhibitable), type II (cyclic GMP-stimulated) and type IV (Ro 20 1724-inhibitable) PDE isoenzymes. Zaprinast caused a selective inhibition of the type Ia PDE isoenzyme (IC_{50} 0.94 μM) with respect to the type II and IV (IC_{50}s 93 μM and 197 μM respectively) isoenzymes.

3 Pretreatment of BTSM strips with zaprinast (10 μM) for 20 min affected neither the initial rate of force development, nor the resultant magnitude of contraction induced by methacholine (10 μM). In addition, zaprinast (10 μM; 20 min) did not affect the cumulative concentration-response relationship induced by methacholine. In contrast, sodium nitroprusside (300 μM) either alone, or in combination with zaprinast (10 μM), significantly attenuated tone induced by low, but not high concentrations of methacholine. This resulted in a non-parallel, rightwards shift of the methacholine concentration-response curves (nitroprusside: 4.0 fold; nitroprusside/zaprinast: 4.8 fold at the EC_{50} values), without a reduction in the maximum tone generated.

4 In BTSM slices, zaprinast (10 or 100 μM) did not influence basal or methacholine (10 μM)-stimulated cyclic GMP accumulation or inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) mass accumulation over a 60 s incubation period, although it did significantly increase cyclic GMP content over longer (30 min) stimulation periods.

5 In [^{3}H]-inositol prelabelled BTSM slices, stimulated in the presence of 5 mM LiCl, methacholine (10 μM) caused a marked increase in total [^{3}H]-inositol phosphate accumulation. This effect was not inhibited by zaprinast (10 μM), sodium nitroprusside (300 μM), or a combination of these drugs despite these agents markedly increasing tissue cyclic GMP content.

6 These findings demonstrate that despite zaprinast being a potent and selective inhibitor of the type Ia PDE isoenzyme in a cell-free system, this drug only increases cyclic GMP content in BTSM following prolonged agonist-stimulation. This may explain its lack of inhibitory effect on methacholine-induced tone. The inability of drugs which increase tissue cyclic GMP content and exhibit anti-spasmogenic activity to inhibit methacholine-stimulated $Ins(1,4,5)P_3$ formation suggests that, unlike vascular smooth muscle, cyclic GMP-dependent mechanisms do not regulate receptor-mediated phosphoinositide hydrolysis in BTSM.

Keywords: Zaprinast (M&B 22948); inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$); cyclic GMP; bovine airway smooth muscle; sodium nitroprusside; cyclic GMP-dependent phosphodiesterase

Introduction

Despite increasing knowledge regarding the major substrates for guanosine 3′:5′-cyclic monophosphate (cyclic GMP)-dependent protein kinase in eucaryotic cells (see Casnellie & Greengard, 1974; Parks *et al.*, 1987), the exact molecular mechanism(s) underlying cyclic GMP-induced airway smooth muscle (ASM) relaxation remains uncertain. In several vascular smooth muscle preparations cyclic GMP (Hirata *et al.*, 1990), glyceryl trinitrate (Ahlner *et al.*, 1988), sodium nitroprusside and atriopeptin II (Rapoport, 1986) and α-human atrial natriuretic peptide (Kajikura & Kuriyama, 1990) have all been shown to attenuate agonist-stimulated phosphoinositide metabolism possibly by inhibiting activation of a guanine nucleotide binding protein, G_p, and uncoupling activated G_p from phosphoinositidase C (Hirata *et al.*, 1990). Since agonist-stimulated hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$) by phosphoinositidase C to form inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and 1,2-diacyglycerol is thought to play a central role in excitation-contraction coupling in smooth muscle (see Abdel-Latif, 1986; Hall & Chilvers, 1989) the inhibition of phosphoinositide metabolism by cyclic GMP has been proposed as an important mechanism underlying its dilator action in vascular smooth muscle (Rapoport, 1986; Hirata *et al.*, 1990).

In contrast to the well-documented effects of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) elevating agents on phosphoinositide responses in ASM (Hall & Hill, 1988; Madison & Brown, 1988; Hall *et al.*, 1989; 1990), little is known about the influence of cyclic GMP in this tissue. In a recent study in guinea-pig tracheal rings, Langlands *et al.* (1989) examined the effects of zaprinast (M&B 22948), a Ca^{2+}/calmodulin-independent, cyclic GMP-specific phosphodiesterase (PDE; type Ia, see Torphy & Cielinski, 1990) inhibitor, on

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cyclic GMP content, Ins(1,4,5)P_3 mass and methacholine (MCh)- and histamine-induced changes in tone. Since zaprinast increased basal and agonist-stimulated cyclic GMP levels and abolished Ins(1,4,5)P_3 generation without influencing the rate or magnitude of tension development, the authors concluded that agonist-induced Ins(1,4,5)P_3 formation was not involved in the contractile response induced by either of these agonists. As these data are complicated by the heterogeneous nature of the tissue studied, conflict with the findings of Hall et al. (1990) who demonstrated no effect of zaprinast on inositol phosphate responses in bovine tracheal smooth muscle (BTSM), and challenge much of the available data that support a pivotal role for Ins(1,4,5)P_3 in initiating ASM contraction (Hashimoto et al., 1985; Chilvers et al., 1989a,b; 1990a; Hall & Chilvers, 1989; Coburn & Baron, 1990) we have re-investigated the potential interaction between cyclic GMP, Ins(1,4,5)P_3 formation and tone in BTSM, an essentially homogeneous ASM preparation (Katsuki & Murad, 1977). A preliminary account of some of these data has been presented to the British Pharmacological Society (Chilvers et al., 1990c).

