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NS 1619 activates Ca^{2+}-activated K^+ currents in rat vas deferens

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Abstract

The effects of NS 1619, a newly developed activator of large-conductance Ca^{2+}-activated K^+ channels, were investigated on single smooth muscle fibers dissociated enzymatically from rat vas deferens and on contractions of the epididymal half of vas deferens. K^+ currents were recorded using whole-cell patch-clamp methods in near-physiological K^+ solutions (5.4 mM extracellular K^+/145 mM intracellular K^+). When cell membrane voltage was stepped to test potentials (−60 to +60 mV) from a holding potential of −10 mV, NS 1619 increased the outwardly rectifying K^+ current in a concentration-dependent manner. The increased portion of the K^+ current by NS 1619 was totally abolished by charybdotoxin (100 nM) but not by glibenclamide (3 μM). NS 1619 reduced electrically stimulated contractile responses of rat vas deferens in a concentration-dependent manner, and charybdotoxin but not glibenclamide partially inhibited the effect of NS 1619. NS 1619 (50 μM) inhibited the noradrenaline-induced contraction. Charybdotoxin (100 nM) partially reduced the NS 1619-induced inhibition while glibenclamide (3 μM) had no effect. NS 1619 (10–100 μM) reduced the high K^+-induced contractions in a noncompetitive manner. The present results indicate that NS 1619 activates charybdotoxin-sensitive Ca^{2+}-activated K^+ channels and probably inhibits Ca^{2+} influx. These two effects might account largely for the observed mechanical inhibition induced by NS 1619 in the epididymal half of isolated rat vas deferens. © 1997 Elsevier Science B.V.

Keywords: NS 1619; Charybdotoxin; Glibenclamide; K^+ channel, Ca^{2+}-activated; Ca^{2+} channel; Vas deferens, rat

1. Introduction

K^+ channel openers such as cromakalim, pinacidil and aprikalim are a group of smooth muscle relaxants. These agents are reported to open ATP-sensitive K^+ channels and ensuing membrane hyperpolarization usually inhibits the activity of voltage-sensitive Ca^{2+} channels (Standen et al., 1989; Edwards and Weston, 1993). More recently, a new class of the benzimidalone derivatives, typified by NS 1619, NS 004 and NS 1608, which also possess smooth muscle relaxant action, has been identified (Olesen and Wätjen, 1992; Olesen et al., 1994a,b). These agents exert their relaxant effects by opening Ca^{2+}-activated K^+ (K_{Ca}) channels. In cell-free patches, NS 004 and NS 1619 directly activate the large-conductance K_{Ca} (BK_{Ca}) channels in cultured bovine tracheal smooth muscle (Macmillan et al., 1995), porcine coronary arterial cells (Hu et al., 1995), and rat cerebral artery smooth muscle (Holland et al., 1996). Charybdotoxin or iberiotoxin inhibits the stimulatory effect of NS 1619 on BK_{Ca} channels in vascular smooth muscle of rat portal vein and cerebral artery (Edwards et al., 1994; Holland et al., 1996). In addition, the inhibitory effects of NS 004 and NS 1619 on whole-cell Ca^{2+} currents have been reported in rat ventricular myocytes (Sargent et al., 1993) and rat vascular smooth muscle (Edwards et al., 1994; Holland et al., 1996). These observations indicate that the benzimidalone derivatives might act at multiple sites to cause smooth muscle relaxation. The aim of the present study was to investigate the effect of NS 1619 on whole-cell K^+ currents in single smooth muscle fibers isolated from the epididymal half of rat vas deferens, on the contractile responses to electric field stimulation, to exogenous noradrenaline and to high extracellular K^+.

