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5-HT_{2A} receptor activation of the external urethral sphincter and 5-HT_{2C} receptor inhibition of micturition: A study based on pharmacokinetics in the anaesthetized female rat

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ABSTRACT

Central and peripheral 5-hydroxytryptamine (5-HT) receptors play a critical role in the regulation of micturition. Bolus doses of 5-$HT_{2A/2C}$ receptor agonists have been shown to activate the external urethral sphincter (EUS) and to inhibit micturition. This study was designed to determine the contribution of these two 5-HT receptor subtypes to activation of the EUS and inhibition of micturition utilising pharmacokinetic knowledge to better control drug exposure. Recordings of urethral and bladder pressure, EUS-Electromyogram (EMG), the micturition reflex induced by bladder filling, blood pressure and heart rate were made in anaesthetized female rats. The effects of intravenous (i.v.) infusions of the 5-HT_2 receptor agonist (2S)-1-(6-chloro-5-fluoroindol-1-yl)propan-2-amine fumarate (Ro 60-0175) in the absence or presence of the selective 5-HT_{2C} receptor antagonist 6-chloro-5-methyl-N-[6-(2-methylpyridin-3-yl)oxypyridin-3-yl]-2,3-dihydroindole-1-carboxamide dihydrochloride (SB 242084) or 5-HT_{2A} receptor antagonist (R)-(2,3-dimethoxyphenyl)-[1-[2-(4-fluorophenyl) ethyl]piperidin-4-yl]methanol (MDL-100,907) were studied on these variables. Continuous infusion of increasing concentrations of Ro 60-0175 only evoked EUS-EMG activity at the highest concentration, which was blocked by co-infusion of MDL-100,907 but not SB 242084. Urethral pressure was unaffected by any drug infusion. Ro 60-0175 at the lowest concentration inhibited the micturition reflex but as the concentration increased this was reversed to facilitation. SB 242084 blocked the inhibition while MDL-100,907 blocked the excitation. Activation of 5-HT_{2A} not 5-HT_{2C} receptors evoked EUS-EMG activity. In conclusion, 5-HT_{2A} receptor activation facilitated the micturition reflex and evoked EUS-EMG while 5-HT_{2C} receptor activation only inhibited the micturition reflex.

1. Introduction

Activation of 5-hydroxytryptamine (5-HT) receptor subtypes, especially 5-HT_{1A}, 5-$HT_{2A/2B/2C}$, 5-HT_7 and to a degree 5-HT_3 receptors, have been shown to affect urethral and bladder variables as well as playing a physiological role in the control of micturition (see Ramage, 2006). In a recent extensive investigation (Mbaki and Ramage, 2008a) into the role of 5-HT_2 receptor subtypes in the control of the bladder and urethra in the female rat utilising bolus doses of agonists and antagonists, it was concluded that activation of central 5-HT_{2C} receptors inhibited micturition, confirming the previous study of Steers and De Groat (1989). However, a physiological role for 5-HT_{2C} receptors could not be identified in the control of micturition (Mbaki and Ramage, 2008a). Further, the activation of 5-HT_{2B} receptors, potentially at the level of the urethra, increased urethral smooth muscle tone. For 5-HT_{2A} receptors, their activation was found to facilitate the micturition reflex as well as playing a small physiological role in the control of the micturition reflex. Although both 5-HT_{2A} and 5-HT_{2C} receptors were implicated in evoking external urethral sphincter-electromyogram (EUS-EMG) activity, confidence in the involvement of both these receptor types was limited due to the level of selectivity of the agonists and antagonists particularly upon intravenous (i.v.) bolus administration which elicited uncontrolled drug exposure.

The present experiments were carried out to determine whether both or one of these subtypes, 5-HT_{2A} and 5-HT_{2C} causes EUS-EMG activation and to confirm the inhibitory effect of 5-HT2C receptor activation on the micturition reflex. This was carried out by administering 5-HT2 receptor ligands by i.v. infusion instead of bolus injection. Infusion avoids the initial high plasma concentrations resulting from bolus administration, thus improving the potential for selective activation and inhibition with respect to these 5-HT_2 receptor subtypes. These infusions were set to target plasma levels that were, by calculation, expected to be selective for either the 5-HT_{2A} or the 5-HT_{2C} receptor subtype. The 5-HT_{2C}-preferring receptor agonist

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(2S)-1-(6-chloro-5-fluoroindol-1-yl)propan-2-amine fumarate (Ro 60-0175; Martin et al., 1998) was infused in the absence and presence of infusions of either the selective 5-HT_{2C} receptor antagonist 6-chloro-5-methyl-N-[6-(2-methylpyridin-3-yl)oxypyridin-3-yl]-2,3-dihydroindole-1-carboxamide dihydrochloride (SB 242084; Kennett et al., 1997) or the selective 5-HT_{2A} receptor antagonist (R)-(2,3-dimethoxyphenyl)-[1-[2-(4-fluorophenyl)ethyl]piperidin-4-yl]methanol (MDL-100,907; Dudley et al., 1990; Kehne et al., 1996; Johnson et al., 1996). Table 1 summarises the relative affinity of these compounds at 5-HT_2 receptor subtypes. A preliminary account of some of these data has been previously published (Mbaki and Ramage, 2008b).

2. Materials and methods

2.1. Animal surgery and preparation

Animal care was in accordance with the UK Animals (Scientific Procedures) Act of 1986. A total of 33 female Sprague–Dawley rats (250–300 g, Charles River Laboratories, UK) were used in this study. Experiments were conducted under terminal general anaesthesia and animals were euthanized at the end of each study by an overdose of sodium pentobarbitone. Anaesthesia was induced (4% in 100% oxygen) and maintained during initial surgery with isoflurane (3% in 100% oxygen). Isoflurane administration was then stopped and anaesthesia was maintained for the duration of the experiment with i.v. administration of urethane (1.2 g/kg). The depth of anaesthesia was assessed by the stability of blood pressure and an absence of hind limb withdrawal in response to paw pinch. Supplementary doses of urethane (0.1 g/kg, i.v.) were given as required. The left jugular vein was cannulated with three separate polyethylene cannulae (each 0.28 mm internal diameter and 0.61 mm outer diameter) inserted via the same incision in the vein. Urethane was administered via one cannula and the other two cannulae were used for the simultaneous infusions of the agonist Ro 60-0175 in saline and the antagonist vehicle respectively, Ro 60-0175 in saline and antagonist respectively, or saline and antagonist vehicle respectively, thus preventing contamination of the cannulae. The trachea was cannulated to maintain a patent airway. The right common carotid artery was cannulated with a heparinised cannula (20 units/ml heparin in 0.9% saline), thus enabling monitoring of the cardiovascular status of the animal and the withdrawal of blood samples (0.2 ml) for analysis of the plasma concentration of the test drugs. Body temperature was maintained between 37 °C and 38 °C using a Harvard homeothermic blanket control unit.

2.2. Measurement of bladder and urethral pressures

The ureters were exposed by retroperitoneal incisions and the proximal end of each ureter was cannulated and allowed to drain externally to prevent the bladder filling with urine during the experiments. The distal ends of the ureters were tied to ensure no backflow and hence no leakage of saline from the bladder. The urinary bladder was exposed by a midline laparotomy and a double lumen cuffed catheter inserted into the lumen through an incision in the dome. The catheter was secured with a suture around its cuff at the tip of the bladder dome, followed by closure of the abdominal incision. The bladder catheter was constructed from polyethylene tubing (1.4 mm internal diameter and 1.9 mm outer diameter) inside which two catheters were sealed. One catheter was connected to a pressure transducer and was used to record bladder pressure and to withdraw residual volume via a 1 ml syringe on a plastic stopcock attached to the transducer. The second catheter was used for bladder infusions and was connected to a syringe infusion pump (KD Scientific, Holliston, MA, U.S.A.). For measurement of urethral pressure a 1.4 F nylon catheter with a side-mounted microtip transducer located 1 mm from the catheter tip (SPR-671, Millar Instruments Inc., Houston, TX, USA) was inserted into the urethra via the urethral orifice and positioned in the mid region of the urethra; where high frequency pressure fluctuations were observed accompanying phasic EUS-EMG firing in addition to urethral relaxation during a micturition reflex (see Figs. 2 and 5). The catheter was secured in place with a strip of masking tape wrapped around the rat's tail to ensure that the catheter did not move during repeated voidings. For EUS-EMG recordings two fine copper wire electrodes (0.2 mm diameter) were used to measure the EMG of the external urethral sphincter. The tip of an electrode was positioned in the bevel of a needle (25 G) and the needle was inserted percutaneously approximately 0.5 mm lateral and 0.5 mm caudal to the urethral orifice. The needle and wire were advanced approximately 5 mm through the skin and the needle slowly withdrawn leaving the wire inserted in the external urethral sphincter. The second electrode was inserted in the same position on the opposite side of the urethral orifice. The electrodes were connected to a Neurolog head stage (NL100; Digitimer, Welwyn Garden City, UK) and the signal was amplified (NL104) and filtered (NL125, 100 Hz low frequency and 30,000 Hz high frequency) and displayed on an oscilloscope (Tektronix, 2205).