Methods

Tissue preparation

Tracheae from 12–18 month old cows were obtained from a local abattoir and transported to the laboratory in oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit (KH) buffer (composition in mM: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.7, $CaCl_2$ 1.3) at 4°C. The cervical trachealis muscle was dissected free of epithelium and surrounding connective tissue and either maintained in oxygenated ice-cold KH buffer for tension, [^{3}H]-InsP and cyclic GMP measurements or snap frozen in liquid nitrogen and stored at −80°C for subsequent phosphodiesterase studies.

Tension measurements

The effects of zaprinast, sodium nitroprusside and the combination of these drugs on methacholine-induced tension development in BTSM were studied as described by Langlands et al. (1989). Essentially BTSM strips measuring approximately 2 × 2 × 10 mm were mounted under an initial tension of 20 mN in 5 ml tissue baths containing oxygenated KH buffer at 37°C. Following a 60–120 min equilibration period, during which time the tension was periodically readjusted to 20 mN and the buffer frequently changed, tissues were pretreated for 20 min with either vehicle (20 μl 0.1 M NaOH), 10 μM zaprinast, 300 μM sodium nitroprusside or the latter two drugs in combination. Cumulative concentration-response curves to methacholine were then constructed according to Van Rossum (1963) from which estimates of agonist potency ($pD_2 = -\log_{10} EC_{50}$) were determined. To account for intrinsic changes in sensitivity (pD_2) of BTSM strips to methacholine between tissues obtained from different animals (see Table 1), the potential inhibitory effects of zaprinast, sodium nitroprusside and zaprinast-plus-sodium nitroprusside on the methacholine concentration-response relationship were performed using a paired experimental design where control (no drug pretreatment) and experimental strips obtained from the same animal were studied in parallel.

In a separate series of experiments designed to assess the effects of zaprinast on the kinetics ($t_{1/2}$) and magnitude (T_{max}) of methacholine-induced tone, BTSM strips were preincubated with 10 μM zaprinast or vehicle prior to challenge with 10 μM methacholine (approx. EC_{90} for tension). Changes in tension were measured isometrically with Grass FT03.c force-displacement transducers and displayed on a Grass 7D curvilinear ink-writing polygraph.

Flurbiprofen (1 μM) was present in the KH buffer throughout the tension, InsP and cyclic GMP experiments to prevent the spontaneous and drug-stimulated release of cyclooxygenase products from the BTSM and to prevent sodium nitroprusside from increasing the tissue cyclic AMP content (Torphy et al., 1985).

Separation and assay of cyclic nucleotide-dependent phosphodiesterase isoenzymic activities

For the separation of PDE isoenzymes, approx. 5 g of BTSM were homogenized (Polytron 2 × 10 s bursts at setting 8) in 10 vol. of ice-cold buffer A (composition in mM: bis-Tris 20 (pH 6.5), sodium acetate 50, dithiothreitol (DTT) 2, EDTA 1, ethylene glycol 30% (v/v) supplemented with benzamidine 2, phenylmethylsulphonylfluoride 0.05, bacitracin (100 μg ml^{-1}) and soybean trypsin inhibitor (10 μg ml^{-1})). To prevent DTT-induced activation of thiol-dependent proteases which may reduce PDE-yield, 60 μM leupeptin was also added to buffer A prior to homogenization (see Degerman et al., 1987). The homogenate was centrifuged at 45000 *g* at 4°C for 30 min to provide soluble (supernatant) and particulate (pellet) fractions. The pellet was resuspended in 2 vol. buffer A containing 300 mM KCl to remove any electrostatically bound PDE and re-centrifuged as above. The combined supernatants were filtered through gauze, diluted in buffer B (composition in mM: bis-Tris 20 (pH 6.5), EDTA 1, ethylene glycol 30% (v/v)) to reduce conductivity to <4 mS at 4°C and applied at a flow rate of 1 ml min^{-1} to a Q-sepharose column (Bio-Rex 1.5 × 1.8 cm) pre-equilibrated in buffer A. The column was then washed with buffer A until the absorbance at 280 nm returned to baseline and then eluted with a linear sodium acetate gradient (50–900 mM, flow rate 0.5 ml min^{-1}, total volume 400 ml). Forty × 10 ml fractions were collected and aliquots assayed for cyclic nucleotide PDE activity as described below. Peak fractions were subsequently pooled and could be stored at −18°C for at least 2 months without loss of Ca^{2+}/calmodulin-independent PDE activity. Cyclic nucleotide-dependent PDE activity was measured by a modification (Schwartz & Passonneau, 1974) of the method described by Thompson & Appleman (1971). The assay is based upon the phosphodiesteric cleavage of [^{3}H]-cyclic AMP or [^{3}H]-cyclic GMP to [^{3}H]-5′-AMP or [^{3}H]-5′-GMP respectively which are dephosphorylated to their corresponding labelled nucleosides and separated from the charged nucleotides by Dowex anion exchange resin. Assays were performed in triplicate at 37°C by adding 30 μl of the relevant column fraction to 270 μl of reaction medium containing (final concentration in mM): triethanolamine 40 (pH 8.0), EGTA 2, DTT 2, $Mg(CH_3COO)_2$ 10, BSA (500 μg ml^{-1}), cyclic AMP (1 μM for type IV PDE isoenzyme) or cyclic GMP (1 μM for type Ia and type II PDE isoenzymes) supplemented with 250000–350000 d.p.m. [^{3}H]-cyclic GMP or [^{3}H]-cyclic AMP, 0.25 u alkaline phosphatase and approximately 5000 d.p.m. [^{14}C]-adenosine to estimate recovery. Reactions were terminated after 10 min by the addition of 1 ml of Dowex AG1-X8 (acetate form, 200–400 mesh, propan-2-ol, water, 1:2:1 (w/v/v)). Samples were thoroughly mixed and after 10 min centrifuged at 12000 *g* for 2 min. The radioactivity in 650 μl aliquots of the resulting supernatant was determined by liquid scintillation counting. One unit of enzyme activity was defined as the amount of PDE which catalysed the formation of 1 pmol 5′-AMP/5′-GMP min^{-1} mg^{-1} protein at 37°C after correction for the recovery (routinely 70–85%) of [^{14}C]-adenosine.