2. Materials and methods

2.1. Preparation

Male Sprague-Dawley rats of approximately 300 g were killed by cervical dislocation. The epididymal half of the vas deferens was dissected out and cleaned of the visible blood vessels and adhering connective tissues. The prepa-

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rations were kept in Krebs-Henseleit solution of the following compositions (mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1, $NaHCO_3$ 25, KH_2PO_4 1.2, Na_2-EGTA 0.03, D-glucose 11.1, ascorbic acid 0.2. Single smooth muscle cells were isolated from the epididymal vas deferens by enzymatic digestion. Briefly, the epithelial sheet of vas deferens was mechanically removed and the tissue was cut into small pieces and placed for 5 min in the oxygenated isolation medium containing (mM): NaCl 110, $MgCl_2$ 2, KCl 5, KH_2PO_4 0.5, NaH_2PO_4 0.5, $NaHCO_3$ 10, $CaCl_2$ 0.16, EGTA 0.49, D-glucose 10, taurine 10, HEPES 10 (pH 7.0 adjusted with NaOH). Tissues were then incubated at 37°C in isolation solution containing 2 mg/ml collagenase, 0.5 mg/ml elastase, 1 mg/ml papain and 100 μM DL-dithiothreitol for about 90 min after which time they were washed with isolation medium to remove digestive enzymes. Single smooth muscle cells were released into Petri dishes by gentle agitation. The dispersed cells were used for experiments within 6 h during which time they were kept at 4°C in isolation solution.

2.2. Whole-cell clamp experiments

Whole-cell K^+ currents in vas deferens smooth muscle were recorded using the voltage-clamp technique (Hamill et al., 1981). Patch pipettes were prepared from 1.5 mm O.D. borosilicate glass capillaries on a micropipette puller (Sutter Instrument), coated with Sylgard elastomer, and fire-polished. The pipettes were filled with electrolyte and their tip resistance ranged between 2–4 MΩ. All recordings were made using an Axopatch-1D amplifier (Axon Instruments, Foster City, CA, USA). The junction potential between electrodes and the bath solution was electronically compensated using the DC offset on the amplifier. About 80% compensation of the series resistance (3.5 MΩ) could be achieved. Leak current was subtracted. Cell capacitance was between 20–25 pF. K^+ current was filtered at 1 kHz and sampled at 5 kHz, and data were analyzed with pClamp software (version 6.0, Axon Instruments).

The whole-cell currents were induced by step voltage pulses (from −60 to +60 mV, 400 ms in duration) and remained stable for 30–40 min, within the time required to complete the experiments. Cells were held at −10 mV in order to inactivate the delayed rectifier current and voltage-sensitive K^+ current. The bath solution contained (mM): NaCl 140, KCl 5.4, $MgCl_2$ 1, HEPES 10, EGTA 0.3, D-glucose 10, pH 7.2 adjusted with NaOH. The high-K^+ intracellular solution contained (mM): KCl 135, $MgCl_2$ 1, $CaCl_2$ 0.4, HEPES 10, EGTA 5.5, pH 7.2 adjusted with KOH (about 145 mM K^+), which gave a free internal Ca^{2+} concentration of approximately 100 nM (calculated by software EQCAL). All experiments were performed at room temperature (about 22°C). The amplitude of the steady-state K^+ currents was measured as the mean value of data points near the end of each command voltage. The cells were continuously superfused at 0.5 ml/min using a minipump and each drug was added to the reservoir connected to the perfusion system.