2.3. Experimental protocols

Animals were left for 1 h to stabilise after completion of surgery. The experimental protocol is illustrated in Fig. 1. Saline was continuously infused into the bladder at a rate of 0.1 ml/min for 15 min to 'prime' the system and cause a series of saline-induced micturition reflexes. The onset of micturition was defined by the point at which bladder pressure was observed to increase, leading to the initiation of a large rapid contraction. This increase in bladder pressure was accompanied by a simultaneous increase in EUS-EMG activity and a decrease in urethral pressure (urethral relaxation). After 'priming the system', infusion was stopped and the bladder emptied. Three minutes later, saline was infused into the bladder at a rate of 0.1 ml/min until micturition was evoked, infusion was discontinued and the residual volume was withdrawn and measured. This discontinuous cystometry with withdrawal of residual volume was repeated with 3 min between the end of one infusion cycle and the start of the next until three consecutive reflex-evoked bladder contractions of similar (±10%) volume threshold (volume of saline required to initiate micturition) and amplitude were obtained, in order to ensure reproducibility of the response (control reflexes). Subsequently, a control test protocol was initiated. After a period of 3 min, the bladder was filled at a rate of 0.1 ml/min to 80% volume threshold. This was calculated for each animal from the average volume threshold measured from the three sequential control cystometric investigations. In order to quantify effects on EUS-EMG activity per se in the presence of a drug, the bladder was filled with a specific volume for a 5 minute period to avoid confounding changes in EUS-EMG activity resulting as a consequence of drug-induced changes in volume threshold during cystometry. The filling to a level of 80% volume threshold was based on previous preliminary experiments in which it was found that the 5-HT_2 receptor agonist *m*-

Table 1
pKi (calculated from Ki) values for agonist and antagonist ligands used in the present study: [a]Hemrick-Luecke and Evans, 2002, [b]Knight et al., 2004, [c]Gleason et al., 2001 and [d]Johnson et al., 1996. h is for human and r is for rat.

Ligand	Receptor subtype		
	5-HT_{2A}	5-HT_{2B}	5-HT_{2C}
[a]Ro 60-0175	r7.49	–	r9.00
[b]Ro 60-0175	h7.44	h8.27	h8.22
[c]MDL-100,907	h9.10	h6.09	h6.95
[d]MDL-100,907	r9.25	–	r6.80
[c]SB 242084	h7.24	h6.90	h9.02

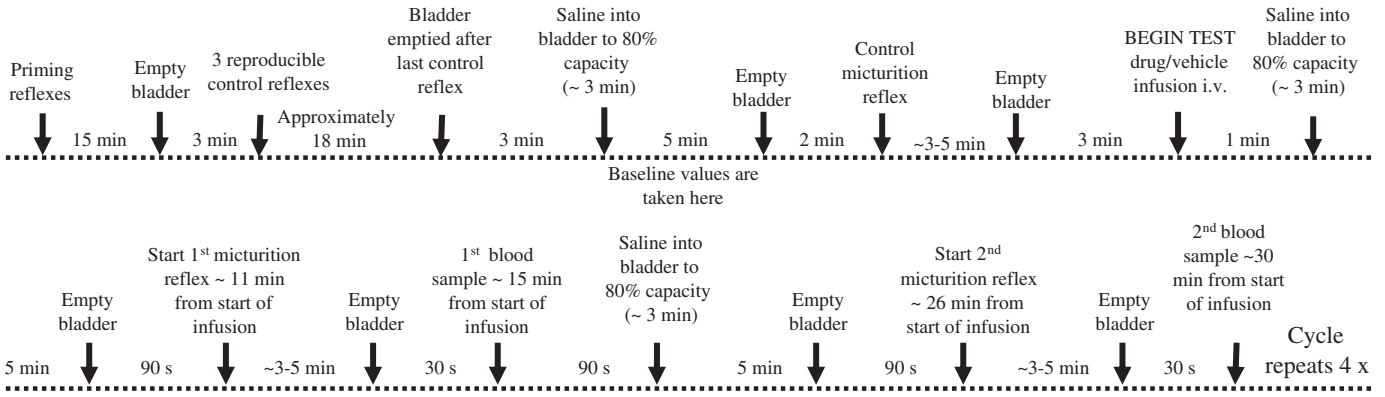

Fig. 1. Diagram showing a time line (not to scale) to illustrate the sequence of events in the experimental protocol. The cycle above following the start of drug/vehicle infusion was repeated for 3 different infusion rates of Ro 60-0175 (27, 90 and 270 μg/kg/min) in the presence of antagonist vehicle or antagonist, or 3 cycle repeats with saline in the presence of antagonist vehicle.

chlorophenylpiperazine (m-CPP; 300 μg/kg, bolus i.v.) did not have any effect on EUS-EMG if the bladder was empty. Baseline EUS-EMG activity, urethral pressure and blood pressure were measured at this time. The bladder was emptied after the 5 minute period and a control reflex was evoked with collection of residual volume thereafter. Intravenous infusion of test drugs or vehicle in a total volume of 0.1 ml/kg/min was commenced 3 min later. One minute later the bladder was filled with saline to a level of 80% volume threshold which took up to 3 min depending on the capacity of the bladder, and held at this capacity for 5 min. The bladder was then emptied and 90 s later the 1st micturition reflex in the presence of test drugs or vehicle was tested. A blood sample (~0.2 ml) was withdrawn from the common carotid artery for analysis of the plasma concentrations of the test compounds, approximately 4 min after the initiation of the micturition reflex test. In each rat, 6 micturition reflexes were tested in the presence of test drug or vehicle at approximate times of 11, 26, 42, 58, 74, 90 min and 6 blood samples were collected at approximate times of 15, 30, 46, 62, 78, 94 min after onset of drug infusion (see Fig. 1). The test compounds or vehicle were infused continuously into the animal for approximately 94 min. It should be noted that blood samples were taken from all animals (including controls) although not all samples were analysed. Control experiments consisted of i.v. infusion of a mixture of 50% cremophor, 40% tetraglycol and 10% ethanol (CTE); then diluted with saline to give a final concentration of 4% CTE. Over the same period blood samples were taken as above but discarded.

2.4. Determination of appropriate drug infusion concentration

Initial pharmacokinetic studies were carried out following i.v. dosing of Ro 60-0175, SB 242084 and MDL-100,907 in female Sprague–Dawley rats. Blood samples were collected as described above and plasma concentrations of compounds measured by liquid chromatography–mass spectrometry (LC–MS), using a Sciex API2000 mass spectrometer (Perkin-Elmer Sciex, Foster City, CA, U.S.A.). Plasma protein binding was measured using a Pierce Biotechnology rapid equilibrium dialysis device (Thermo Fisher Scientific). From the measured pharmacokinetic parameters infusion protocols were designed to target specific free plasma drug concentrations in the present studies. Given the known Ki of 1 nM for Ro 60-0175 at the cloned rat 5-HT_{2C} receptor (Hemrick-Luecke and Evans, 2002), it was decided to target 3 increasing concentrations of 10, 30 and 100 nM free plasma concentrations using infusions of 27, 90 and 270 μg/kg/min of Ro 60-0175 respectively, in order to investigate both selective (5-HT_{2C}) and non-selective (5-HT_{2A}/5-HT_{2B}) effects. Similarly, given the reported Ki for SB 242084 at human 5-HT_{2C} receptors (0.94 nM, Gleason et al., 2001), infusion regimens were designed to target 3 and 10 nM free plasma concentrations using 15 minute loading dose infusions of 22.5 μg/kg/min and 67.5 μg/kg/min respectively, in order to reach the target concentration by the end of the infusion, followed for both regimens by a maintenance dose of 3.5 μg/kg/min (infused for approximately 75 min). The reported Ki for MDL-100,907 was 0.78 nM (Gleason et al., 2001) at the human 5-HT_{2A} receptor and 0.56 nM (pKi 9.25) at the rat 5-HT_{2A} receptor (Johnson et al., 1996) and it was estimated that targeting a free plasma concentration of 3 nM would maintain selectivity for 5 HT_{2A} receptors. It was calculated that an initial loading dose infusion of 16.7 μg/kg/min for 15 min, followed by maintenance at 0.83 μg/kg/min would provide sufficient exposure.