Measurement of cyclic GMP

BTSM slices (300 μm × 300 μm) were prepared as previously described (Chilvers et al., 1989a) and preincubated for 60 min in 100 ml oxygenated (95% O_2:5% CO_2) KH buffer at 37°C. Two ml of gravity packed slices were then transferred to 50 ml Erlenmeyer flasks containing 10 ml oxygenated KH buffer and incubated for a further 60 min at 37°C. After this second incubation period, 50 μl aliquots of gravity-packed BTSM slices were transferred to flat bottom vials containing 230 μl KH

buffer. Zaprinast (final concentration 10 μM) or vehicle (0.3 mM NaOH) were added in a 10 μl volume 20 min before stimulation with methacholine (10 μM). Reactions were terminated at appropriate times with 300 μl ice-cold 1 M trichloroacetic acid and extracts neutralized according to Downes *et al.* (1986). Aliquots (250 μl) of the neutralized tissue extracts were diluted 2 fold in 100 mM sodium acetate (pH 6.2) and acetylated by the consecutive addition of triethylamine (10 μl) and acetic anhydride (5 μl). Cyclic GMP levels were then measured by radioimmunoassay as described by Brooker *et al.* (1979) and Lappin *et al.* (1984). In brief, 100 μl of acetylated sample were added to 25 μl guanosine-3', 5'-cyclic monophosphate, 2-0-succinyl 3-[^{125}I]-iodotyrosine methyl ester in 0.1% BSA (approx. 2000 d.p.m.) and 100 μl anti-cyclic GMP antibody in 0.1% BSA. After vortex mixing, samples were incubated overnight at 4°C and free and antibody-bound cyclic GMP separated by charcoal precipitation in 100 mM phosphate buffer (pH 7.4). The detection limit and sensitivity of this assay were approximately 2 fmol and 15 fmol cyclic GMP respectively.

Measurement of Ins(1,4,5)P_3 and [^{3}H]-InsPs

Ins(1,4,5)P_3 mass was measured in the same neutralized trichloroacetic acid tissue extracts used for the above cyclic GMP estimations with a radioreceptor assay exactly as described previously (Challiss *et al.*, 1988). The protein content of each tissue pellet was determined according to Lowry *et al.* (1951).

The accumulation of total [^{3}H]-InsPs in the presence of 5 mM LiCl was used as an index of agonist-stimulated phosphoinositide hydrolysis (Berridge *et al.*, 1982). BTSM slices were prepared and pre-incubated in KH buffer for 60 min as detailed above and labelled with 0.1 μM *myo*-[^{3}H]-inositol for 60 min in the absence of agonist, exactly as described previously (Chilvers *et al.*, 1989a). Tissue slices were dispensed, pretreated with zaprinast (10 μM), sodium nitroprusside, (300 μM), vehicle or zaprinast in combination with sodium nitroprusside for 20 min in the presence of LiCl (5 mM) and stimulated with methacholine (10 μM). Trichloroacetic acid extracts were prepared as outlined above and total [^{3}H]-InsPs separated by Dowex AG1-X8 anion-exchange resin (100–200 mesh, Cl^- form; 0.5 cm × 1.75 cm columns) (Chilvers *et al.*, 1989a). Unlabelled samples were run in parallel to assess changes in tissue cyclic GMP content.

Materials

Acetyl-β-methylcholine, alkaline phosphatase (P-4252), bacitracin, benzamidine, bovine serum albumin (grade III), cyclic AMP (sodium salt), cyclic GMP (sodium salt), dithiothreitol, EDTA, EGTA, fluorbiprofen, leupeptin, phenylmethylsulphonylfluoride, sodium nitroprusside and soybean trypsin inhibitor were purchased from Sigma. Zaprinast (M&B 22948) was obtained from Rhone-Poulenc Rorer Inc., Dagenham, Essex. Anti-cyclic GMP antibody, cyclic GMP 2'-O-succinyl 3-[^{125}I]-iodotyrosine methyl ester (1980 Ci mmol^{-1}), [2,8-^{3}H]-cyclic AMP (36.1 Ci mmol^{-1}), [8-^{3}H]-cyclicGMP (23.7 Ci mmol^{-1}), [8-^{14}C]-adenosine (55 Ci mmol^{-1}) and D-inositol 1,4,5-trisphosphate were from Amersham International Dowex AG1-X8 (200–400 mesh, acetate form and 100–200 mesh, chloride form) were from BioRad. *myo*-[2-^{3}H]-inositol (17 Ci mmol^{-1}) and D-[^{3}H]-inositol 1,4,5-trisphosphate (17–20 Ci mmol^{-1}) were purchased or received as gifts from NEN (DuPont). All other reagents were of analytical grade obtained from commercial sources.

