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On the mechanism of the differential effects of NS004 and NS1608 in smooth muscle cells from guinea pig bladder

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Abstract

Our recent study revealed that the reported BK_{Ca} channel openers NS004 (5-trifluoromethyl-(5-chloro-2-hydroxyphenyl)-1,3-dihydro-2*H*-benzimidazole-2-one) and its analog NS1608 (*N*-(3-(trifluoromethyl)phenyl)-*N'*-(2-hydroxy-5-chlorophenyl)urea) produced a differential pattern of action on the BK_{Ca} channel in porcine coronary arterial cells (Hu, S., H.S. Kim and C.A. Fink, 1995, Differential effects of the BK_{Ca} channel openers NS004 and NS1608 in porcine coronary arterial cells, Eur. J. Pharmacol. 294, 357). In this study, using the patch-clamp method on the smooth muscle cells from guinea pig bladder detrusor, the activity profile of NS004 and NS1608 on the whole-cell BK_{Ca} current (I_{BK}) was examined and found to be similar to that in coronary arterial cells. NS004 did not significantly modify I_{BK} at concentrations below 5 μM, but robustly augmented I_{BK} at concentrations greater than 20 μM. With a minimum effective concentration of 0.5 μM, NS1608 caused potentiation of I_{BK} with a bell-shaped concentration-response relationship. The activation was maximized between 5–10 μM and substantially attenuated at higher concentrations. The behavior of single BK_{Ca} channels in the presence of NS004 and NS1608 was scrutinized to elucidate the mechanism underlying their distinct patterns of action. The channel open-state probability (NP_o) was increased by NS004 at 0.5, 5 and 50 μM to a respective 1.75 ± 0.38, 4.05 ± 0.90, and 15.01 ± 3.66 fold ($n = 7$) of the control by increasing the open time and the frequency of opening while having no effect on the single BK_{Ca} channel conductance. NS1608 at 0.5 and 5 μM increased NP_o to 1.69 ± 0.43 and 18.03 ± 6.64 fold of the control ($n = 4$), respectively. NS1608 at higher concentrations repeatedly blocked channel openings as evidenced by the highly flickering open state, though the overall open time and frequency of opening remained high. The diminished activation of I_{BK} by 50 μM NS1608 manifested therefore a net result of these two opposing actions on the single channels. Thus, the two structurally related BK_{Ca} channel openers interact distinctly with the channel gating components.

Keywords: Bladder detrusor, guinea pig; Patch-clamp; BK_{Ca} channel; Whole-cell recording; Inside-out single channel recording; Open-state probability

1. Introduction

The large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) are reportedly present in most types of vascular and non-vascular smooth muscle. Since the channels are activated by membrane depolarization and an increase in intracellular Ca^{2+} concentration, they may serve as a negative feedback pathway to control the Ca^{2+} homeostasis and cell excitability (Brayden and Nelson, 1992). Given the large unitary conductance and the dense distribution, opening of the BK_{Ca} channel is a potentially powerful intervention leading to muscle relaxation.

Recently, two organic molecules (Fig. 1), NS004 (5-trifluoromethyl-(5-chloro-2-hydroxyphenyl)-1,3-dihydro-2*H*-benzimidazole-2-one, Olesen and Watjen, 1992) and a conformationally less restricted analog NS1608 (*N*-(3-(trifluoromethyl)phenyl)-*N'*-(2-hydroxy-5-chlorophenyl)urea, Olesen, 1994a), have been described to directly activate the BK_{Ca} channels (Olesen, 1994b; Olesen, 1995). In an earlier study, we investigated the actions of NS004 and NS1608 in cells from porcine coronary artery, and found that these two structurally related molecules potentiated the whole-cell BK_{Ca} current (I_{BK}) with distinct patterns of action (Hu et al., 1995). Speculation was raised for the mechanism(s) underlying the differential activity profile, but direct evidence is lacking.