2.5. Drug delivery

Simultaneous infusions (0.1 ml/kg/min) of the agonist Ro 60-0175 in saline and the vehicle for the antagonist (see Drugs and solutions below), Ro 60-0175 in saline and antagonist, or saline and antagonist vehicle were performed in these experiments. As mentioned above, Ro 60-0175 was administered as three different doses; 27, 90 and 270 μg/kg/min targeting 10, 30 and 100 nM free plasma concentrations. Ro 60-0175-vehicle experiments were performed initially where a solution of low dose Ro 60-0175 in saline (27 μg/kg/min) was infused via one cannula and a solution of the vehicle for the antagonist was infused via the other cannula. Simultaneous infusions of low dose Ro 60-0175 and vehicle lasted approximately 30 min with the infusion of the middle dose of Ro 60-0175 (90 μg/kg/min) commencing thereafter. At approximately 60 min after the onset of drug infusion, 270 μg/kg/min was infused for a further 30 min. The total time for drug infusion was between 90 and 94 min depending on the duration of the micturition reflexes.

With the Ro 60-0175-antagonist experiments, Ro 60-0175 was infused as described above. With the antagonists, as previously mentioned, initial pharmacokinetic studies postulated the requirement of both antagonists SB 242084 and MDL-100,907 to be administered as an initial loading dose followed by a maintenance dose. Therefore, the low dose of Ro 60-0175 (27 μg/kg/min) was infused via one cannula while the loading dose of the antagonist was infused via the other cannula. Infusion of the loading dose of antagonist lasted for 15 min and was thereafter replaced with the maintenance dose for the remainder of the experiment.

Experiments were also performed with the antagonists alone, testing the low dose regimen of SB 242084 (22.5 μg/kg/min for 15 min; 3.5 μg/kg/min for 30 min) followed by the high dose regimen (67.5 μg/kg/min for 15 min; 3.5 μg/kg/min for 30 min) and MDL-100,907 at 1.67 μg/kg/min for 15 min; 0.83 μg/kg/min for 15 min

followed by 5.01 μg/kg/min for 15 min; 0.83 μg/kg/min for 15 min and then 16.7 μg/kg/min for 15 min; 0.83 μg/kg/min for 15 min.

Blood samples were analysed for compound plasma concentration as above and corrected for plasma protein binding to provide free plasma concentrations which are presented in Table 2.

2.6. Data capture and analysis

Arterial blood pressure (with heart rate derived from the blood pressure signal), bladder pressure, urethral pressure, amplified EUS-EMG and integrated EUS-EMG were recorded using a Cambridge Electronic Design (CED) 1401+ interface and Spike2 (version 5.06) data collection software which was used for off-line analysis.

2.7. Analysis of results

The total integrated EUS-EMG signal using the root mean square of the raw EUS-EMG was used for analysis as it produced waveforms that were easier to analyse than the noisy raw EMG signal. The raw EUS-EMG signal was rectified and smoothed at a time constant of 0.1 s using Spike2 functions, in order to smooth the curve but still maintain enough data points. The total integrated EUS-EMG signal following deduction of background noise was calculated. Baseline values of the rectified and smoothed EUS-EMG signal, as well as urethral pressure, mean arterial pressure and heart rate were recorded as the mean values obtained over a 5 minute period. Drug effects on baseline integrated EUS-EMG signal, urethral pressure and blood pressure were measured over 1 min intervals at approximate times of 20–25, 50–55 and 80–85 min (see Fig. 1). Onset of drug effect was calculated from appearance of the first spike of continuous and regular EUS-EMG activity.

For the micturition reflex, urethral pressure although recorded in this study was not analysed any further as no overt changes were observed in response to the drugs tested as previously reported (Mbaki and Ramage, 2008a). The following variables were measured: volume threshold (ml) — defined as the volume of saline required to elicit voiding, pressure threshold (mm Hg) — bladder pressure recorded at the start of a micturition contraction and residual volume (ml) — withdrawn using a 1 ml syringe after micturition had occurred. These variables were analysed when the micturition reflex was tested at time 11, 26, 42, 58, 74, and 90 min after the onset of drug infusion.

2.8. Statistical analysis

Drug and vehicle effects were expressed as percentage change from baseline control values for the integrated EUS-EMG signal, urethral pressure, and micturition variables of volume threshold, pressure threshold and residual volume and were expressed as absolute change from baseline values for mean arterial pressure and heart rate. Data are presented in the text and graphical format as mean ± standard error of the mean (S.E.M.) of the calculated percentage change from baseline control values for the measured variables. Changes in mean integrated EUS-EMG signal, urethral pressure, mean arterial pressure and heart rate caused by test drugs were compared with time matched vehicle controls using two-way analysis of variance and the Bonferroni correction test. Drug evoked changes on the micturition reflex were compared with vehicle controls using one-way analysis of variance and the Bonferroni's multiple comparison test. Values of $P<0.05$ were considered to be statistically significant.

2.9. Drugs and solutions

Drugs and chemicals were obtained from the following sources: Isoflurane from Abbott Animal Health, Queenborough, Kent, UK; Urethane

Table 2
Summary of measured free plasma concentrations of 5-HT_{2C} agonist Ro 60-0175, 5-HT_{2C} antagonist SB 242084 and 5-HT_{2A} antagonist MDL-100,907 administered as infusion doses i.v. Free plasma concentrations are presented as geometric means (N.B. not all blood samples were analysed).

Associated reflex test	Time of sample min (approx.)	Ro 60-0175 infusion μg/kg/min	Ro 60-0175 Target and measured [Free plasma] nM (n = 4)		SB 242084 infusion μg/kg/min	SB 242084 Target and measured [Free plasma] nM (n = 5)		MDL-100,907 infusion μg/kg/min	MDL-100,907 Target and measured [Free plasma] nM (n = 3)	
1	15	27	10	8	22.5	3	5	16.7	3	22 (n = 2)
2	30		10	8	3.5	3	4	0.83	3	7
3	46	90	30	24	3.5	3	5	0.83	3	4
4	62		30	34		3	5		3	3
5	78	270	100	84	3.5	3	4	0.83	3	3
6	94		100	126		3	4		3	4

Table 3
Baseline values for integrated EUS-EMG signal, urethral pressure, mean arterial pressure (MAP) and heart rate (HR) during bladder filling to 80% volume threshold and control values for volume threshold, pressure threshold and residual volume for the bladder micturition reflex. Values are presented as mean ± S.E.M.

Experimental group	n	EUS-EMG μV	Urethral pressure mm Hg	Bladder reflex			MAP mm Hg	HR bpm
				Volume threshold ml	Pressure threshold mm Hg	Residual volume ml		
Saline + 4% CTE	5	0.27 ± 0.02	27 ± 0.1	0.49 ± 0.11	11 ± 1	0.25 ± 0.09	97 ± 0.2	348 ± 3
Ro 60-0175 + 4% CTE	5	0.22 ± 0.06	23 ± 0.1	0.49 ± 0.11	11 ± 1	0.25 ± 0.09	111 ± 2	345 ± 2
Ro + SB 242084 (22.5; 3.5 μg/kg/min)	5	0.40 ± 0.03	26 ± 0.3	0.43 ± 0.11	9 ± 1	0.26 ± 0.09	112 ± 0.2	369 ± 3
Ro + SB 242084 (67.5; 3.5 μg/kg/min)	3	0.10 ± 0.01	28 ± 0.2	0.31 ± 0.06	9 ± 1	0.11 ± 0.06	99 ± 1	387 ± 1
Ro + MDL-100,907 (16.7; 0.83 μg/kg/min)	5	0.30 ± 0.01	25 ± 0.1	0.44 ± 0.06	9 ± 1	0.21 ± 0.07	104 ± 0.3	372 ± 1
SB 242084 (22.5 and 67.5; 3.5 μg/kg/min)	5	0.10 ± 0.02	28 ± 1	0.40 ± 0.07	8 ± 1	0.17 ± 0.1	106 ± 1	353 ± 2
MDL-100,907 (1.67, 5.01 and 16.7; 0.83 μg/kg/min)	5	0.48 ± 0.03	26 ± 0.1	0.35 ± 0.05	8 ± 1	0.10 ± 0.03	112 ± 1	346 ± 2

from Sigma-Aldrich, Poole, Dorset, UK; (2S)-1-(6-chloro-5-fluoroindol-1-yl)propan-2-amine fumarate (Ro 60-0175) from Tocris Cookson Ltd, Avonmouth, Bristol, UK; 6-chloro-5-methyl-N-[6-(2-methylpyridin-3-yl)oxypyridin-3-yl]-2,3-dihydroindole-1-carboxamide dihydrochloride (SB 242084) and (R)-(2,3-dimethoxyphenyl)-[1-[2-(4-fluorophenyl) ethyl]piperidin-4-yl]methanol (MDL-100,907) were synthesised by Pfizer Global Research and Development, Sandwich, Kent, UK. Ro 60-0175 was dissolved in 0.9% saline. SB 242084 and MDL-100,907 were dissolved in a mixture of 50% cremophor, 40% tetraglycol and 10% ethanol; (CTE) then diluted with saline to give a final concentration of 4% CTE. All drugs or combination of drugs and vehicle were infused i.v. in a total volume of 0.1 ml/kg/min.