Data analysis

Significant differences between means were assessed by unpaired *t* test for non-contractile data and non-parametric analysis (Mann-Whitney U-test) for contractile studies. The null hypothesis was rejected when $P < 0.05$; IC_{50} and slope factor values were determined by computer-assisted iterative curve fitting (ALLFIT).

Results

Effect of zaprinast on cyclic nucleotide phosphodiesterase activity

Three peaks of Ca^{2+}/calmodulin-independent, cyclic-nucleotide hydrolytic activity were obtained following anion exchange chromatography of a crude BTSM supernatant eluting at 14, 41 and 57 mS (measured at 27°C). Using the classification of Torphy & Cielinski (1990), and the substrate specificity, kinetics of cyclic AMP/cyclic GMP hydrolysis and sensitivity of the PDE activity of each peak to a range of selective inhibitors (rolipram, Ro 20 1724, SK 94120, imazodan and zaprinast), these peaks corresponded to type Ia, II and IV PDE isoenzymes respectively (M.A. Giembycz & P.J. Barnes, unpublished results). The specific activity of these peaks, measured in the absence of Ca^{2+}/calmodulin and with 1 μM cyclic GMP (type Ia and II PDE) and 1 μM cyclic AMP (type IV PDE) as substrate and allowing correction for [^{14}C]-adenosine recovery was 4.3, 3.8 and 7.8 pmol 5'-GMP/5'-AMP formed min^{-1} mg^{-1} protein for types Ia, II and IV respectively.

Zaprinast caused a concentration-dependent inhibition of all three PDE activities (Figure 1), but exhibited marked selectivity (>100 fold) as an inhibitor of the Ca^{2+}/calmodulin-independent, cyclic GMP-specific (type Ia) isoenzyme (IC_{50} 941 ± 111 nM) over both the type II isoenzyme (IC_{50} 93 ± 10 μM) and the type IV isoenzyme (IC_{50} 197 ± 28 μM). The concentration of zaprinast chosen for the subsequent studies (10 μM) gave an 84.2 ± 0.6% inhibition of type Ia PDE hydrolytic activity and 5.9 ± 2.3 and 13.7 ± 3.8% inhibition of types II and IV PDE activity respectively and hence, in the cell-free assay system employed, this concentration of zaprinast was the most discriminating to permit selective inhibition of type Ia (Ca^{2+}/calmodulin-independent, cyclic GMP specific) PDE activity.

Effect of zaprinast and sodium nitroprusside on methacholine-induced tone

Pretreatment of BTSM strips with zaprinast (10 μM) for 20 min in the presence of flurbiprofen (1 μM) failed to attenuate the

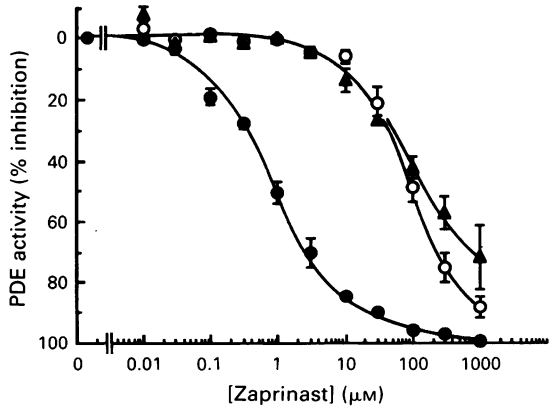

Figure 1 Effect of zaprinast on type Ia, II and IV phosphodiesterase (PDE) activities in the soluble fraction of BTSM homogenates. PDE isoenzymes were separated from 45 000 *g* soluble fractions of BTSM homogenates by Q-sepharose anion exchange chromatography and cyclic nucleotide-dependent PDE activity measured in the presence of various concentrations of zaprinast. Cyclic AMP (1 μM) was used as substrate for type IV (▲), and cyclic GMP (1 μM) for types Ia (●) and II (○) PDE isoenzymes respectively. Values are presented as mean for 8 determinations, each performed in triplicate, from 4 separate tissue preparations; vertical bars shows s.e.mean.

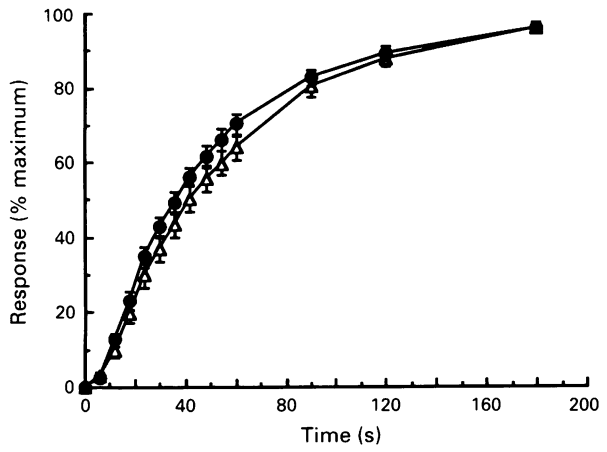

Figure 2 Effect of zaprinast on kinetics of methacholine-induced tone in BTSM strips. Strips were incubated in the absence (△) or presence (●) of zaprinast (10 μM) for 20 min, prior to addition of methacholine (10 μM; approx. EC_{90} for tension development). Data are presented as mean for 10 tissue samples in 4 separate experiments; s.e.mean shown by vertical bars.