In the present study, we have investigated the effects of NS004 and NS1608 on I_{BK} in enzymatically isolated cells from guinea pig bladder detrusor, a non-vascular smooth

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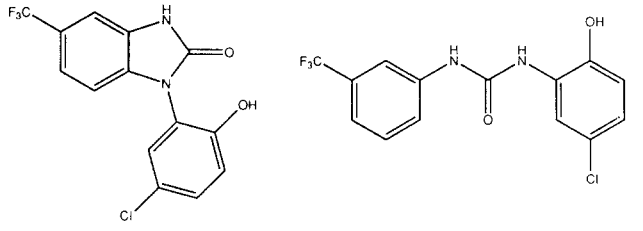

NS004 NS1608

Fig. 1. Chemical structures of NS004 and NS1608.

muscle. The results showed that these two compounds also produced a differential concentration-response relationship as that observed in coronary vascular smooth muscle cells. The mechanism of action was further investigated using the single BK_{Ca} channel recordings. The results revealed that NS1608 exerted a dual (stimulatory and inhibitory) action whereas NS004 monotonically activated the BK_{Ca} channel in the same concentration range.

2. Materials and methods

2.1. Cell isolation

Male guinea pigs (~ 300 g) were killed by exposure to CO_2 for 2 min. The urinary bladder was removed and rinsed in a Ca^{2+}-free medium composed of (in mM) 135 NaCl, 5.4 KCl, 2 $MgSO_4$, 5 glucose, 10 Hepes, and pH 7.3 adjusted with NaOH. The bladder detrusor muscle was cut and cleaned free of connective tissue and blood vessels. Pieces of tissue were then incubated for two periods of 35 min at 36°C in an enzyme medium containing (in mM) 130 K glutamate, 20 taurine, 5 pyruvate, 5 creatine, 10 Hepes and 0.5 $CaCl_2$ complemented with 1 mg/ml collagenase (Sigma, C2139), 0.2 mg/ml pronase (Sigma, P5147), 1 mg/ml fatty acid free albumin. During incubation, tissue pieces were gently stirred to assure a full exposure to the enzymes. After a total of 70 min enzyme digestion, single bladder smooth muscle cells were obtained by gentle agitation of the preparation through a Pasteur pipette into the 'KB' (Kraftbruhe) solution (Isenberg and Klockner, 1982). Single cells were stored at 4°C for 1 h before recording. The cell capacitance was 34.8 ± 2.5 pF ($n = 16$).

2.2. Experimental setting and current recording

Experiments were performed at 22°C using the whole-cell or inside-out configuration of the patch-clamp technique (Hamill et al., 1981) in enzymatically dispersed smooth muscle cells from guinea pig bladder detrusor. Patch electrodes were pulled from Kimax-51 capillary tubes (Kimble Products, USA). The resistance of electrodes after fire polishing was around 2 MΩ (larger inner diameter) for the whole-cell mode to promote the dialysis of pipette solution and around 5 MΩ (smaller inner diameter) for the inside-out mode to reduce the number of channels recorded. Whole-cell currents were amplified by a List EPC-7 amplifier (Adams&List Assoc.), digitized at 2 kHz with a TL-1-125 DMA interface (Axon Instruments) and stored on a Compaq DeskPro/66 M microcomputer for later analysis with pClamp version 6.0 (Axon Instruments). The inside-out single channel recordings were first low-pass-filtered at 2 kHz by a 8-poles Bessel filter (Frequency Devices 902 LPF), and video-taped with a Toshiba Pulse-Code Modulation data recorder (DX-900). After each experiment, the recordings were played back through a window discriminator (AI2020, Axon Instruments) at a sampling rate of 4 kHz and stored on the computer for analysis. The junction potential between the electrodes and the bath solution was compensated using the DC offset on the amplifier. No leak subtraction was applied.

The open-state probability of single BK_{Ca} channels was calculated as the total open duration at the first level divided by the total duration of the recording, which was typically 40 s or more for all experiments. As most of the patches studied contained multiple BK_{Ca} channels of identical conductance, the open-state probability was therefore referred to as NP_o.