3. Results

The absolute values for all recorded baseline variables and the control reflex (the mean ± S.E.M. of three control micturition reflexes) are presented in Table 3.

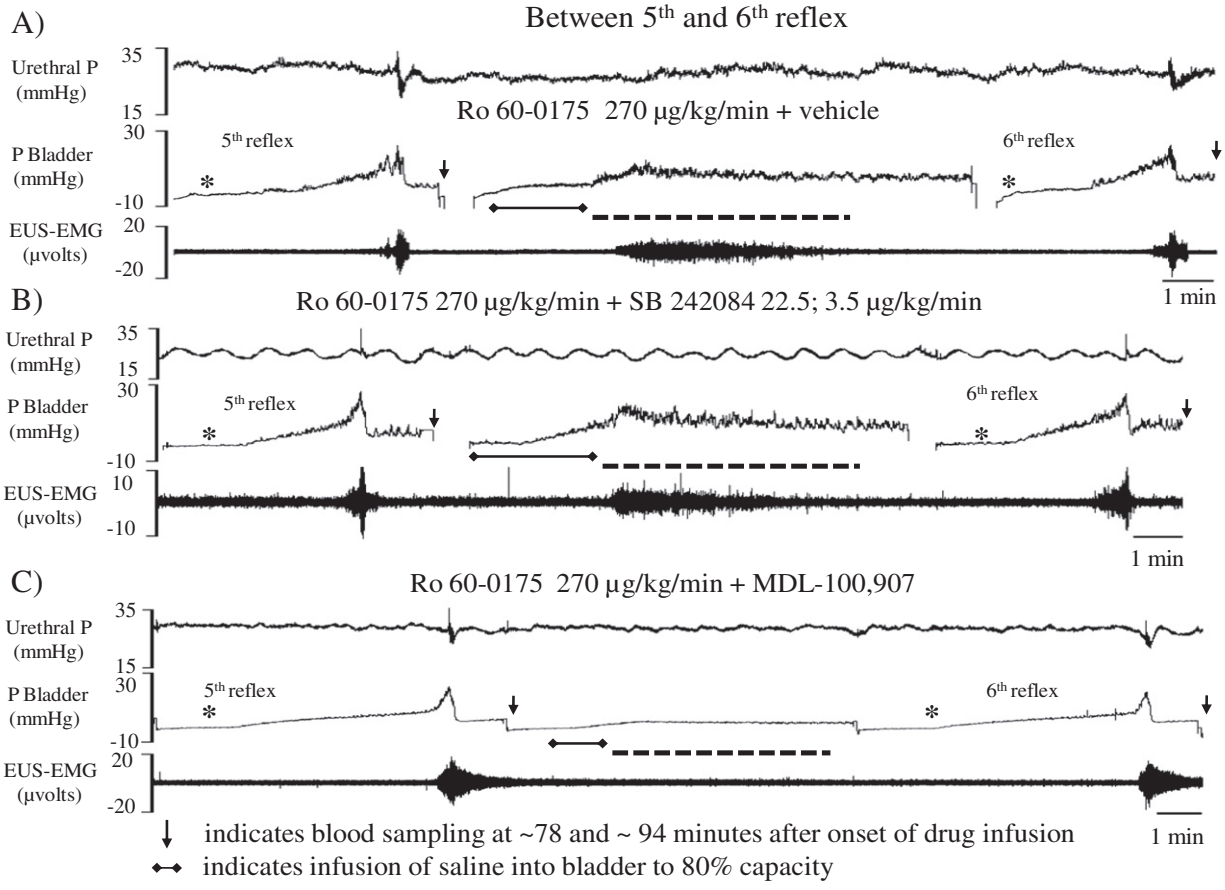

Fig. 2. Representative continuous traces showing recordings of urethral pressure (P), bladder pressure, and external urethral sphincter (EUS) EMG from 3 experiments for the 5th to 6th micturition reflex approximately 74 and 90 min from the start of drug/vehicle infusions. Each panel shows effects of co-infusions of Ro 60-0175 with (A) antagonist vehicle (4% CTE), (B) SB 242084 and (C) MDL-100,907. * shows start of infusion of saline into the bladder to initiate a micturition reflex; shows when measurements were taken after infusion of saline into the bladder to give 80% of volume required for micturition.

3.1. Controls — effect of vehicle infusions

Intravenous infusions (0.1 ml/kg/min; n = 5) of saline plus antagonist vehicle (4% CTE) had no effect on EUS-EMG signal, urethral pressure or mean arterial pressure when the bladder was filled to 80% of volume threshold (Figs. 3 and 4), and these variables remained stable for the duration of the experiment. Additionally, no changes were observed on the micturition reflex (Figs. 6 and 7).

3.2. EUS-EMG, urethral pressure and mean arterial pressure (bladder filled to 80% volume threshold)

3.2.1. Effect of Ro 60-0175

Ro 60-0175 (n = 5) at low doses (27 and 90 μg/kg/min) in the presence of the antagonist vehicle (4% CTE) evoked no significant effect on EUS-EMG signal or urethral pressure over the duration of the experiment. However, during infusion of the highest dose (270 μg/kg/min) Ro 60-0175 between the 5th and 6th reflex tests when the bladder was filled with saline equivalent to 80% of the volume required for micturition, EUS-EMG activity was now observed, reaching a maximum of 1239 ± 467% by 1 min and then declining, becoming non significant after 4 min. Representative traces of this part of the experiment are shown in Fig. 2A, while mean data for the complete dose range is shown in Figs. 3 and 4. The mean onset of appearance of EUS-EMG activity from filling the bladder to 80% capacity was 83 ± 14 s. Mean urethral pressure was still unaffected by the highest infusion dose of Ro 60-0175. Over this high dose period there was also an associated significant increase in mean arterial pressure, which reached a maximum of 21 ± 3 mm Hg (Fig. 3) but there was no effect on heart rate (data not shown).

3.2.2. Effect of Ro 60-0175 in the presence of 5-HT_{2C} antagonist SB 242084

Ro 60-0175 again at 27 μg/kg/min and 90 μg/kg/min in the presence of SB 242084 infused at 22.5; 3.5 μg/kg/min (n = 5) or at the higher concentration regimen of 67.5; 3.5 μg/kg/min (n = 3) had no effect on EUS-EMG signal or urethral pressure. However, during infusion of the highest dose (270 μg/kg/min) Ro 60-0175 between the 5th and 6th reflex tests when the bladder was filled with saline equivalent to 80% of the volume required for micturition, EUS-EMG activity was still observed in the presence of low and high concentration regimens of SB 242084. The level of EUS-EMG activity with the lower concentration regimen of SB 242084 was larger reaching a maximum within the first minute of 2276 ± 539% although not significantly different from Ro 60-0175 alone, and although activity declined it now remained significant over the entire 5 min period. In the presence of the high concentration regimen of SB 242084 EUS-EMG activity was similar to that of Ro 60-0175 alone reaching a maximum by the first minute of 1282 ± 605% but there was no decline in the level of activity over the 5 minute period. Representative traces of this part of the experiment are shown in Fig. 2B, while mean data for the complete dose range in the presence of SB 242084 are shown in Fig. 3. The mean onset of appearance of EUS-EMG activity in the presence of SB 242084 for the low and high dose regimens was 100 ± 27 s and 113 ± 16 s, respectively. Again this was similar to that observed with Ro 60-0175 alone. Urethral pressure again was unaffected by the combination of Ro 60-0175 and SB 242084. SB 242084 also failed to block the pressor effect of the high