2.3. Organ bath experiments

Segments of the vasal duct of about 2 cm in length were placed in organ baths with one end attached to myograph force-displacement transducers (Grass Instrument). The bath solution was constantly oxygenated with 95% O_2/5% CO_2. Tissues were allowed to equilibrate under 0.5 g initial tension for 1.5 h during which time the bath medium was changed every 30 min. All experiments were conducted at 37 ± 1°C. Electric field stimulation was repetitively delivered to the preparations with use of Grass SD9 Stimulator via two platinum electrodes (0.2 Hz, 0.5 ms pulse duration, 60 V). The electrically stimulated monophasic contractions were abolished by 3 μM tetrodotoxin or by combination of 10 μM phentolamine and 3 μM α,β-methylene ATP (30 min contact time), indicating the neurogenic origin of the contraction. NS 1619 was added cumulatively to the bath to induce concentration-dependent inhibition of the evoked contractions in the absence and presence of K^+ channel blockers. The mean amplitude of five consecutive twitches was calculated at the start of application of the next dose. In the second set of experiments, biphasic contractions were evoked by a train stimulation for 10 s (0.5 ms pulse duration, 40 V) with varying frequency (1–16 Hz). The effect of NS 1619 on both purinergic and adrenergic components was examined. NS 1619 at each concentration was applied 10 min before delivery of electric stimulation.

In the last series of experiments, the preparations were contracted with noradrenaline (30 nM to 30 μM) or external K^+ (5 to 80 mM) to construct the first concentration–response curves. Once the maximum response to each agonist had been reached, tissues were rinsed with Krebs solution every 20 min until the tension fell to the basal level. Tissues were then equilibrated with different concentrations of NS 1619 for 10 min and another concentration–response curve to noradrenaline or external K^+ was repeated. The high K^+-induced contraction was unaffected by 10 μM phentolamine, indicating noradrenergic nerve terminals were not involved in the response. In experiments using high K^+ solution, the equimolar amount of Na^+ was replaced by K^+ to maintain a constant ion strength. Results were expressed as a percentage of the maximal response at 60 mM K^+ in control.

2.4. Chemicals

The following chemicals and drugs were used: NS 1619, phentolamine mesylate (Research Biochemicals International, Natick, MA, USA), tetrodotoxin, charybdotoxin, glibenclamide, (−)-noradrenaline bitartrate, α,β-methylene ATP, collagenase type II, elastase, papain, DL-dithiothreitol (Sigma, St. Louis, MO, USA). All drugs

were dissolved in Krebs solution except for NS 1619 and glibenclamide in dimethyl sulfoxide (0.2%).

2.5. Statistics

Results were expressed as mean ± S.E.M from *n* experiments. Cumulative concentration–response relationships were analyzed with a non-linear curve fitting by a logistic equation (Grafit, Erithacuc Software). Student's *t*-test was used to evaluate the significant difference between mean values ($P < 0.05$).

3. Results

3.1. Activation of outward K^+ current by NS 1619

The whole-cell K^+ currents were recorded in single smooth muscle fibers freshly isolated from rat vas deferens under near-physiological K^+ concentrations (5.4 mM K^+ outside and 145 mM K^+ inside) in Ca^{2+}-free bath solution. The major currents were outwardly rectifying non-inactivating currents elicited by 400-ms test pulses (−60 mV to +60 mV) at a holding potential of −10 mV. This clamped potential would minimize the involvement of other voltage- and time-dependent K^+ currents (Edwards et al., 1994; Holland et al., 1996). The outward current was slightly reduced by 100 nM charybdotoxin in two out of five recordings. Exposure to NS 1619 immediately increased the level of outward currents with a maximal effect within 2–3 min in a concentration-dependent fashion (Fig. 1). NS 1619 (10 μM) failed to enhance the outward K^+ current in the absence of intracellular Ca^{2+} (zero Ca^{2+}, 3 mM Na_2EGTA added in the pipette solution, $n = 3$). Fig. 1D shows current-voltage relationships for 0, 1, 3 and 10 μM NS 1619, respectively. Fig. 2A shows that 3 μM NS 1619 significantly enhanced the outward currents from the control values (Fig. 2A). Glibenclamide (3 μM, 5 min contact time) did not significantly affect the amplitude of the current (Fig. 2C), whilst charybdotoxin (100 nM, 5 min contact time) totally reversed the stimulatory effect of NS 1619 (Fig. 2D). The