The whole-cell I_{BK} was measured when cells were subjected to depolarization steps from −60 to +60 mV in a 20 mV increment from a holding potential of 0 mV. The bath solution contained (in mM) 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Hepes and 5 glucose; and the pipette solution had (in mM) 140 KCl, 1 $MgCl_2$, 0.1 $CaCl_2$, 0.6 EGTA, 2 Na_2UDP, 2 K_2ATP, 5 glucose, and 10 Hepes, in which the free Ca^{2+} concentration was estimated to be 10^{-8} M (Imai and Takeda, 1967). In inside-out single channel recordings, the pipette and the bath solutions had the (same) composition of (in mM) 140 KCl, 5 NaCl, 1 $MgCl_2$, 10 Hepes and 5 glucose; except for the $CaCl_2$ concentration, which was 10^{-3} M in the pipette and 10^{-8} M in the bath buffered with EGTA.

NS004 and NS1608 were synthesized in the Research Department of Ciba-Geigy Corp. The drugs were first dissolved in 95% ethyl alcohol to form a stock solution of 50 mM, which was then diluted with saline to the desired concentrations shortly before experiments. In this study, vehicle control experiments were performed and showed that ethyl alcohol at a maximal concentration of 0.1% had no discernible electrophysiological effects on K^+ channels.

3. Results

3.1. Effects of NS004 on whole-cell I_{BK} and single BK_{Ca} channels

The whole-cell I_{BK} was recorded when voltage steps ranging between −60 mV and +60 mV with an incre-

ment of 20 mV were imposed on the cells from a holding potential of 0 mV, at which other voltage-dependent K^+ currents were inactivated allowing the demonstration of isolated I_{BK} (Olesen et al., 1994c). To minimize the contamination of K_{ATP} current, the pipette solution contained 2 mM ATP, a concentration considerably higher than 40–50 μM, the concentration generally found to half-maximally inhibit the K_{ATP} channels. Step depolarizing pulses generated a family of time-dependent and non-inactivating current (see Fig. 2A and Fig. 4A), of which over 80% were blockable by 80 nM iberiotoxin and 1 mM tetraethylammonium, indicating a prominent contribution of the BK_{Ca} channels. In most of the cells tested, significant stimulation of the whole-cell I_{BK} by NS004 was not seen at concentrations below 5 μM. However, a robust augmentation of I_{BK} was consistently observed within 2 min of the application of 50 μM NS004 (see Fig. 2A). These observations suggest a steep concentration-response relationship of NS004. Fig. 2C shows the concentration-response curve of I_{BK} measured at +60 mV from a total of 6 experiments. The steady-state I_{BK} at +60 mV was 1.09 ± 0.07, 1.46 ± 0.23 and 3.16 ± 0.26 fold of the control in the presence of 0.5, 5 and 50 μM NS004, respectively. The current-voltage relationship curves in the absence and presence of NS004 at 5 and 50 μM, derived from the recordings of Fig. 2A, are illustrated in Fig. 2B. It is evident that sizable activation of I_{BK} was only seen at the concentration of 50 μM. The effects of NS004 were readily reversible within a few minutes.

The effect of NS004 on single BK_{Ca} channel was investigated in inside-out patches exposed to symmetrical K^+ solutions (140 mM). The BK_{Ca} channel in guinea pig bladder muscle cells had a conductance of 208 ± 6 pS ($n = 23$). NS004 applied in bath did not modify channel conductance but caused an increase in duration of open state and frequency of opening in a concentration-dependent manner (Fig. 3). The values of NP_o (patch held at +40 mV) in the presence of 0.5, 5 and 50 μM of NS004 were, respectively, 1.75 ± 0.38, 4.05 ± 0.90 and 15.01 ± 3.66 fold ($n = 7$) of the control. The onset and offset of the action in inside-out patches were rapid, and the activation was seen in less than 2 min.

