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Effects of NS1608, a BK_{Ca} channel agonist, on the contractility of guinea-pig urinary bladder *in vitro*

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1 The functional effects of NS1608 ((*N*-(3-(trifluoromethyl)phenyl)-*N*′-(2-hydroxy-5-chlorophenyl)urea), an opener of the large conductance, Ca^{2+}-activated K^+ (BK_{Ca}) channel, on the contractility of guinea-pig urinary bladder muscle are described.

2 NS1608 (0.3–30 μM) had no significant effect on the integrated myogenic activity (tension integral) or the electrically evoked twitches of detrusor muscle strips. Possible mechanisms for the discrepancy between the lack of functional effects of NS1608 *per se* on detrusor contractility and this drug's agonistic effect on BK_{Ca} currents in isolated bladder myocytes are discussed.

3 4-Aminopyridine (1 mM), a blocker of voltage-gated K^+ (K_V) channels, increased the tension integral 2.7-fold, on average. NS1608 (30 μM) counteracted this effect.

4 Apamin (100 nM), a selective blocker of the small conductance, Ca^{2+}-activated K^+ (SK_{Ca}) channel, increased the tension integral 1.7-fold, on average. This effect was reversed by NS1608 (30 μM).

5 Ryanodine (10 μM), a modulator of the sarcoplasmic reticulum (SR) Ca^{2+}-release channel, increased the tension integral 1.9-fold, on average. This effect was reversed by NS1608 (30 μM).

6 Iberiotoxin (IbTX, 50 nM), a selective blocker of the BK_{Ca} channel, caused additional increases in the tension integral of detrusor strips pretreated with apamin or ryanodine and prevented the inhibitory effects of NS1608 (30 μM) in detrusor contractility.

7 The present study shows that blockade of repolarizing currents carried by, respectively apamin- and 4-aminopyridine-sensitive K^+ channels unmasks an activation of BK_{Ca} in guinea-pig urinary bladder smooth muscle strips.

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Abbreviations: BK_{Ca}, high-conductance Ca^{2+}-activated potassium channel; IbTX, iberiotoxin; K_{ATP} channel, ATP-dependent potassium channel; K_V channels, voltage-gated potassium channels; NS1608, *N*-(3-(trifluoromethyl)phenyl)-*N*′-(2-hydroxy-5-chlorophenyl)urea; SK_{Ca}, small-conductance Ca^{2+}-activated potassium channel; SR, sarcoplasmic reticulum

Introduction

Potassium channels, by virtue of their effects on resting membrane potential and membrane repolarization during the course of action potentials, modulate the excitation–contraction coupling process in smooth muscle fibres. The marked variety of K^+ channels in smooth muscle provides a plethora of targets for therapeutic drug development, which has so far been most successful in relation to the ATP-dependent K^+ (K_{ATP}) channel, openers of which are in current clinical practice. In contrast, agonists of the large conductance, Ca^{2+}-activated K^+ (BK_{Ca}) channel have not yet provided useful therapeutic agents, despite the unequivocal evidence that (i) several small molecules, obtained from plant extracts (McManus *et al*., 1993) and fermentation broths (Singh *et al*., 1994) or by synthetic chemistry (Olesen *et al*., 1994; Hu *et al*., 1997; Li *et al*., 1997; Butera *et al*., 2001), enhance BK_{Ca} channel activity in electrophysiological experiments (reviewed by Garcia & Kaczorowski, 2001); (ii) selective BK_{Ca} antagonists, such as iberiotoxin (IbTX) (Suarez-Kurtz *et al*., 1991) and paxilline (DeFarias *et al*., 1996) markedly affect excitation–contraction (EC) coupling in smooth muscle and (iii) the absence of functional BK_{Ca} currents enhances basal and nerve-mediated contractility of smooth muscle (Meredith *et al*., 2004). In the present study, we examined the effects of the BK_{Ca} channel opener, NS1608 ((*N*-(3-(trifluoromethyl)phenyl)-*N*′-(2-hydroxy-5-chlorophenyl)urea; Olesen, 1994) on the contractility of urinary bladder strips from guinea-pigs. Patch clamp data from vascular and nonvascular smooth muscle cells indicate that NS1608 enhances BK_{Ca} channel activity by increasing the open time and the frequency of openings, and by shifting the midpoint of channel activation to more negative potentials, leading to hyperpolarization of the resting membrane potential (Hu *et al*., 1995; Hu & Kim, 1996; Siemer *et al*., 2000). These effects are reported to occur at micromolar concentrations of NS1608,