initial rate or magnitude of tone induced by 10 μM methacholine (approx. EC_{90}). There was no significant difference in the lag-time between addition of agonist and the onset of contraction in control (4.2 ± 0.7 s) or zaprinast pretreated tissues (3.8 ± 0.3 s) and no difference ($P > 0.05$) in the time to achieve half maximal ($t_{1/2}$) contraction (control: $t_{1/2} = 39.6 \pm 3.9$ s; zaprinast pretreatment: $t_{1/2} = 37.3 \pm 2.4$ s) or in the maximum tension developed (control: 204.8 ± 12.6 mN; zaprinast pretreatment: 243.5 ± 18.9 mN; Figure 2). In addition, the cumulative concentration-response relationship induced by methacholine was also unaffected by pretreatment of tissue with zaprinast (Figure 3a). In contrast, pretreatment of BTSM for 20 min with sodium nitroprusside (300 μM) alone or in combination with zaprinast (10 μM) significantly attenuated tone induced by low, but not high concentrations of methacholine (Figures 3b, 3c; Table 1). Thus, the methacholine concentration-response curve was shifted to the right in a non-parallel manner by both nitroprusside (4.0 fold at EC_{50}) and by nitroprusside and zaprinast in combination (4.8 fold at EC_{50}) without a reduction in the maximum response attained (Table 1).

Effect of zaprinast on methacholine-stimulated Ins(1,4,5)P_3 and cyclic GMP accumulation

Methacholine (10 μM) caused a rapid, transient increase in Ins(1,4,5)P_3 mass in BTSM slices similar to that observed with carbachol (Chilvers *et al.*, 1989b) with a maximal response (175% over basal) observed at 5 s (Table 2). The increase in Ins(1,4,5)P_3 caused 5 s after methacholine addition was about 70% of that caused by a maximally-effective concentration of this agonist (100 μM, see Table 2). Pretreatment of slices with zaprinast (10 μM) for 20 min prior to stimulation with methacholine did not influence the time course of methacholine-induced Ins(1,4,5)P_3 accumulation (Table 2).

Table 2 Effect of zaprinast on methacholine-stimulated accumulation of inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) in BTSM slices

		Ins(1,4,5)P_3 (pmol mg^{-1} protein)	
Time (s)	*+/− MCh*	*Control*	*+Zaprinast*
5	−	10.0 ± 0.5	9.8 ± 0.7
	+	17.1 ± 0.6	17.4 ± 0.9
10	+	14.7 ± 0.7	14.9 ± 0.8
15	+	11.9 ± 0.9	12.5 ± 0.8
30	+	10.2 ± 0.8	10.2 ± 0.8
60	−	10.4 ± 0.4	10.7 ± 0.4
	+	8.6 ± 0.7	8.1 ± 0.8

BTSM slices (300 μm × 300 μm) were pre-incubated with vehicle or zaprinast (10 μM) for 20 min prior to addition of vehicle or methacholine (MCh, 10 μM). Incubations were terminated at the indicated time-points by addition of trichloroacetic acid and samples neutralised and assayed for Ins(1,4,5)P_3 concentrations. Addition of 100 μM methacholine for 5 s increased Ins(1,4,5)P_3 accumulation to 21.4 ± 0.8 pmol mg^{-1} protein. Increasing the concentration of zaprinast (100 μM) had no effect on the maximal methacholine (10 μM) stimulation of Ins(1,4,5)P_3 accumulation (5 s: 17.4 ± 1.0 pmol mg^{-1} protein). Data represent mean ± s.e.mean, for 3 separate experiments, each performed in triplicate.

Methacholine (10 μM) also caused a time-dependent increase in the cyclic GMP content (Figure 4) with levels increasing from control values of 105 ± 21 fmol mg^{-1} protein under resting conditions, to 453 ± 29 fmol mg^{-1} protein by 30 s post-methacholine challenge. Zaprinast pretreatment did not significantly affect either basal or methacholine-stimulated (0–60 s) cyclic GMP accumulation (Figure 4) although significant increases in cyclic GMP were observed in the presence of zaprinast with a longer (30 min) methacholine-stimulation period (Figure 5a). In two further experiments pretreatment of BTSM slices with zaprinast (100 μM) for 20 min likewise failed to attenuate methacholine-stimulated increases in Ins(1,4,5)P_3 or cyclic GMP at 5 s (data not shown).