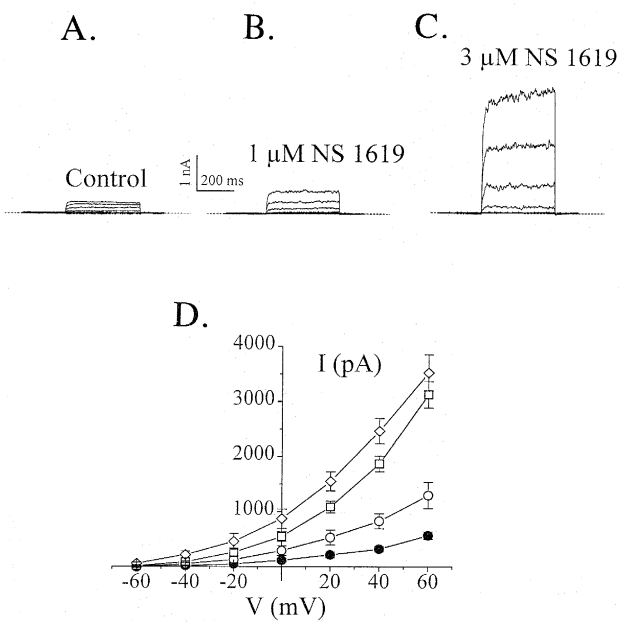

Fig. 1. Concentration-dependent effect of NS 1619 on whole-cell outward current. The pipette solution contained approximately 100 nM free Ca^{2+}. The outward currents were induced by step-voltage pulses (−60 to +60 mV) from a holding potential of −10 mV in control (A) and in the presence of 1 μM NS 1619 (B) and 3 μM NS 1619 (C). Dashed line indicates the zero current level. (D) Current–voltage relationships in the absence (●) and presence of NS 1619 (○, 1 μM; □, 3 μM; ◇, 10 μM). The recorded current displays strong outward rectification. Values are mean ± S.E.M. from 5–8 experiments.

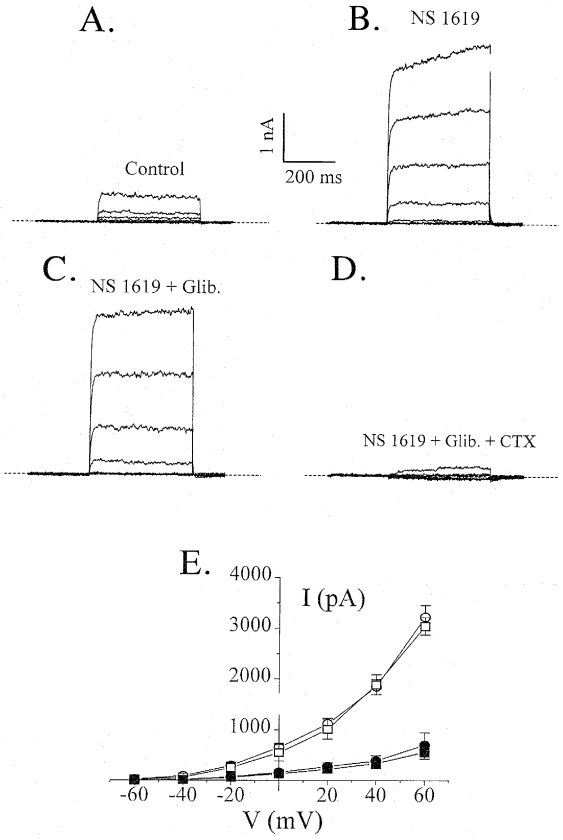

Fig. 2. Effect of glibenclamide and charybdotoxin (CTX) on the NS 1619-activated outward currents. The cell membrane was held at −10 mV and pulsed from −60 to +60 mV. (A) Control outwardly rectifying current recorded with pipette solution containing 100 nM Ca^{2+}. (B) 3 μM NS 1619 immediately increased this current, an effect which was particularly striking at test pulses more depolarized than 0 mV. (C) Subsequent exposure to 3 μM glibenclamide (Glib.) only slightly reduced the current. (D) Application of 100 nM CTX completely abolished the NS 1619-induced increase of the outward current. Dashed line indicates the zero current level. (E) Current-voltage relationships were plotted in the control (●) and in the presence of K^+ channel regulators (3 μM NS 1619, ○; NS 1619 + 3 μM glibenclamide, □; NS 1619 + glibenclamide + 100 nM CTX, ■). Values are mean ± S.E.M. from 5 experiments.