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which do not activate voltage-gated (K_V) or ATP-dependent K^+ channels, nor block L-type Ca^{2+} channels (Hu *et al*., 1995; Hu & Kim, 1996; Siemer *et al*., 2000). This selectivity profile led us to make use of NS1608 as a pharmacological tool for exploring the functional role of BK_{Ca} channels in modulating the contractility of bladder smooth muscle.

Methods

Preparations

Experiments were performed at 37°C on isolated urinary bladder strips obtained from adult guinea-pigs. Animals were kept following the precepts of humane care, in rooms with temperature control and light/dark cycle, and were asphyxiated by CO_2 inhalation. For recording muscle tension, strips (2–3 mm width, 6–9 mm length) were mounted vertically between two metal stirrups, of which the lower was fixed and the upper was attached to a rigid wire connected to a force-displacement transducer (Grass FT-03; Grass Instruments Co., Quincy, MA, U.S.A.). Six strips were studied simultaneously. A 1-g load was initially applied to the preparations, as previous studies from our laboratory have shown that this allows stable tension to be recorded for several hours (Suarez-Kurtz *et al*., 1991; 1999). The transducer signals were amplified and recorded on a Grass polygraph (Model 7) and stored on a PC computer, using a DigiData 1200 interface and the software Axoscope (Axon Instruments, Foster City, CA, U.S.A.). Integration of the isometric tension data, performed with Fetchan provided by pClamp 10.1 (Axon Instruments), was used to quantify drug effects on the spontaneous myogenic activity of the bladder segments (Suarez-Kurtz *et al*., 1991). The zero level for integration was set at 5% of the average amplitude of the spontaneous tension oscillations, and 5–10-min periods were integrated at various times during the experiments. The data are expressed relative to the 'basal' tension integral, that is, 45–60 min after the initial equilibration of the preparations with drug-free, physiological saline solution (see below). In one series of experiments, designed to evaluate the effects of NS1608 on electrically evoked twitches, the strips were stimulated with trains (3 s, 1–10 Hz) of square wave pulses of 0.2 ms duration applied *via* platinum ring electrodes. The twitches were blocked by 1 μM tetrodotoxin, and therefore result from stimulation of intramural excitatory neurones.

Solutions and chemicals

The physiological saline solution, a modified Krebs–Henseleit solution, had the following composition (in millimolar): NaCl 120, KCl 5.9, $CaCl_2$ 2.5, $MgCl_2$ 1.1, $NaHCO_3$ 15, NaH_2PO_4 1.2, glucose 11, and HEPES 10. The pH of this solution after equilibration with 95% O_2 and 5% CO_2 was 7.3 at 37°C. Stock solutions of NS1608 (kindly provided by NeuroSearch, Ballerup, Denmark) and ryanodine (Sigma Chemical Co., St Louis, MO, U.S.A.) were prepared in DMSO, and all subsequent dilutions were made in the experimental salines. The highest concentration of DMSO in the bath was 0.2%, which has no effect on the functional parameters under study. Stock solutions of apamin (Sigma) and IbTX (Peninsula Laboratories, Belmont, CA, U.S.A.) were made in 100 mM NaCl solution containing 0.1% bovine serum albumin, whereas 4-aminopyridine (Sigma) was stored in aqueous solution.