Effect of zaprinast and sodium nitroprusside on [^{3}H]-InsP and cyclic GMP accumulation

Since zaprinast (10 or 100 μM) did not influence basal or short-term (60 s) methacholine-stimulated cyclic GMP levels, but was able to enhance the accumulation of cyclic GMP over longer periods of methacholine stimulation (Figure 5a), the effect of zaprinast (10 μM) on methacholine-stimulated phosphoinositide metabolism was examined 30 min after methacholine challenge. Since only a transient increase in

Table 1 Effect of zaprinast and sodium nitroprusside on methacholine-induced contraction: cumulative concentration-response curves to methacholine were constructed using BTSM strips, pretreated for 20 min with vehicle, zaprinast (10 μM), sodium nitroprusside (300 μM) or a combination of the latter two agents as indicated

	Methacholine concentration-response curve				
		Fold rightwards shift at			T_{max}
	pD_2	EC_{20}	EC_{50}	EC_{80}	(mN)
Control	6.48 ± 0.10				352 ± 32
+ zaprinast	6.42 ± 0.09	1.0	1.2	1.2	323 ± 31
Control	6.89 ± 0.21				324 ± 21
+ sodium nitroprusside	6.29 ± 0.07*	5.1	4.0	2.3	328 ± 16
Control	6.59 ± 0.07				376 ± 34
+ zaprinast + sodium nitroprusside	5.91 ± 0.13**	5.2	4.8	3.4	331 ± 31

Data are presented as means ± s.e.mean, derived from 8 tissue samples in 4 separate experiments. Statistical significance is indicated as *$P < 0.05$; **$P < 0.01$.

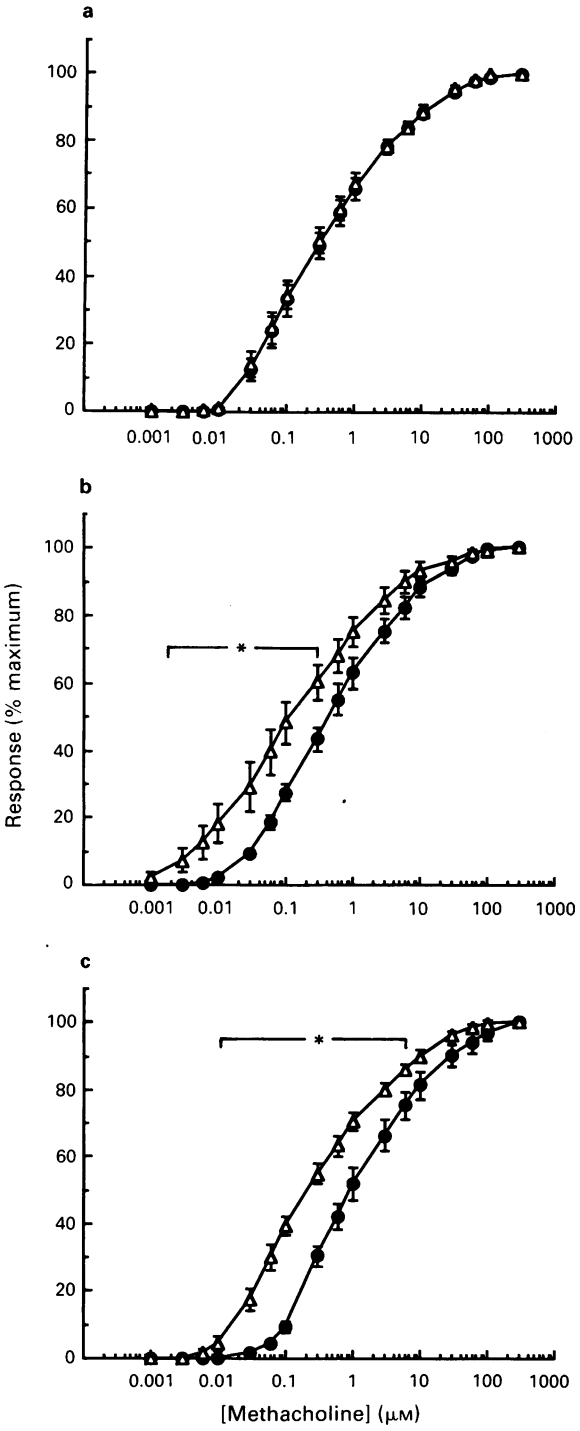

Figure 3 Effect of zaprinast and sodium nitroprusside on methacholine-induced tone in BTSM strips. Cumulative concentration-response curves to methacholine were constructed in BTSM strips pretreated for 20 min in the absence (△) or presence (●) of (a) zaprinast (10 μM); (b) sodium nitroprusside (300 μM); or (c) a combination of zaprinast (10 μM) and sodium nitroprusside (300 μM). pD_2 and T_{max} values and the degree of rightward shift observed are presented in Table 1. Values are presented as mean of 8 observations in 4 separate experiments; s.e.mean shown by vertical bars. Statistical significance is indicated as $*P < 0.05$.

Ins(1,4,5)P_3 accumulation is observed following muscarinic receptor-stimulation in this tissue (Table 2), despite continued $PtdInsP_2$ hydrolysis (Chilvers *et al.*, 1990b), total [^{3}H]-InsP accumulation in the presence of Li^+ was used as an index of receptor-mediated phosphoinositide hydrolysis. In addition, to investigate whether the elevation of cyclic GMP levels by other means (i.e. through direct stimulation of soluble guanylyl cyclase) was able to influence phosphoinositide