current-voltage relationship under various pharmacological manipulations is shown in Fig. 2E. The stimulatory effect of NS 1619 can be partially (about 70%) reversed after a 15 min washing period.

3.2. Effect of NS 1619 on electrically stimulated contractile response

Monophasic contractions of the epididymal half of rat vas deferens were evoked by repetitive electric field stimulation (0.2 Hz, 0.5 ms pulse duration, 60 V). Traces in Fig. 3A show that NS 1619 concentration-dependently reduced the amplitude of evoked contractions with an IC_{50} of 52.7 ± 5.8 μM ($n = 9$, Fig. 3A). 300 μM NS 1619 reduced contractions by approximately 85%. Charybdotoxin significantly shifted the concentration–response curve for the inhibitory effect of NS 1619 to the right (IC_{50} of 101.5 ± 9.3 μM in 100 nM charybdotoxin and 138.8 ± 9.4 μM in 200 nM charybdotoxin, respectively, $n = 6$, Fig. 3B). Charybdotoxin by itself enhanced the electrically stimulated contractions, therefore, the strength of stimulation was lowered to between 30 and 40 V in an attempt to match the initial levels of contractions in the absence of charybdotoxin, whereas glibenclamide (10 μM) did not alter the NS 1619-induced inhibition of electrically stimulated contractions (IC_{50} of 46.6 ± 7.1 μM, $n = 5$).

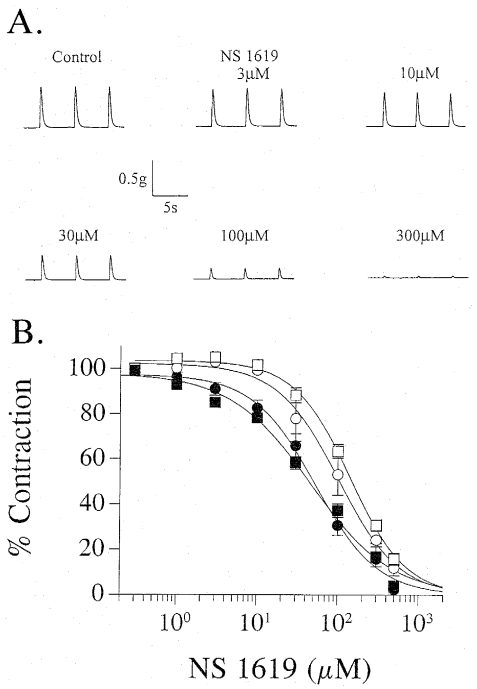

Fig. 3. Effect of NS 1619 on electrically stimulated contractions. (A) Monophasic contractile response was evoked by repetitive electric field stimulation (0.2 Hz, 0.5 ms pulse duration, 60 V). NS 1619 concentration-dependently reduced the amplitude of evoked contractions. NS 1619 was added cumulatively. Calibration bars apply to all traces. (B) Charybdotoxin partially reduced the inhibitory effect of NS 1619 (●, $n = 9$, in control; ○, $n = 6$, in 100 nM; □, $n = 6$, in 200 nM charybdotoxin) while glibenclamide (10 μM) was without effect (■, $n = 5$). Each of the K^+ channel blockers was added 10 min before application of NS 1619. Results were expressed as percentage of control values and curves were drawn by fitting the data point to a logistic equation to give respective IC_{50} values.