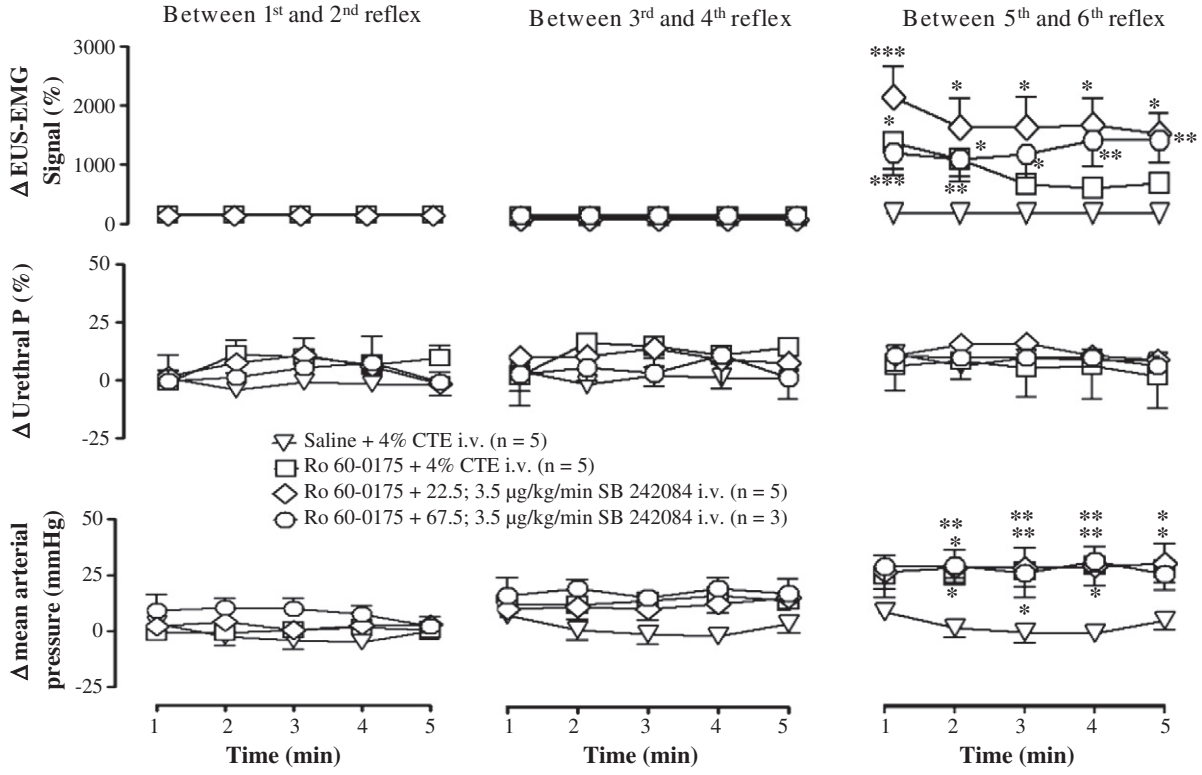

Fig. 3. A comparison of the effect of co-infusions of agonist vehicle (saline) plus 4% CTE (n = 5), Ro 60-0175 plus 4% CTE (n = 5) and Ro 60-0175 plus SB 242084 (22.5 μg/kg/min; n = 5 and 67.5 μg/kg/min; n = 3) on changes (Δ) in baseline EUS-EMG activity (%), urethral pressure (P; %) and mean arterial pressure (mm Hg). Panels from left to right show these changes in baseline values recorded between 20–25, 50–55 and 80–85 min, after the start of the co-infusions. Each point represents the mean and vertical bars show the S.E.M. of the calculated percentage change from baseline control values for the measured variables (except for mean arterial pressure). Changes (*) caused by Ro 60-0175 in the presence of 4% CTE or Ro 60-0175 in the presence of SB 242084 are compared to vehicle using two-way analysis of variance and the Bonferroni correction test. *P<0.05, **P<0.01, ***P<0.001. NB — Changes caused by Ro 60-0175 in the presence of 4% CTE with Ro 60-0175 in the presence of both doses of SB 242084 have also been be carried out but found to be non-significant.

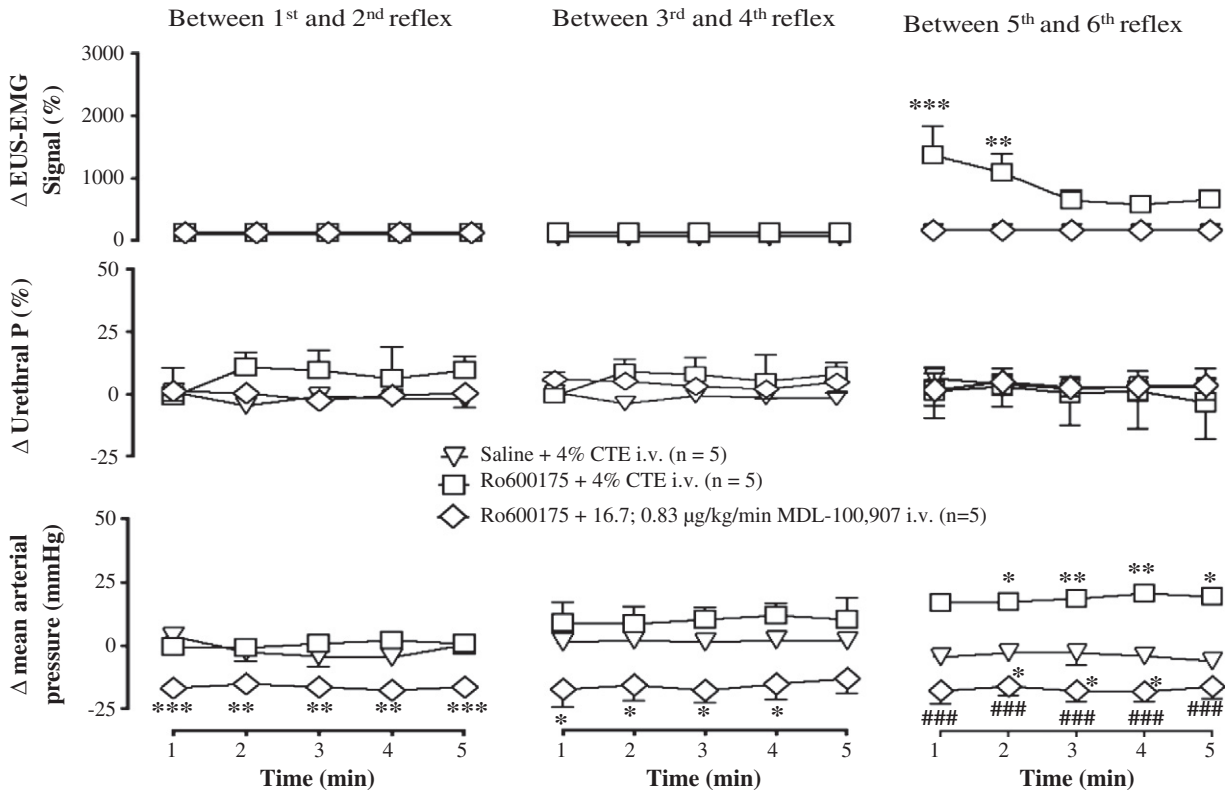

Fig. 4. A comparison of the effect of co-infusions of agonist vehicle (saline) plus 4% CTE (n = 5), Ro 60-0175 plus 4% CTE (n = 5) and Ro 60-0175 plus MDL-100,907 (n = 5) on changes (Δ) in baseline EUS-EMG activity (%), urethral pressure (P; %) and mean arterial pressure (mm Hg). Panels from left to right show these changes in baseline values recorded between 20–25, 50–55 and 80–85 min respectively, after the start of the co-infusions. Each point represents the mean and vertical bars show the S.E.M. of the calculated percentage change from baseline control values for the measured variables (except for mean arterial pressure). Changes (*) caused by Ro 60-0175 in the presence of 4% CTE or Ro 60-0175 in the presence of MDL-100,907 are compared to vehicle (saline plus 4% CTE) and changes (#) by Ro 60-0175 in the presence of 4% CTE are compared with changes caused by Ro 60-0175 in the presence of MDL-100,907 using two-way analysis of variance and the Bonferroni correction test. *P<0.05, **P<0.01 ***, ###P<0.001. NB — Only 1 dose of MDL-100,907 was used in this study.

dose (270 μg/kg/min) of Ro 60-0175, which caused maximum rises of 22 ± 8 and 23 ± 4 mm Hg during low and high concentration regimens of SB 242084, respectively. Mean data for the effects of Ro 60-0175 on urethral pressure and mean arterial pressure in the presence of both concentration regimens of SB 242084 are shown in Fig. 3. Heart rate was unaffected by the combination of Ro 60-0175 and SB 242084 (data not illustrated).

3.2.3. Effect of Ro 60-0175 in the presence of the 5-HT_{2A} antagonist MDL-100,907

Again the low doses of Ro 60-0175 (27 and 90 μg/kg/min) in the presence of MDL-100,907 (n = 5) had no effect on the EUS-EMG signal or urethral pressure. However, in the presence of MDL-100,907 during infusion of the highest dose (270 μg/kg/min) of Ro 60-0175, the expected appearance of EUS-EMG activity on filling the bladder with saline equivalent to 80% of the volume required for micturition was blocked. Representative traces of this part of the experiment are shown in Fig. 2C, while mean data for the complete dose range in the presence of MDL-100,907 are shown in Fig. 4. Further, the expected rise in blood pressure was now reversed to a decrease of 18 ± 4 mm Hg (Fig. 4) reflecting decreases observed with MDL-100,907 alone (see below). Heart rate was unaffected by the combination of Ro 60-0175 and MDL-100,907 (data not illustrated).