Experimental protocol

After 45–60 min equilibration in PSS, the detrusor strips were submitted to one of three experimental protocols: (i) exposure to NS1608 for 60 min, in the experiments designed to quantify the effects of this drug *per se* on the tension integral and on the electrically induced twitches; (ii) exposure to 4-aminopyridine, apamin or ryanodine for 30 min, prior to addition of NS1608 to the medium for 60 min, in the experiments designed to explore the pharmacological interaction of NS1608 with each one of these drugs; (iii) 30-min exposure to apamin or ryanodine, followed by addition of IbTX, and after 30 min, addition of NS1608 for 60 min, in the experiments designed to explore the influence of IbTX on the interaction of NS1608 with apamin and ryanodine. Time control experiments, in which the strips were not exposed to NS1608, were performed for each protocol. The reported drug effects on tension integral refer to measurements taken after 30-min exposure to apamin, 4-aminopyridine or ryanodine, after 30-min exposure to apamin or ryanodine plus IbTX and/or after 60 min exposure to NS1608, in the absence or the presence of the other drugs. Pooled data from n identical experiments are presented as means $\pm$ s.d. Comparison of the tension integral values relative to different drug treatments was performed using two-way ANOVA followed by the Student–Newman–Keuls test, using the software package SPlus-5 (Mathsoft, Seattle, WA, U.S.A.). The level of significance was set at $P<0.05$.

Results

Effects of NS1608 on the contractility of urinary bladder strips

NS1608 (0.3–30 μM, 60 min exposures) had no significant effect on the integrated myogenic activity (tension integral) or the electrically evoked twitches of urinary bladder strips (Figure 1a–c). For comparison, under identical experimental conditions, the K_{ATP} channel agonist, cromakalin (10 μM, $N=5$) abolished the spontaneous motility and reduced the amplitude of the electrically evoked twitches (10 Hz) by $66\pm15\%$. The apparent discrepancy between the lack of effects of NS1608 on detrusor contractility and the electrophysiological evidence (Hu & Kim, 1996) that this drug stimulates BK_{Ca} currents in urinary bladder myocytes led us to explore experimental variables that might occlude the functional consequences of the stimulatory effect of NS1608 on the BK_{Ca} channel.

Effects of NS1608 on urinary bladder strips pretreated with 4-aminopyridine or apamin

Initially, we explored the possibility that parallel repolarizing currents through small-conductance Ca^{2+}-activated K^+ channels (SK_{Ca}) and/or K_V channels present in the detrusor muscle could indirectly affect the contractile response to NS1608. The experimental protocol consisted of exposing the strips to either 4-aminopyridine – a blocker of K_V channels – or apamin – a