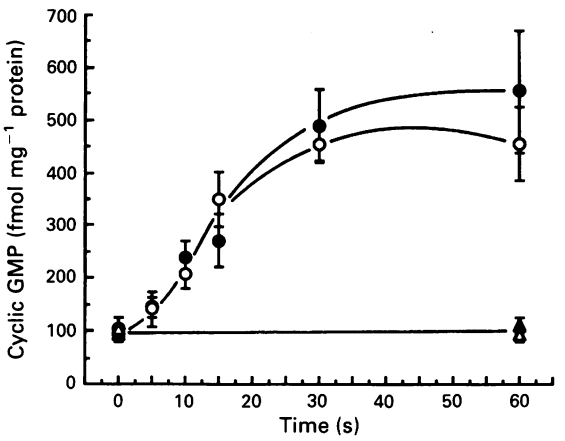

Figure 4 Effect of zaprinast on methacholine-stimulated accumulation of cyclic GMP in BTSM slices. Slices (300 μm × 300 μm) were pre-incubated with vehicle (○, △) or zaprinast (10 μM) (●, ▲) for 20 min prior to addition of methacholine (10 μM) (○, ●) or KH buffer (△, ▲). Incubations were terminated at the indicated time-points by addition of trichloroacetic acid and samples neutralised and assayed for cyclic GMP concentration. Data represent mean for 3 separate experiments, each performed in triplicate; s.e.mean shown by vertical bars.

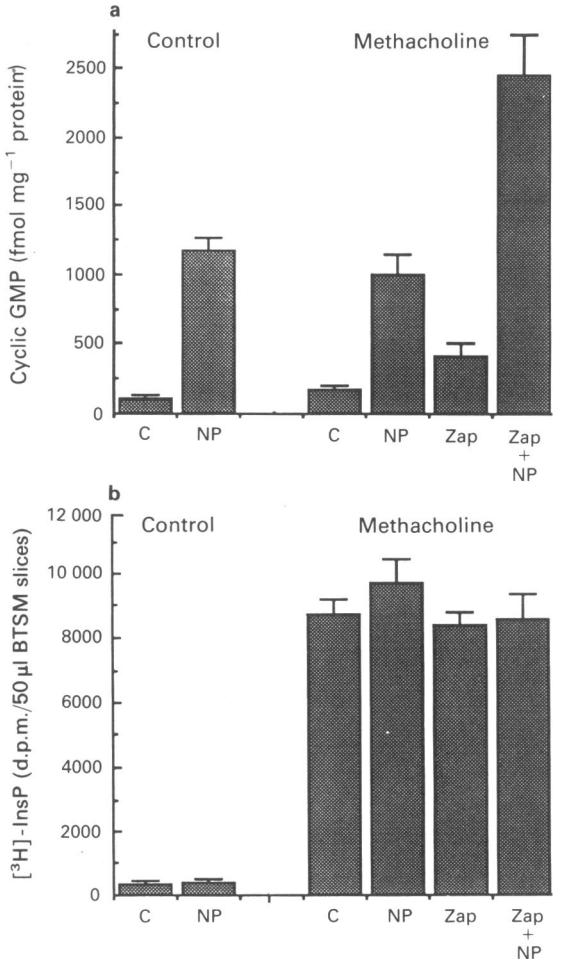

Figure 5 Effect of zaprinast and sodium nitroprusside on 30 min methacholine-stimulated cyclic GMP and [^{3}H]-inositol phosphate (InsP) accumulations. Unlabelled (a) or [^{3}H]-inositol pre-labelled (b) BTSM slices were pre-incubated with vehicle (c), zaprinast (Zap) (10 μM), sodium nitroprusside (NP) (300 μM) or a combination of these agents as indicated for 20 min prior to stimulation with methacholine (10 μM) or KH buffer for 30 min. Cyclic GMP (a) and [^{3}H]-InsP (b) accumulations were determined in neutralised trichloroacetic acid extracts as described in the Methods section. Data are presented as means for 3 separate experiments performed in triplicate; vertical bars shown s.e.mean.

hydrolysis, the effects of sodium nitroprusside alone at a concentration (300 μM) known to inhibit methacholine-induced tone, or in combination with zaprinast, on cyclic GMP and [^{3}H]-InsP accumulation were examined.

Figure 5a shows that incubation of BTSM slices for 30 min with sodium nitroprusside caused a marked increase in basal and methacholine-stimulated cyclic GMP levels. When added in combination, sodium nitroprusside and zaprinast acted synergistically producing an 8 fold increase in the cyclic GMP content. Despite these marked increases in cyclic GMP observed with zaprinast, sodium nitroprusside or a combination of these agents in methacholine-stimulated tissue, no effect on methacholine-stimulated [^{3}H]-InsP formation was observed (Figure 5b).

Discussion

These data indicate that in the soluble fraction of BTSM homogenates, zaprinast appears to be an effective inhibitor of the type Ia (Ca^{2+}/calmodulin-independent, cyclic GMP-specific) PDE isoenzyme with an approximate 100 and 200 fold selectivity over the type II and IV PDE isoenzymes, respectively. The potency of zaprinast in inhibiting Type Ia PDE activity in BTSM (IC_{50} 0.94 μM) is very similar to that previously reported in canine trachealis (Silver *et al.*, 1988) and bovine photoreceptors (Gillespie & Beavo, 1989). The concentration of zaprinast (10 μM) used for the studies employing tissue slices was chosen not only to allow a direct comparison with the study by Langlands *et al.* (1989) but also because this concentration caused near maximal inhibition of type Ia PDE without major effects on either the type II or IV isoenzymes in BTSM.