3.2.4. Effect of 5-HT_{2C} antagonist SB 242084 alone

SB 242084 (loading dose 22.5 and 67.5 μg/kg/min; maintenance dose 3.5 μg/kg/min; n = 5) evoked no significant changes in EUS-EMG signal or urethral pressure. SB 242084 had no effect on mean arterial pressure apart from a transient decrease (−4 ± 1 mm Hg) in the first minute of the lower dose infusion. Neither of the two concentrations of SB 242084 had any effect on heart rate (data for SB 242084 alone not illustrated).

3.2.5. Effect of the 5-HT_{2A} antagonist MDL-100,907 alone

MDL-100,907 (loading doses of 1.67, 5.01 and 16.7 μg/kg/min; maintenance dose for each infusion regimen of 0.83 μg/kg/min; n = 5) evoked no significant changes in baseline EUS-EMG signal or urethral pressure. MDL-100,907 evoked a significant dose related decrease in mean arterial pressure of 11 ± 0.2, 16 ± 2 and 25 ± 0.4 mm Hg respectively at each of the infusion doses. Heart rate was unaffected (data for MDL-100,907 alone not illustrated).

3.3. Micturition reflex

3.3.1. Effect of infusions of Ro 60-0175

Ro 60-0175 (n = 5) at the lowest infusion concentration of 27 μg/kg/min inhibited the micturition reflex as shown by a significant increase in volume threshold (59 ± 8%) for the 2nd reflex tested. However, at 90 μg/kg/min Ro 60-0175 no longer evoked an increase in volume threshold and by 270 μg/kg/min there was now a significant decrease in the volume threshold of both reflexes (5th and 6th) tested (−43 ± 9% and −50 ± 10%). Pressure threshold was increased for all 6 reflex tests but it was only significant for the 6th reflex tested increasing by 53 ± 22%. Surprisingly this was when volume threshold

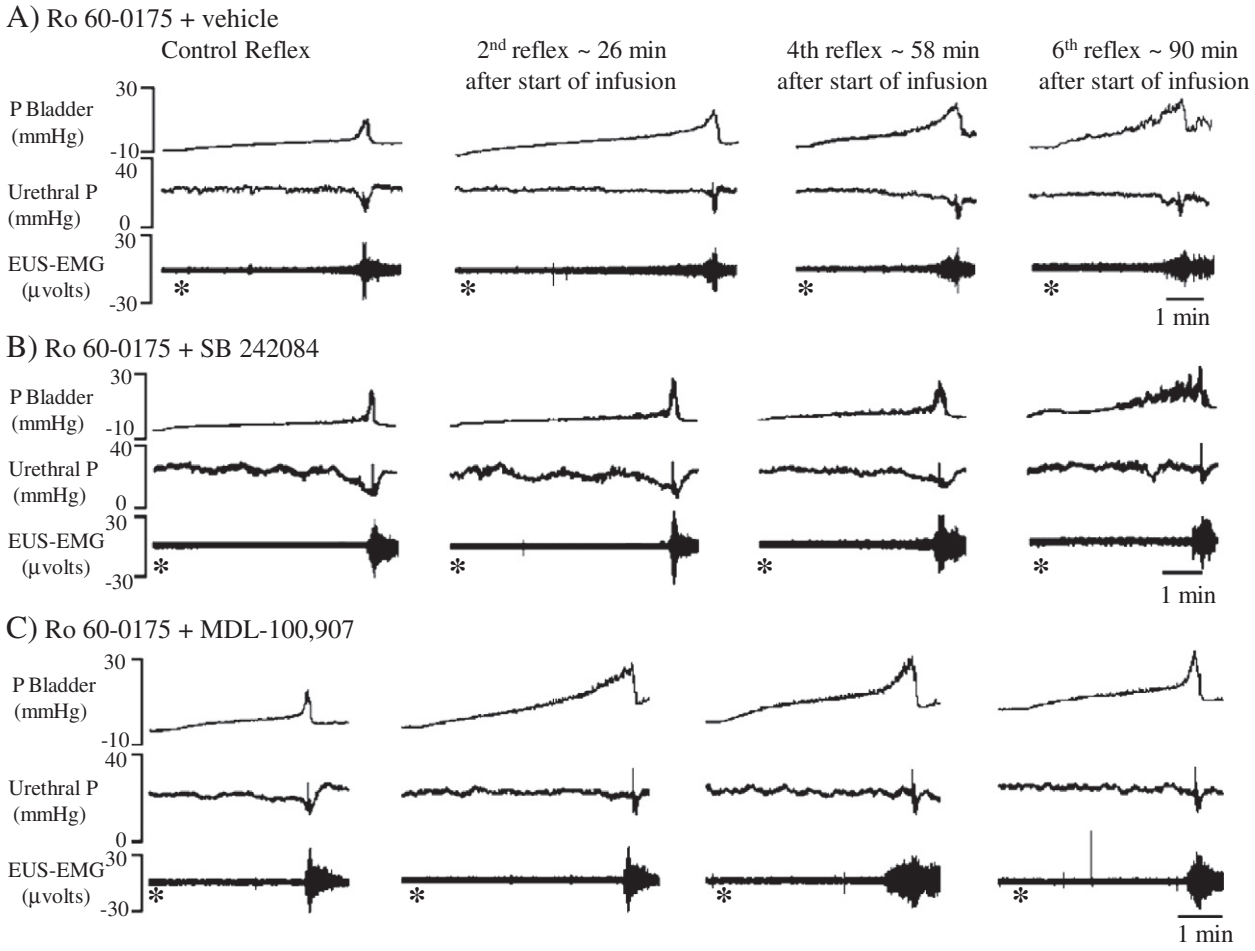

Fig. 5. Representative traces showing recordings of bladder and urethral pressure (P) and external urethral sphincter (EUS)-EMG from 3 separate experiments in female anaesthetized rats. Each panel shows the effects of co-infusions of Ro 60-0175 with (A) the antagonist vehicle (4% CTE), (B) SB 242084 and (C) MDL-100,907 on the micturition reflex. * denotes onset of saline infusion into the bladder to evoke the reflex.

was significantly reduced. Residual volume was only significantly increased (105 ± 27%) by Ro 60-0175 at the 2nd reflex test during the lowest infused concentration, while after this, Ro 60-0175 tended to cause a reduction in residual volume. Representative recordings of the effect of Ro 60-0175 on the micturition reflex are shown in Fig. 5A and mean data in Figs. 6 and 7.

3.3.2. Effect of infusion of Ro 60-0175 in the presence of the 5-HT_{2C} antagonist SB 242084

The infusion of both low (22.5; 3.5 µg/kg/min; n = 5) and high (67.5; 3.5 µg/kg/min; n = 3) concentration regimens of SB 242084 blocked the inhibitory effect of Ro 60-0175 on volume threshold (2nd reflex tested) reversing this to an excitatory action i.e. a reduction in volume threshold (Fig. 6). This was observed to be significant when compared with vehicle for the low dose SB 242084 infusion, and significant for both concentration regimens of SB 242084 compared with the increase in volume threshold evoked by Ro 60-0175 in the absence of the antagonist. Thus the ability of Ro 60-0175 to cause a significant reduction in the volume threshold was earlier in onset in the presence of both infusion doses of SB 242084 at the 2nd reflex (compared to 5th reflex test with Ro 60-0175 alone) (Fig. 6). Recordings from one of these experiments are shown in Fig. 5B. The significant increase in pressure threshold caused by Ro 60-0175 that was observed at the 6th reflex was only blocked by the high dose infusion of SB 242084 (Fig. 6). The increased residual volume observed for the 2nd reflex tested at 27 µg/kg/min Ro 60-0175 was blocked by both the low and high concentration regimens of SB 242084.

3.3.3. Effect of infusion of Ro 60-0175 in the presence of the 5-HT_{2A} antagonist MDL-100,907

In the presence of MDL-100,907 the ability of Ro 60-0175 to excite the reflex at higher concentrations was inhibited (Figs. 5C and 7). Volume threshold was observed to be increased and never reduced over the complete infusion period reaching statistical significance compared with vehicle at the 5th reflex tested during infusion of 270 µg/kg/min Ro 60-0175. Similarly, a comparison of volume threshold with Ro 60-0175 alone was significant during infusion of both 90 µg/kg/min and 270 µg/kg/min Ro 60-0175 in the presence of MDL-100,907. MDL-100,907 did not inhibit the increase in pressure threshold evoked by 270 µg/kg/min Ro 60-0175 and increases in pressure threshold at 90 µg/kg/min Ro 60-0175 were now significant when combined with MDL-100,907. Residual volume was now observed to be increased except for the 1st reflex tested over the infusion period, although never significant in the presence of MDL-100,907 compared with vehicle. However, the increase in residual volume with the combination of MDL-100,907 and Ro 60-0175 was statistically significant compared with the tendency of Ro 60-0175 alone to decrease residual volume only at the 4th and 5th reflexes tested (Fig. 7).