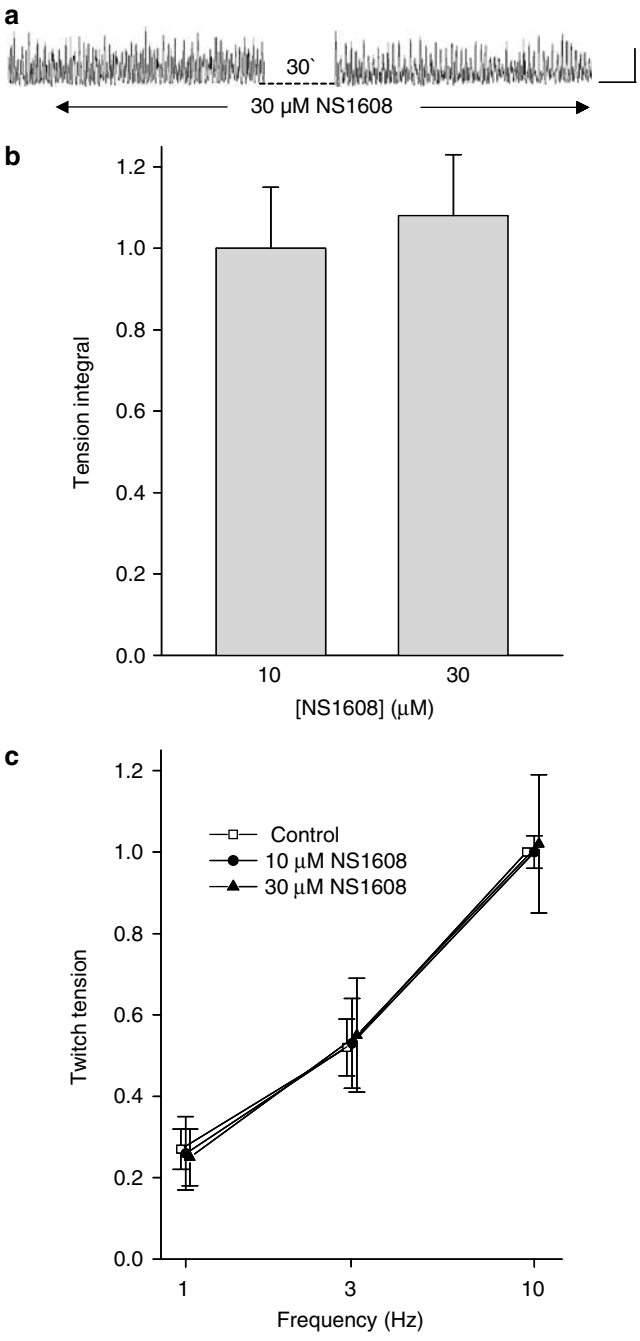

Figure 1 Effects of NS1608 on the contractility of guinea-pig detrusor strips. (a) Representative isometric tension recordings from a strip exposed to 30 μM NS1608 for 60 min. Calibration bars: horizontal, 3 min; vertical, 1 g. (b, c) Concentration–response plots for the effects of 10 and 30 μM NS1608 on the tension integral and on the amplitude of electrically evoked twitches. Each data point is derived from 8–10 experiments. The data are normalized to the control (predrug) values of the tension integral (b) or the amplitude of twitches elicited at 10 Hz (c), and are expressed as means $\pm$ s.d.

selective blocker of the SK_{Ca} channel – for 30 min before and also during 60-min exposures to 1–30 μM NS1608.

Figure 2a shows recordings from a representative experiment in a 4-aminopyridine-treated bladder strip, and pooled data from 16 similar experiments are shown in Table 1. The tension integral was significantly greater after 30-min exposure to 4-aminopyridine (1 mM), as compared to the control values. This effect persisted during the subsequent 60 min in the time-control experiments (not shown). Addition of 1–10 mM NS1608 to the medium between 30 and 90 min of exposure to 4-aminopyridine had no effect on the tension integral, whereas 30 μM NS1608 significantly decreased the tension integral, practically reversing the effect of 4-aminopyridine (Table 1).

Tension recordings from a representative experiment with apamin (100 nM) are reproduced in Figure 2b, and pooled data from 15 similar experiments are shown in Table 2. Apamin caused a significant increase of the tension integral, which persisted throughout the 90 min observation period in the time-control experiments, and was not affected by addition of 1–10 μM NS1608 to the medium between 30 and 90 min (not shown). In contrast, 30 μM NS1608 completely reversed the effect of apamin on the tension integral (Table 2).

Effects of NS1608 on urinary bladder strips pretreated with ryanodine

The evidence that Ca^{2+} release from the sarcoplasmic reticulum (SR) through ryanodine-sensitive channels modulates the activity of BK_{Ca} and SK_{Ca} channels in bladder urinary muscle (Imaizumi *et al*., 1998; Herrera *et al*., 2000) led us to investigate the pharmacological interaction between ryanodine and NS1608. The tension integral of detrusor muscle strips increased significantly throughout 90-min exposure to 10 μM ryanodine (Figure 2c, Table 3). Addition of 1–10 μM NS1608 to the medium between 30 and 90 min had no effect on the tension integral (not shown), whereas a significant reduction was observed in the presence of 30 μM NS1608 (Figure 2c, Table 3).