3.2. Effects of NS1608 on whole-cell I_{BK} and single BK_{Ca} channels

The effects of NS1608 were examined in parallel at whole-cell and single-channel levels. Whole-cell I_{BK} was

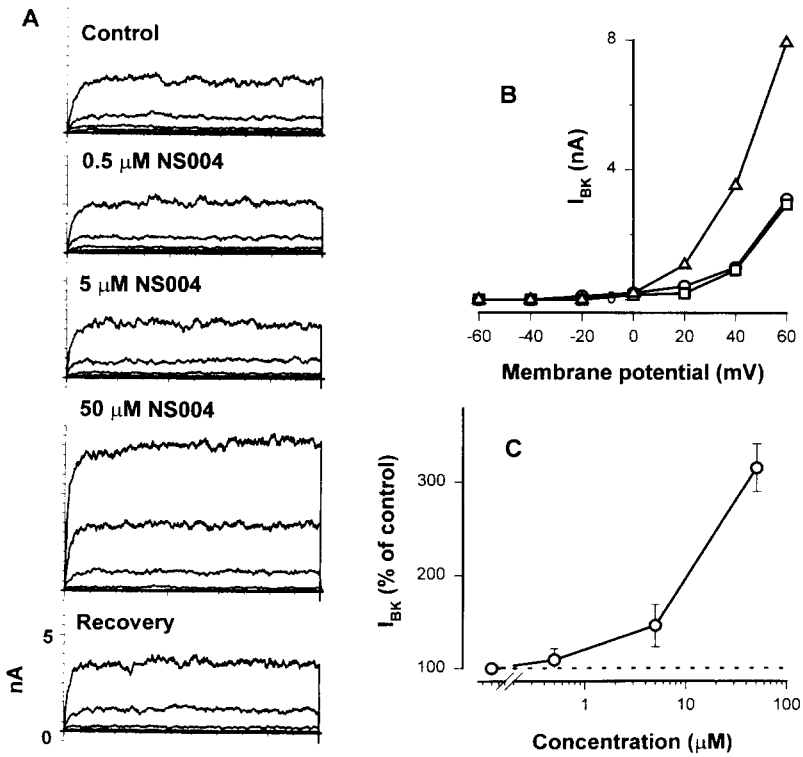

Fig. 2. Effects of NS004 on whole-cell I_{BK}. (A) Representative recordings of the whole-cell I_{BK} in response to a voltage step protocol ranging from −60 mV to +60 mV with an increment of 20 mV and a duration of 1 s in control (top of A), and during subsequent exposure to NS004 at 0.5 (second of A), 5 (third of A), and 50 μM (fourth of A) and after washout of NS004 (bottom of A). Holding potential was 0 mV. Cell was bathed in physiological K^+ solution ($[K^+]_i$ 140 mM/$[K^+]_o$ 5 mM). The pipette contained 2 mM ATP and 10^{-8} M free Ca^{2+}. Ordinate in the bottom panel shows the current scale. (B) Current-voltage curves in control (circle), in the presence of 5 μM (square), and 50 μM NS004 (triangle). Ordinate shows the amplitude of the steady-state I_{BK} (nA) and abscissa is the membrane potential (mV), at which I_{BK} was generated. (C) Concentration-response curve for I_{BK} measured at +60 mV (circle). Ordinate shows the amplitude of the steady-state I_{BK} expressed as a percentage of the control value, which is considered as 100%. The left most point and the dotted line indicate the control level. Abscissa is NS004 concentration (μM) in a logarithmic scale.

elicited by a step voltage protocol identical to that used in the experiments with NS004. As shown in Fig. 4A, NS1608 exerted a stimulatory effect on I_{BK} with a minimum effective concentration of 0.5 μM. The effect reached a maximum at concentrations between 5 and 10 μM. Subsequent application of NS1608 at concentrations higher than 10 μM led to a transient activation followed by a gradual and substantial reduction of the amplitude and slowdown of the onset rate of I_{BK}. The stimulatory effect consistently preceded the blocking effect by several minutes. In the meantime, the current traces became increasingly noisier. The suppression was especially prominent upon prolonged exposure (> 5 min) to the drug. The current-voltage curves derived from the recordings in Fig. 4A are illustrated in Fig. 4B, in which I_{BK} is greater in 5 μM than in 50 μM NS1608 in the entire voltage range tested. Fig. 4C shows the bell-shaped concentration-response curve of I_{BK} measured at +60 mV from a total of 9 experiments. The amplitude of steady-state I_{BK} at +60 mV was, respectively, 1.28 ± 0.14, 2.91 ± 0.38 and 1.84 ± 0.22 fold of the control in the presence of 0.5, 5 and 50 μM of NS1608. During washout of 50 μM NS1608, I_{BK} underwent an augmentation before returning to a level close to the control level, an observation which would be expected as the drug concentration gradually decreased. Compared with NS004, NS1608 had a considerably slower onset of action and wash out of the drug could take 10 min or longer.