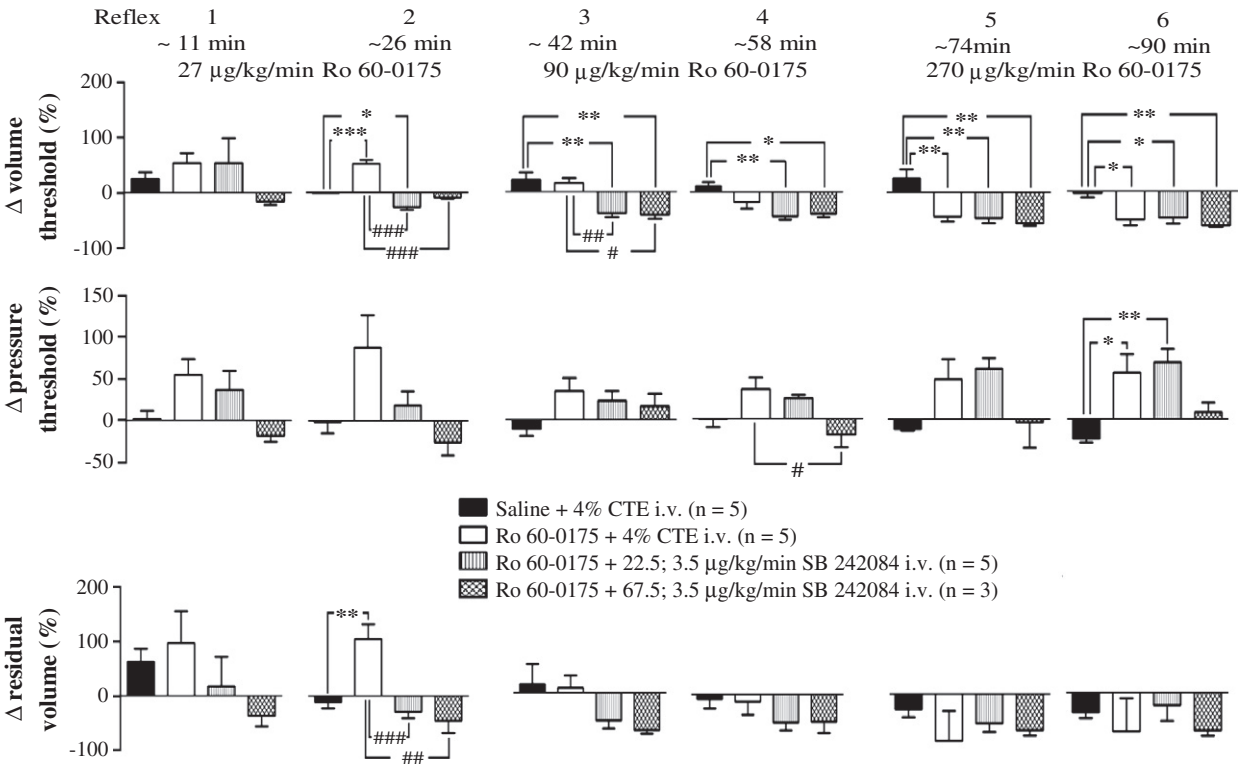

Fig. 6. Histograms comparing the effects of co-infusions of saline (agonist vehicle) plus 4% CTE, Ro 60-0175 plus antagonist vehicle (4% CTE) and Ro 60-0175 plus SB 242084 on the micturition reflex showing percentage (%) changes (Δ) in volume and pressure threshold and residual volume. Two micturition reflexes were tested during each infusion concentration of Ro 60-0175. From left to right the first panel shows the effect of infusion of drugs/vehicles on micturition reflex variables after 11 and 26 min, the next after 42 and 58 min and the last after 74 and 90 min. Each bar represents the mean and vertical bars show the S.E.M. of the calculated percentage change from baseline control values for the measured variables. Comparisons have been carried out using one-way analysis of variance and the Bonferroni's multiple comparison test. *,#P<0.05, **,##P<0.001, ***,###P<0.001.

3.3.4. Effect of the 5-HT$_{2C}$ antagonist SB 242084 and MDL-100,907 alone

Micturition variables were not significantly affected by infusion of SB 242084 (n = 5) or MDL-100,907 (n = 5) alone (data not illustrated).

4. Discussion

It was previously demonstrated that the 5-HT$_{2C}$ receptor agonist Ro 60-0175 administered as a bolus dose of 300 µg/kg (i.v.) evoked EUS activity and caused increases in urethral pressure in anaesthetized rats (Mbaki and Ramage, 2008a). In the present experiments, administering Ro 60-0175 by infusion evoked EUS activity, although only at the highest i.v. concentration (270 µg/kg/min; free plasma concentration between 84 and 126 nM) after the 5th reflex test. This evoked EUS activity was not associated with urethral pressure changes. The effect of Ro 60-0175 on EUS activity was abolished in the presence of selective 5-HT$_{2A}$ receptor antagonist MDL-100,907 at a free plasma concentration of 3 to 4 nM, which was calculated as selective for 5-HT$_{2A}$ receptors. While the selective 5-HT$_{2C}$ receptor antagonist SB 242084 failed to block the excitatory action of Ro 60-0175 on the EUS at a measured free plasma concentration of 4 and 10 nM for the lower and higher infusion regimen respectively, both doses did block the increase in volume threshold evoked by Ro 60-0175 at the 2nd reflex test. This is in agreement with previous data showing that inhibition of the micturition reflex is via activation of 5-HT$_{2C}$ receptors (Mbaki and Ramage, 2008a). Thus from this antagonist data plus the observation that Ro 60-0175 only causes EUS excitation at a high dose, it can be concluded that this action in the rat is due to activation of 5-HT$_{2A}$ not 5-HT$_{2C}$ receptors.

The ability of Ro 60-0175 to evoke EUS-EMG activity required bladder filling with saline to 80% of the volume necessary to evoke micturition (see Mbaki and Ramage, 2008a) thus suggesting this phenomenon requires activation of bladder afferents. However, the volume range of bladder filling and thus the level of bladder afferent activation enabling Ro 60-0175-evoked EUS activity are yet to be determined. In previous experiments, a bolus i.v. dose of 300 µg/kg Ro 60-0175 (Mbaki and Ramage, 2008a) evoked EUS activity that was maintained for over 10 min. In the present experiments, evoked EUS activity declined by the 4th minute, although in the presence of SB 242084 this decline was prevented and the level of evoked EUS activity was observed to be larger. This suggests that the additional activation of 5-HT$_{2C}$ receptors tends to inhibit the 5-HT$_{2A}$ receptor-mediated EUS excitation. Whether this relates to the ability of central activation of 5-HT$_{2C}$ receptors to inhibit micturition remains to be determined. Both 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor protein have been located in various brain regions known to be involved in micturition (Clemett et al., 2000; Cornea-Hébert et al., 1999; Fay and Kubin, 2000;) and in the spinal cord of the rat (Doly et al., 2004; Sharma et al., 1997), the 5-HT$_{2A}$ receptor being detected intensely in the ventral horn (Maeshima et al., 1998), specifically in Onuf's nucleus (Xu et al., 2007) which contains neurons innervating the EUS. A peripheral location for 5-HT$_{2C}$ receptors is lacking, from inconsistent reports on the existence of mRNA for the receptor in rat lumbar DRG neurons (Nicholson et al., 2003; Pierce et al., 1996). Maintenance of EUS-EMG activity over 10 min following the bolus dose of Ro 60-0175 (Mbaki and Ramage, 2008a) was probably due to the sudden increase in plasma concentration resulting in powerful activation of 5-HT$_{2A}$ receptors, thus masking any 5-HT$_{2C}$ inhibitory effect on EUS activity.