IbTX prevents the relaxing effects of NS1608 in apamin- or ryanodine-treated urinary bladder strips

The highly selective BK_{Ca} channel blocker, IbTX was used to explore the functional role of these channels in the pharmacological interaction between NS1608 and either apamin or ryanodine. IbTX (50 nM) increased markedly the tension integral of detrusor strips pretreated with apamin (Figure 3a, Table 2) or ryanodine (Figure 3b, Table 3). As previously shown by Suarez-Kurtz *et al*. (1991), this effect persisted throughout the 90-min exposure to IbTX, in time-control experiments. In the continuous presence of IbTX and either apamin or ryanodine, NS1608 (30 μM) had no significant effect on the tension integral of detrusor muscle strips (Tables 2, 3).

Discussion

The ability of NS1608 to activate BK_{Ca} channels in urinary bladder myocytes from various species has been convincingly demonstrated (Hu & Kim, 1996; Siemer *et al*., 2000). In guinea-pig bladder myocytes, the concentration–response curve for the stimulatory effect of NS1608 on BK_{Ca} was bell shaped, with a minimum effective concentration of 0.5 μM and maximum effects at 5–10 μM. At higher concentrations, NS1608 caused transient activation, followed by a time-dependent reduction of the BK_{Ca} currents. These electrophysiological results contrast with the lack of functional effects of NS1608 *per se* on the contractility of guinea-pig detrusor