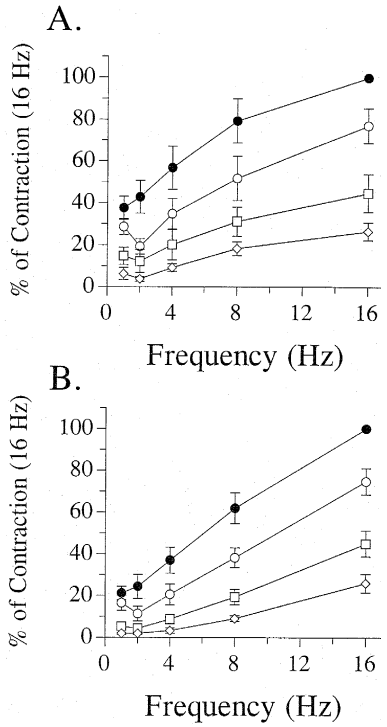

Fig. 4. Effect of NS 1619 on frequency-dependent contractions evoked by field stimulation. Biphasic contractile responses were evoked by field stimulation (0.5 ms pulse duration, 40 V, 10 s) with varying frequency (1–16 Hz). The peak amplitude of the first purinergic (A) and the following adrenergic (B) components was measured in the absence (●, $n = 5$) and presence of NS 1619 (○, 10 μM, $n = 4$; □, 50 μM, $n = 5$; ◇, 100 μM, $n = 5$). Results were expressed as a percentage of the contractile response evoked by 16 Hz. Curves were drawn by joining adjacent data points. Values are mean ± S.E.M. from n experiments.

In another group of experiments, the vas deferens when electrically stimulated (1–16 Hz, 0.5 ms pulse duration, 40 V, 10 s), produced an initial purinergic contraction followed by a sustained adrenergic contraction. NS 1619 (10–100 μM) inhibited the frequency-dependent contractile responses (both purinergic and adrenergic components) in a concentration-dependent manner (Fig. 4A and B).

3.3. Effect of NS 1619 on the agonist-induced contractile responses

Noradrenaline caused contractions of rat vas deferens consisting of intermittent spikes superimposed upon a tonic component with an EC_{50} value of 0.59 ± 0.06 μM ($n = 7$) and a maximal increase in tension of 1.02 ± 0.06 g ($n = 7$, measured as the mean height of intermittent spikes in the presence of 10 μM noradrenaline). NS 1619 did not affect the basal tension at concentrations of up to 100 μM. Following construction of a control concentration–re-

sponse curve for noradrenaline, the preparations were incubated for 10 min with 50 μM NS 1619 and another concentration–response curve for noradrenaline was obtained. Fig. 5 shows that NS 1619 caused an insurmountable inhibition of noradrenaline-induced tension. Neither charybdotoxin (100 nM) nor glibenclamide (3 μM) affected the noradrenaline-induced contractile responses. Charybdotoxin significantly reduced the inhibitory effect of NS 1619 on contractions induced by noradrenaline at concentrations greater than 0.3 μM (Fig. 5A) when applied 10 min before addition of NS 1619. In contrast, glibenclamide had no effect (Fig. 5B).