To clarify the mechanism underlying the effect with a bell-shaped concentration-response curve, single BK_{Ca} channel recording in inside-out patches was made in the presence of NS1608 in the same concentration range as in the whole-cell recording. The results in Fig. 5 showed that NS1608 at 0.5 μM increased the open dwell time hence

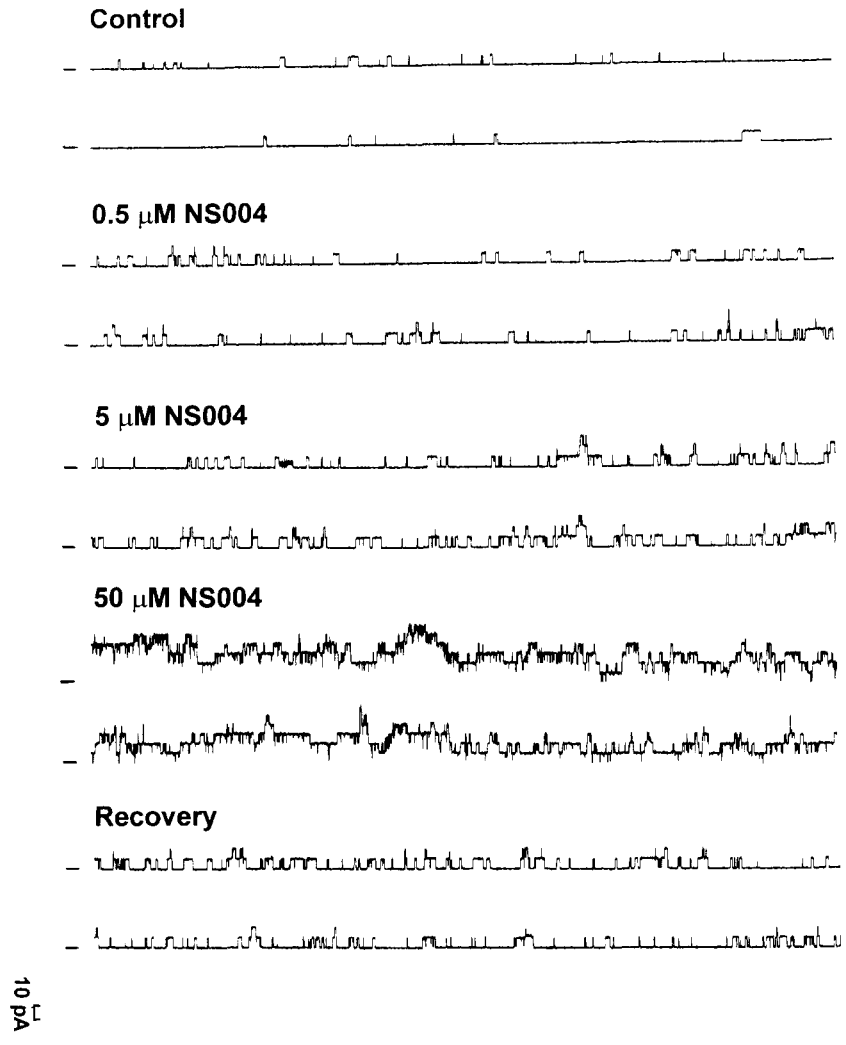

Fig. 3. Effects of NS004 on single BK_{Ca} channels. Representative single BK_{Ca} channel recordings in control (top), during sequential exposure to NS004 at 0.5 (second), 5 (third), and 50 μM (fourth) and 5 min after washout of NS004 (bottom). Recordings were made from an inside-out patch held at +40 mV and exposed to symmetrical 140 mM K^+ solutions. NS004 was applied in the bath solution with a free Ca^{2+} concentration adjusted to 10^{-8} M. In all panels, two current traces are consecutive recordings with a total duration of 20 s. Upward deflections are the channel opening events. The closed state of the channels is indicated by the short lines at the left of the traces. The amplitude of unitary current in all panels was approximately 8.2 PA. Current was filtered at 2 kHz.