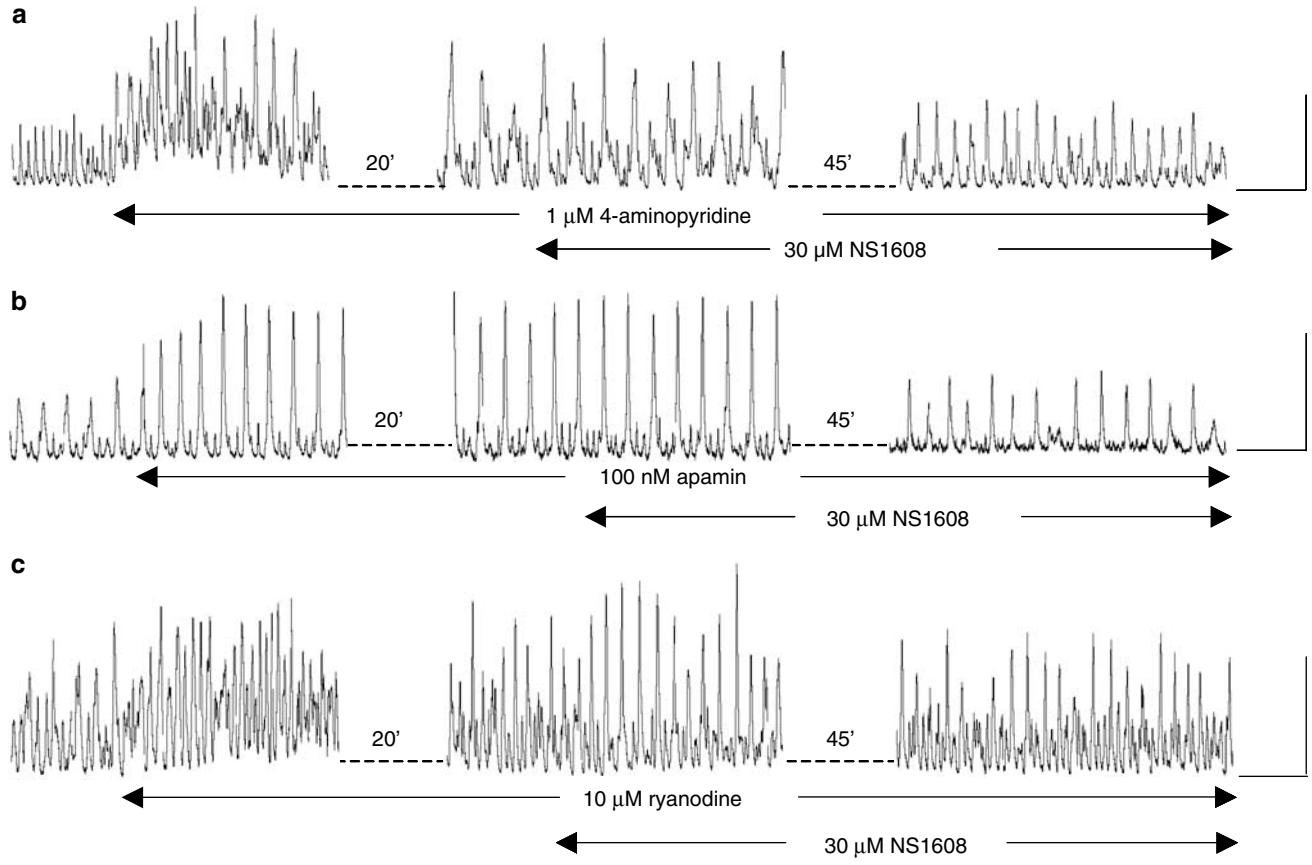

Figure 2 Pharmacological interaction between NS1608 and 4-aminopyridine, apamin or ryanodine on the contractility of guinea-pig detrusor strips. (a–c) Representative tension recordings from strips exposed to 1 mM 4-aminopyridine (4-AP, panel a), 100 nM apamin (b) or 10 μM ryanodine (c) for 30 min followed by addition of 30 μM NS1608 to the bathing media for 60 min. Calibration bars: horizontal, 2 min; vertical, 1 g.

Table 1 Effects of 4-aminopyridine, NS1608 and IbTX on the tension integral of guinea-pig detrusor muscle

	n	*Tension integral (mean±s.d.)*
Control	11	1.00±0
4-Aminopyridine 1 mM	11	2.57±0.68
4-Aminopyridine + NS1608 10 μM	8	2.37±0.78
4-Aminopyridine + NS1608 30 μM	8	1.41±0.80

**: NS; *: NS; NS

n = number of experiments; *$P<0.05$, **$P<0.01$, pairwise comparison, Student–Newman–Keuls test.

Table 2 Effects of apamin, NS1608 and IbTX on the tension integral of guinea-pig detrusor muscle

	n	*Tension integral (mean±s.d.)*
Control	22	1.00±0
Apamin 100 nM	22	1.67±0.55
Apamin + NS1608 30 μM	15	1.13±0.53
Apamin + IbTX 50 nM	7	2.61±0.79
Apamin + IbTX + NS1608 30 μM	7	2.92 ±1.45

**: *; NS; NS; *

n = number of experiments; *$P<0.05$, **$P<0.01$,

Table 3 Effects of ryanodine, NS1608 and IbTX on the tension integral of guinea-pig detrusor muscle

	n	*Tension integral (mean±s.d.)*
Control	22	1.00±0
Ryanodine 10 μM	22	1.97±0.87
Ryanodine + NS1608 30 μM	15	0.93±0.41
Ryanodine + IbTX 50 nM	7	4.49±2.02
Ryanodine + IbTX + NS1608 30 μM	7	4.56 ±2.82

**: **; NS; *; NS

n = number of experiments; *$P<0.05$, **$P<0.01$, pairwise comparison, Student–Newman–Keuls test.

strips, observed in the present study. Thus, exposure to 0.3–30 μM NS1608 for 60 min periods had no effect on the tension integral or the electrically evoked twitches of detrusor muscle strips. This apparent discrepancy might result from the vastly different experimental conditions in which the patch clamp and the tension results were obtained. For example, the low solubility of NS1608 in aqueous media led us to limit the drug's maximal concentration in the bathing medium to 30 μM, to avoid spurious influence of the solvent, DMSO. Although low micromolar concentrations of NS1608 cause significant stimulation of BK_{Ca} in isolated myocytes (Hu & Kim, 1996; Siemer *et al*., 2000), it is possible that, due to diffusion barriers in the detrusor strip preparation, the concentrations reached at