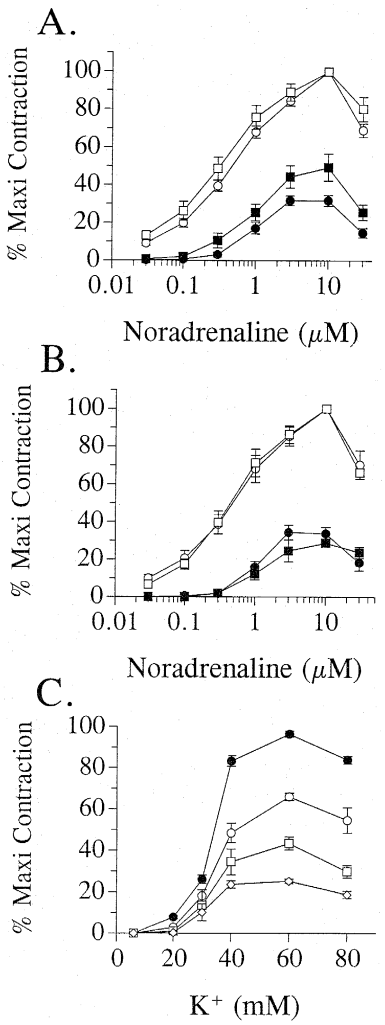

Fig. 5. Inhibitory effects of NS 1619 on the noradrenaline- and high K^+-induced contractions. Concentration-response curves for noradrenaline ranging from 10 nM to 30 μM were obtained under different manipulations. Symbols represent: ○, the first dose–response curve in control, $n = 7$; ●, for the second dose–response curve in 50 μM NS 1619, $n = 7$; □, the first dose–response curve in 100 nM charybdotoxin, $n = 6$ (A) or in 3 μM glibenclamide, $n = 4$ (B); ■, the second dose–response curve in charybdotoxin + NS 1619, $n = 6$ (A) or in glibenclamide + NS 1619, $n = 4$ (B). (C) Concentration–response curve for KCl in the absence (●, $n = 13$) and presence of NS 1619 (○, 10 μM, $n = 4$; □, 30 μM, $n = 5$; ◇, 100 μM, $n = 4$). Drug was incubated for 10 min before repeating the second concentration-response curve. Results were expressed as a percentage of the maximal response obtained in the first (control) dose-response curve. Curves are drawn by connecting the adjacent points. Values are mean ± S.E.M. from n experiments.

Since charybdotoxin only partially inhibited the NS 1619-induced inhibition of either electrically stimulated or noradrenaline-induced contractions, it is possible that NS 1619 might also interact with other sites, therefore, its effect on contractions induced by various concentrations of external K^+ was examined. The magnitude of tension development corresponded with a graded increase in K^+ (5 to 80 mM) in the bath solution. Fig. 5C shows that NS 1619 (10–100 μM) non-competitively inhibited the high K^+-induced contraction.

4. Discussion

In this study, the effects of NS 1619 on whole-cell current, electric stimulation-, noradrenaline- and high K^+-evoked contractions in the epididymal half of rat vas deferens were examined. The cell membrane was clamped at −10 mV to minimize involvement of voltage-sensitive and time-dependent K^+ currents and to optimize the condition for detection of K_{Ca} current under near-physiological K^+ concentrations. Control currents were slightly reduced by charybdotoxin. Exposure of NS 1619 to single smooth muscle fibers markedly enhanced the outwardly rectifying K^+ currents in a concentration-dependent manner. The effect observed is in agreement with similar activator effects of NS 1619 and other benzimidalone derivatives such as NS 004 and NS 1608 on K_{Ca} channels recorded from smooth muscle cells of rat portal veins (Edwards et al., 1994), porcine coronary artery (Hu et al., 1995), rat cerebral artery (Holland et al., 1996) and from cultured bovine aortic smooth muscle (Olesen et al., 1994a). NS 1619 has been recently found to activate single BK_{Ca} channel activity in rat hypothalamic and cortical neurons (Sellers and Ashford, 1994; Lee et al., 1995) and in smooth muscles (Macmillan et al., 1995; Holland et al., 1996). The NS 1619-induced increase of outward current is likely due to its activation of K_{Ca} channels since this increase was entirely reversed by charybdotoxin, a selective blocker of BK_{Ca} channels (Miller et al., 1985). Furthermore, NS 1619 did not affect the outward K^+ currents when recordings were made in the absence of intracellular Ca^{2+}. By contrast, glibenclamide, the selective ATP-sensitive K^+ channel blocker in smooth muscle (Standen et al., 1989; Quast and Cook, 1989), did not influence the effect of NS 1619. Similarly, abolition of the stimulatory actions of NS 1619 or NS 004 on BK_{Ca} channels in Ca^{2+}-free solution in aortic or tracheal smooth muscle has been previously reported (Olesen et al., 1994a; Macmillan et al., 1995). These observations suggest that NS 1619 and its related compounds are unable to substitute for Ca^{2+} as channel openers. Charybdotoxin and other K_{Ca} channel