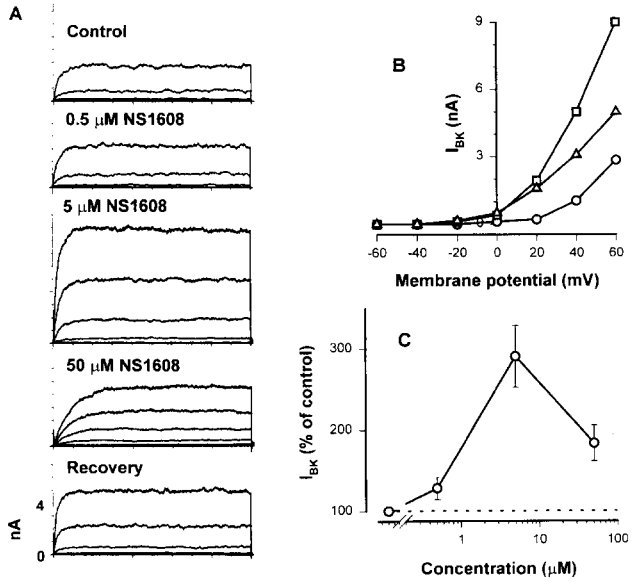

Fig. 4. Effects of NS1608 on whole-cell I_{BK}. (A) Representative recordings of the whole-cell I_{BK} in response to a voltage step protocol ranging from −60 mV to +60 mV with an increment of 20 mV and a duration of 1 s in control (top of A), and during subsequent exposure to NS1608 at 0.5 (second of A), 5 (third of A), and 50 μM (fourth of A) and after washout of NS1608 (bottom of A). Holding potential was 0 mV. All currents were measured at the end of 6 min exposure to the drug. Cell was bathed in physiological K^+ solution ($[K^+]_i$ 140 mM/$[K^+]_o$ 5 mM). The pipette contained 2 mM ATP and 10^{-8} M free Ca^{2+}. Ordinate in the bottom panel shows the current scale. (B) Current-voltage curves in control (circle), in the presence of 5 μM (square), and 50 μM NS1608 (triangle). Ordinate shows the amplitude of steady-state I_{BK} (nA) and abscissa is the membrane potential (mV), at which I_{BK} was generated. (C) Concentration-response curve for I_{BK} measured at +60 mV (circle). Ordinate shows the amplitude of steady-state I_{BK} expressed as a percentage of the control value, which is considered as 100%. The left most point and the dotted line indicate the control level. Abscissa is the concentration of NS1608 (μM) in a logarithmic scale.

NP_o without altering the amplitude of the unitary current. While NP_o was continuously increased by 5 μM NS1608, the channel open state became increasingly flickery, a phenomenon often observed when channel openings are rapidly blocked and unblocked by drug molecules. In 50 μM NS1608, the blocking effect became so predominant that the channels were virtually in a state of frequent on-off transition, though the overall NP_o remained to be high. These observations indicated the appearance of an inhibitory action (through physically blocking open channels) concomitant to an opening effect (by increasing channel open time and open frequency). The values of NP_o (patch held at +40 mV) were 1.69 ± 0.43 and 18.03 ± 6.64 fold of the control ($n = 4$), respectively, in 0.5 and 5 μM NS1608. An accurate quantification of rapid on-off events in a burst induced by 50 μM NS1608 was not possible due to the limitation of the resolution of our recording system. Nevertheless, the number of the channel being open and the overall open time appeared to be further increased by 50 μM NS1608. The apparent amplitude of unitary current was found unchanged, suggesting that NS1608 did not alter single BK_{Ca} channel conductance. Given the two opposing effects of NS1608 on the BK_{Ca} channel, the observed augmentation by 5 μM NS1608 or subsequent attenuation by 50 μM NS1608 of the whole-cell I_{BK} (Fig. 4) was, in fact, a net result of these effects depending on which one outplayed the other. All effects were reversible after prolonged (> 10 min) washout of the drug.