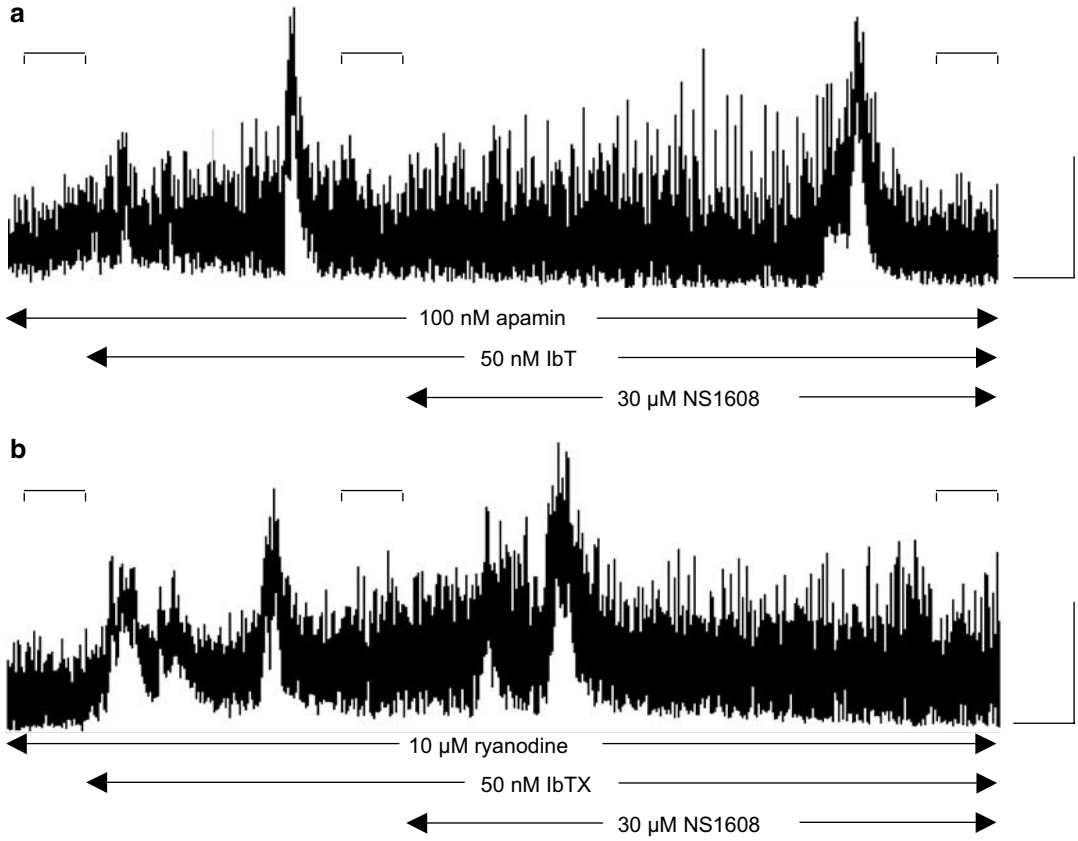

Figure 3 Effects of IbTX on the functional interaction between NS1608 and apamin or ryanodine in guinea-pig detrusor strips. (a, b) Tension recordings from two strips, which, at the beginning of the tracings, had been exposed to either 100 nM apamin (a) or 10 μM ryanodine (b) for 25 min. IbTX (50 nM) was added to the bathing media for 30 min, followed by addition of NS1608 for 60 min. The brackets indicate the times when the tension integrals were calculated. Calibration bars: horizontal, 5 min; vertical, 1 g.