The complete lack of effect of zaprinast on basal and methacholine-stimulated Ins(1,4,5)P_3 accumulation and on cyclic GMP levels over the first 60 s following stimulation with methacholine contrasts markedly with the findings of Langlands *et al.* (1989) in guinea-pig tracheal rings where an identical experiment protocol resulted in a significant elevation in tissue cyclic GMP and a complete inhibition of methacholine- and histamine-stimulated increase in Ins(1,4,5)P_3. Our additional experiments demonstrating no effect of higher concentrations of zaprinast (100 μM) on methacholine-stimulated Ins(1,4,5)P_3 accumulation, or any effect of zaprinast on phosphoinositide hydrolysis over longer stimulation periods, where significant increases in tissue cyclic GMP levels were observed, and the lack of effect of sodium nitroprusside which induced large increases in tissue cyclic GMP levels on [^{3}H]-InsP responses all argue against a significant role of cyclic GMP-dependent mechanisms in regulating muscarinic-cholinoceptor-mediated phosphoinositide hydrolysis in BTSM. This conclusion is supported by the observations by Hall *et al.* (1989; 1990) that pretreatment of [^{3}H]-inositol-labelled BTSM slices with zaprinast (100 μM) for 20 min failed to influence either carbachol (1 μM)- or histamine (1 mM)-stimulated accumulation of [^{3}H]-InsPs, despite marked attenuation of both responses by a range of cyclic AMP elevating agents. These findings in BTSM are also consistent with the recent demonstration in rat glioma C_6Bu_1 cells (Kim *et al.*, 1989) that dibutyryl cyclic AMP, but not 8-bromo-cyclic GMP, causes the phosphorylation of PLC-γ, a mechanism which is thought to be involved in the regulation of phosphoinositidase C by cyclic AMP-dependent protein kinase.

As it has been demonstrated that cyclic GMP is an important regulator of phosphoinositide metabolism in other bovine tissues, notably mesenteric arterial smooth muscle (Ahlner *et al.*, 1988), it is unlikely that a species effect alone explains the differences that exist between our own study and that of Langlands *et al.* (1989). It is probable that since ASM is a relatively minor cellular component in intact guinea-pig tracheal rings, in contrast to BTSM preparations which are >95% smooth muscle (Katsuki & Murad, 1977), that some of the biochemical responses observed in the former tissue reflect changes in non-airway smooth muscle cells. However, since it is clear that major differences do exist in the ability of cyclic GMP-elevating agents and non-hydrolysable cyclic GMP analogues to influence phosphoinositide metabolism in smooth muscle in different tissues, a specific species difference cannot be fully excluded. In addition, our study does not exclude the possibility that the apparent differences that exist between vascular and airway smooth muscle with regard to the sensitivity of agonist-stimulated inositol phosphate response to cyclic GMP elevating agents are dependent on the type of receptor activated, since studies in vascular tissue have examined the modulation of α_1-adrenoceptor responses, while investigations in airway tissue have used agonists that interact with histamine H_1-receptors and M_3-muscarinic-cholinoceptors.

In contrast, therefore, to vascular smooth muscle where cyclic GMP-mediated inhibition of phosphoinositide metabolism may well be an important mechanism mediating relaxation (Rapoport *et al.*, 1983; Ahler *et al.*, 1988; Hirata *et al.*, 1990); an alternative mechanism(s) appears to be responsible for cyclic GMP-induced relaxation in BTSM (Ishii & Murad, 1989) as judged by the dissociation between the effect of sodium nitroprusside on methacholine-induced tone and on phosphoinositide metabolism. Although BTSM is known to contain relatively large amounts of cyclic GMP-dependent protein kinase (Torphy *et al.*, 1982) and have a high cyclic GMP to cyclic AMP-dependent protein kinase ratio (Fiscus *et al.*, 1984; Felbel *et al.*, 1988) the mechanisms underlying the ability of 8-bromo-cyclic GMP, and cyclic GMP-dependent protein kinase, to inhibit carbachol-induced intracellular Ca^{2+} mobilisation in dissociated BTSM cells (Felbel *et al.*, 1988) are largely unknown. In BTSM, activation of cyclic GMP-dependent protein kinase is known to phosphorylate several sarcolemmal-associated proteins (Hogaboom *et al.*, 1982), two of which correspond to GS_1 and GS_2 subtrates identified in guinea-pig vas deferens, uterus and ileum (Casnellie & Greengard, 1974). In addition, cyclic GMP-dependent protein kinase promotes the monophosphorylation of myosin light chain kinase (Nishikawa *et al.*, 1984), but unlike the diphosphorylation caused by cyclic AMP-dependent protein kinase has no effect on the Ca^{2+}/calmodulin dependence of myosin light chain phosphorylation and consequently little functional effect. Activation of cyclic GMP-dependent protein kinase has been shown to stimulate Ca^{2+} extrusion (Kobayashi *et al.*, 1985) and Ca^{2+} uptake into the sarcoplasmic reticulum (probably via the phosphorylation of phospholamban; Raemaekers *et al.*, 1988) in aortic smooth muscle cells and bovine pulmonary artery respectively. The agonist-stimulated increases in cyclic GMP which result from Ca^{2+} activation of the soluble guanylyl cyclase or through protein kinase C-induced phosphorylation of guanylyl cyclase (Zwiller *et al.*, 1985) may therefore play an important role in the feedback control of Ca^{2+} movements in ASM although evidence that this occurs via modulating agonist-stimulated phosphoinositide metabolism in bovine ASM is lacking.

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