In the present study, 5-HT$_{2A}$ receptor-evoked increases in EUS activity were surprisingly not associated with changes in urethral pressure. This may be due to the increases in EMG activity being due to pelvic floor rather than EUS muscle activity. However, evoking a micturition reflex does cause the expected changes in urethral pressure along with the increase in EUS activity (see Fig. 5),

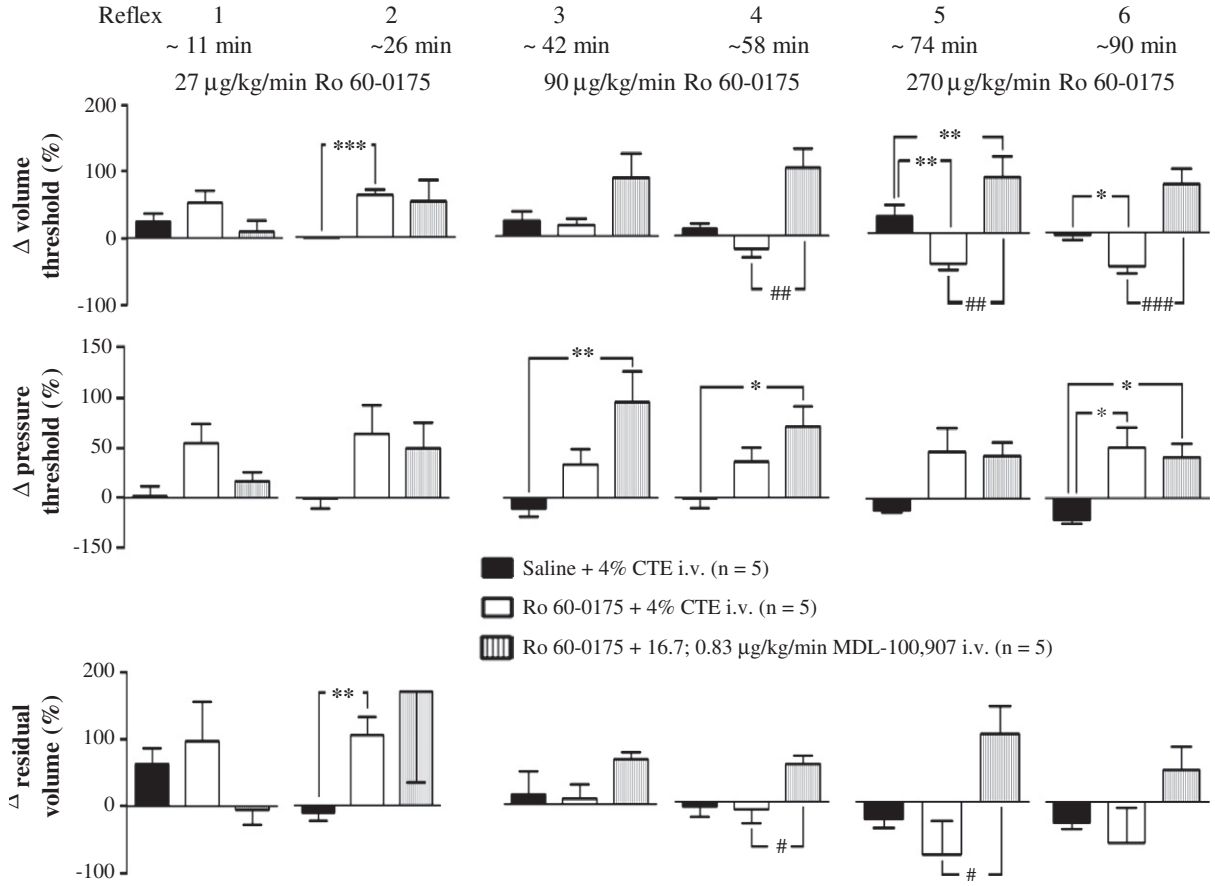

Fig. 7. Histograms comparing the effects of co-infusions of saline (agonist vehicle) plus 4% CTE, Ro 60-0175 plus antagonist vehicle (4% CTE) and Ro 60-0175 plus MDL-100,907 on the micturition reflex showing percentage (%) changes (Δ) in volume and pressure threshold and residual volume. Two micturition reflexes were tested during each infusion concentration of Ro 60-0175. From left to right the first panel shows the effect of infusion of drugs/vehicles on micturition reflex variables after 11 and 26 min, the next after 42 and 58 min and the last after 74 and 90 min. Each bar represents the mean and vertical bars show the S.E.M. of the calculated percentage change from baseline control values for the measured variables. Comparisons have been carried out using one-way analysis of variance and the Bonferroni's multiple comparison test. *,#P <0.05, **,##P<0.001, ***P<0.001.

indicating that the electrodes do detect EUS-EMG. Furthermore, this increase in EUS-EMG activity was observed with the bladder filled to 80% threshold volume, when the urethral smooth muscle would have been constricted to prevent incontinence, thus EUS firing per se may not have any significant effect on urethral pressure. Nevertheless, failure of Ro 60-0175 to cause the expected (see Mbaki and Ramage, 2008b) 5-HT_{2B} receptor-mediated increase in urethral pressure observed following a bolus dose of this agonist is surprising. The binding affinity of Ro 60-0175 to 5-HT_{2B} receptors is unpublished for the rat but it has good affinity for human 5-HT_{2B} receptors (see Table 1). Whether this reflects species differences remains to be determined, but there does not appear to be any overt species differences for other 5-HT_2 receptor subtypes (see Table 1). Another explanation could be related to the different methods of drug administration, bolus versus infusion. Urethral smooth muscle constriction may require a high receptor occupancy of 5-HT_{2B} receptors that is achieved on bolus but not infusion dosing, and is further compounded by background constriction of urethral smooth muscle as indicated above. Interestingly, the selective 5-HT_{2B} receptor agonist BW723C86 (Knight et al., 2004) was reported (Mbaki and Ramage, 2008a) not to affect urethral pressure. Thus the role of the 5-HT_{2B} receptor in controlling the urethra needs further investigation. The present study does however demonstrate that it is 5-HT_{2A} not 5-HT_{2C} receptor activation that is responsible for the increase in EUS-EMG evoked by Ro 60-0175 in partially filled bladders.

The failure of Ro 60-0175 to increase blood pressure at the low dose, although causing inhibition of micturition as indicated by the significant increase in volume threshold for the 2nd reflex, supports the view that a plasma concentration of 8 nM Ro 60-0175 is active only at 5-HT_{2C} receptors. Increases in blood pressure along with EUS activation were only observed between the 5th and 6th reflex tests during infusion of the highest dose of Ro 60-0175 (free plasma concentration between 84 and 126 nM). Plasma concentrations between the 1st/2nd reflex and between the 5th/6th were calculated to be equivalent to pKi values of 8.09 and 7.89 respectively indicating that in vivo selectivity is broadly in agreement with human 5-HT_{2C} and 5-HT_{2A} receptor pKi values of 8.22 and 7.44 respectively (Knight et al., 2004). However, Ro 60-0175-induced increase in volume threshold was reversed to a decrease in the presence of SB 242084 at the 2nd reflex tested (plasma concentration 8 nM) suggesting that a 5-HT_{2A} excitatory action was already present. Further, from the 4th reflex tested (plasma concentration 34 nM), decreases in volume threshold induced by Ro 60-0175 alone were reversed to an increase following co-infusion with MDL-100,907. This 5-HT_{2A} excitatory action is believed to be due to increased bladder smooth muscle tone (Mbaki and Ramage, 2008a), and is supported by in vitro data in rat bladder strips (Kodama and Takimoto, 2000). Detection of 5-HT_{2A} mRNA in rat lumbar DRG (Pierce et al., 1996) and therefore potential presence of the receptor on bladder afferent nerve terminals suggests another location for an excitatory action of Ro 60-0175. Therefore peripheral excitation can mask the inhibitory action of Ro 60-0175 on the central control of volume threshold. In spite of the reversal of volume threshold to decrease with increasing concentration of Ro 60-0175, pressure threshold increased at all 6 reflex tests, although

only significant for the 6th reflex tested. The mechanism for this opposing effect on pressure threshold is difficult to explain but does indicate that there has been a decrease in bladder compliance, hence bladder wall tension has increased. This could be caused by decreased sympathetic drive to the bladder (Yoshiyama and de Groat, 2002) as well as the 5-HT_{2A} mediated increase in bladder tone at higher concentrations of the agonist. This sympathoinhibition would be expected to be mediated by 5-HT_{2C} receptors, as 5-HT_{2A} receptor activation causes sympathoexcitation (see Ramage, 2001). In this respect, the increase in pressure threshold was blocked by the high dose of SB 242084.

In the above, it is argued that Ro 60-0175 may have a 5-HT_{2A} agonist action by the 2nd reflex and in this respect blockade of 5-HT_{2A} receptors by MDL-100,907 exposes a significant Ro 60-0175-induced increase in pressure threshold by the 3rd reflex. One surprising action of MDL-100,907 is that by the 2nd reflex when Ro 60-0175 alone causes an increase in volume threshold, in the presence of MDL-100,907 this increase is no longer significant. The overshoot of MDL-100,907 from the target plasma concentration of 3 nM to 22 nM, may be the cause of this, with activation of another receptor subtype. However, the 3 nM plasma concentration of MDL-100,907 has been confirmed to be selective for the 5-HT_{2A} over 5-HT_{2C} receptor as the excitatory action of Ro 60-0175 on volume threshold was blocked, leaving only the 5-HT_{2C}-mediated inhibitory action. Overall, the effects on micturition and blood pressure indicate that the antagonist doses chosen are selective and effective at their target receptors, but caution should be applied when ascribing effects of Ro 60-0175 to 5-HT_{2C} receptors alone.

5. Conclusion

In conclusion, this study presented in anaesthetized female rats demonstrates that activation of 5-HT_{2A} receptors causes EUS excitation and facilitates micturition, while activation of 5-HT_{2C} receptors inhibits micturition, and supports the view that 5-HT_2 receptor subtypes are not physiologically involved in EUS activity or the micturition reflex.

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