the drug's postulated side of action are insufficient for functional stimulation of the BK_{Ca} currents. In addition, two alternative, but not mutually exclusive possibilities might account for the lack of functional effects of NS1608 in guinea-pig detrusor contractility. The first relates to the membrane potential range in which the electrophysiological effects of NS1608 occur: The published patch clamp data from guinea-pig bladder myocytes (Hu & Kim, 1996) show that the stimulatory effects of NS1608 on BK_{Ca} currents are only evident at inside-positive membrane potential. Extrapolation of these data to the contour of the action potentials recorded from guinea-pig bladder myocytes, which overshoot by ca. 20 mV (Klöckner & Isenberg, 1985; Heppner *et al*., 1997), suggests that there is a limited time and membrane potential window for the occurrence of the NS1608-induced activation of BK_{Ca}, which might not translate into functional contractile effects. This interpretation might also apply to other BK_{Ca} channel openers that are effective at inside-positive membrane potentials (Hu *et al*., 1997; Butera *et al*., 2001). However, we observed no effect of NS1608 (10 μM) on the tension integral of rat detrusor muscle (Mora & Suarez-Kurtz, unpublished observations), whereas in rat bladder myocytes, the same concentration of NS1608 shifted the activation voltage of BK_{Ca} channels by $\sim$100 mV towards more negative potentials (Siemer *et al*., 2000).

Regarding the second possibility, Herrera *et al*. (2000) suggested that the membrane depolarization and the subsequent elevation of intracellular Ca^{2+} concentration during the action potential of guinea-pig detrusor myocytes are sufficient to cause a significant activation of BK_{Ca} currents, a notion which is consistent with the large increase in contractility induced by selective BK_{Ca} blockers (Suarez-Kurtz *et al*., 1991; DeFarias *et al*., 1996) and by deletion of the gene (*mSlo1*) for the pore-forming subunit of the BK_{Ca} channel (Meredith *et al*., 2004). Thus, it is plausible that the additional stimulation induced by BK_{Ca} channel openers, such as NS1608, is insufficient to affect the excitation-coupling process in functional experiments, when other repolarizing K^+ currents are operative. Accordingly, blockade of currents through SK_{Ca} channels or K_V channels by apamin or 4-aminopyridine, respectively, increased the contractility of detrusor muscle (Fujii *et al*., 1990; Herrera *et al*., 2000; Imai *et al*., 2001) and uncovered inhibitory effects of NS1608 on the tension integral. Although increased neurotransmitter release from intramural neurons (Hanani & Maudlej, 1995) might contribute to the contractile effects of 4-aminopyridine, it is significant that a neurogenic blocking cocktail did not prevent the striking contractile response of rabbit detrusor strips to 3-4-diaminopyridine (Davies *et al*., 2002), which points to the smooth muscle fibres as the major target underlying this response.

Relaxing effects of NS1608 were also observed in ryanodine-treated detrusor muscle. Ryanodine *per se* increased the spontaneous myogenic activity, which is consistent with a previous report by Herrera *et al*. (2000). These authors ascribed the contractile effects of ryanodine to the operation of complex negative-feedback loops involving the ryanodine-sensitive

SR-Ca^{2+}-release channel and the Ca^{2+}-activated channels BK_{Ca} and SK_{Ca}, such that the major role of the SR Ca^{2+}-release is to decrease contraction, *via* stimulation of BK_{Ca} and SK_{Ca} in the muscle cell membrane. The intrinsic mechanism whereby ryanodine unmasks the relaxant effects of NS1608 cannot be deduced from our experiments, but it is conceivable that relative changes in the activity of BK_{Ca} and SK_{Ca} channels following blockade of the ryanodine-sensitive channels are involved.

The relaxant effects of NS1608 in detrusor strips pretreated with apamin- or ryanodime-treated were completely abolished by IbTX, a specific blocker of the BK_{Ca} channel (Galvez *et al*., 1990). These results provide strong support for the notion that NS1608 relaxes urinary bladder detrusor and other smooth muscles by acting as a BK_{Ca} channel opener. Nevertheless, the possibility that ancillary effects on Ca^{2+} and/or Cl^{-} channel activity might contribute to the relaxant effects of 30 μM NS1608 in detrusor strips treated with apamin, 4-aminopyridine or ryanodine cannot be excluded, in view of previous results with other, chemically related BK_{Ca} openers (Edwards *et al*., 1994; Sheldon *et al*., 1997).

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