blocking agents such as iberiotoxin and tetraethylammonium ions antagonized the effect of NS 1619 in vascular smooth muscle and central neurones (Olesen et al., 1994a; Sellers and Ashford, 1994; Edwards et al., 1994; Holland et al., 1996). In addition, lack of effect of glibenclamide indicates that NS 1619 does not activate the ATP-sensitive K^+ channels as previously described in other cell types (Olesen et al., 1994a; Sellers and Ashford, 1994; Holland et al., 1996). These results demonstrate that NS 1619 activates charybdotoxin-sensitive K^+ channels in smooth muscle cells isolated from rat vas deferens as in other types of smooth muscle (Olesen et al., 1994a; Edwards et al., 1994; Holland et al., 1996).

When electrically stimulated, the rat vas deferens produced contractile responses which consisted of purinergic and adrenergic components (Sneddon and Burnstock, 1984; Allcorn et al., 1986). NS 1619 reduced these contractions in a concentration-dependent manner. NS 1619 at 3 μM reduced the electrically stimulated response ($< 10\%$) compared to its marked activator action on the outward currents in single smooth muscle fibers. NS 1619 (1–10 μM) started to significantly increase the K^+ current at above -40 mV and the effect of NS 1619 became more evident at membrane potentials more depolarized than 0 mV. Therefore, it is possible that a higher concentration of NS 1619 is needed to open the K_{Ca} channels in the intact vas deferens to induce muscle relaxation. NS 1619 hyperpolarized aortic smooth muscle by about 25 mV at 40 μM (Olesen et al., 1994a) and NS 1619 at 30 μM inhibited by approximately 30% the spontaneous mechanical activity of rat portal vein (Edwards et al., 1994). This percentage of inhibition is comparable to approximately 34% reduction by NS 1619, at the same concentration of electrically stimulated monophasic contractions seen in the present study. Charybdotoxin at 100 nM partially but significantly reduced the inhibitory effect of NS 1619 on evoked contractions while glibenclamide had no effect; however, the same concentration of charybdotoxin totally abolished the NS 1619-increased outward currents. NS 1619 decreased the exogenous noradrenaline-induced contraction and charybdotoxin caused a partial inhibition of the effect of NS 1619. These results suggest that NS 1619 might act at multiple sites on smooth muscle. Indeed, NS 1619 decreased the contractile response evoked by high external K^+ concentrations in the same preparations. The inhibitory action of the related molecule, NS 004, on Ca^{2+} currents in rat ventricular myocytes was previously described (Sargent et al., 1993). More recently, NS 1619 has been shown to inhibit the L-type Ca^{2+} channels and high K^+-induced contraction in rat arteries (Holland et al., 1996). Furthermore, the relaxant effect of NS 1619 on spontaneous mechanical activity of rat portal vein was not inhibited by charybdotoxin at concentrations identical to, or even greater than, those which entirely suppressed the NS 1619-induced increase of BK_{Ca} currents (Edwards et al., 1994; Holland et al., 1996). These results suggest that activation of BK_{Ca} channels might play little part, if any, in the spasmolytic effect of NS 1619 in the whole vascular tissue.

Taken together, results of the present investigation show that NS 1619 activates charybdotoxin-sensitive K_{Ca} current in single smooth muscle fiber of rat vas deferens, which might account for only a small portion of the observed inhibition of contractile responses evoked by either electric field stimulation or by exogenous noradrenaline. As in rat portal vein and cerebral artery, inhibition of Ca^{2+} influx probably through Ca^{2+} channels appears to play the major role in the relaxant effect of NS 1619 in isolated rat vas deferens.

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