4. Discussion

4.1. Differential pattern of action on whole-cell I_{BK} by NS004 and NS1608

In the present study, the effects of two structurally related BK_{Ca} channel openers NS004 and NS1608 on the whole-cell I_{BK} in intact smooth muscle cells from guinea pig bladder were evaluated when conditions were optimized for the detection of isolated I_{BK}. While the activation of I_{BK} by NS004 was reported in various types of cells including bovine aortic cell (Olesen, 1994b), mouse cerebellar granule cell (Olesen et al., 1994d), rat pituitary tumor cell (McKay et al., 1994), canine and porcine coronary arterial cell (Xu et al., 1994; Hu et al., 1995), and rabbit pulmonary arterial cell (Vandier and Bonnet, 1996), the investigation of the effect of NS1608 has not been documented until very recently (Olesen, 1995; Hu et al., 1995). Consistent with the results from these earlier studies that NS004 activates the whole-cell I_{BK} with a moderate to low potency as indicated by the concentration (between 5 and 20 μM) required to see the effect, we observed a marginal but significant stimulation of I_{BK} (×1.46) in bladder cells at the concentration of 5 μM. The activation was intensified in a steep concentration-dependent manner.

At the whole-cell level, NS1608 appeared to be a more potent BK_{Ca} channel opener than NS004. A weak stimulation of I_{BK} (×1.28) was detected at the drug concentration of 0.5 μM. At 5 μM, NS1608 augmented I_{BK} with a magnitude (×2.91) considerably greater than that by NS004 (×1.46). An interesting observation was the substantial attenuation of I_{BK} and retardation of the current activation by 50 μM NS1608 in cells from bladder and porcine coronary artery (Hu et al., 1995), which was not reported with NS004 in any cell types. The effect can not be attributed to the contamination of the effects of NS1608 on other types of K^+ channels like K_V and K_{ATP}, since in this study I_{BK} was investigated in isolation after minimizing all other current components. Besides, NS1608 was found to have minimal effect on K_V and K_{ATP} currents in porcine coronary arterial cells as was seen with NS004 (Xu et al., 1994; Hu et al., 1995). Thus, NS1608 was likely to exert a concentration-related dual effect (stimulatory and inhibitory) on I_{BK}, a speculation raised in

our earlier study in coronary arterial cells (Hu et al., 1995). It seemed necessary to investigate the effects of NS004 and NS1608 on single BK_{Ca} channels to elucidate the mechanism that caused distinct forms of concentration-response relationship (see Fig. 2C and Fig. 4C).

4.2. Differential pattern of action on single BK_{Ca} channels by NS004 and NS1608

In the present study, the BK_{Ca} channels in bladder smooth muscle cell were identified on the basis of the Ca^{2+}-sensitivity (a 10-fold increase in $[Ca^{2+}]_i$ caused a 31 mV negative shift of the voltage where NP_o was 50%) and the single channel conductance that was 208 pS in inside-out patches exposed to symmetrical 140 mM K^+ solutions. Bath applied NS004 in the entire concentration range studied did not alter the amplitude of unitary current but merely increased opening frequency and the mean open time. NP_o values were elevated in a concentration-dependent manner with a steep rise occurring between 5 and 50 μM, a result in agreement with that from the whole-cell recordings. The onset of NS004 action in inside-out patches was rapid (< 2 min) and not significantly different from that in the whole-cell recordings.

The results with NS1608 in single-channel recordings in inside-out patches showed that, in addition to an increase in the opening frequency and mean open time as NS004 did, the drug also induced a concentration-dependent increase in open-state noise. The latter observation indicates a repeated and stochastical blockade of the channels by NS1608, resulting in a flickering conductance as drug molecules bind and unbind to the sites of action. The time-scale of a drug-receptor reaction is set by the mean life time of a single drug-receptor complex or, in other words, the residency time of a drug molecule in the channel (Hille, 1992). The recordings shown in Fig. 5 in

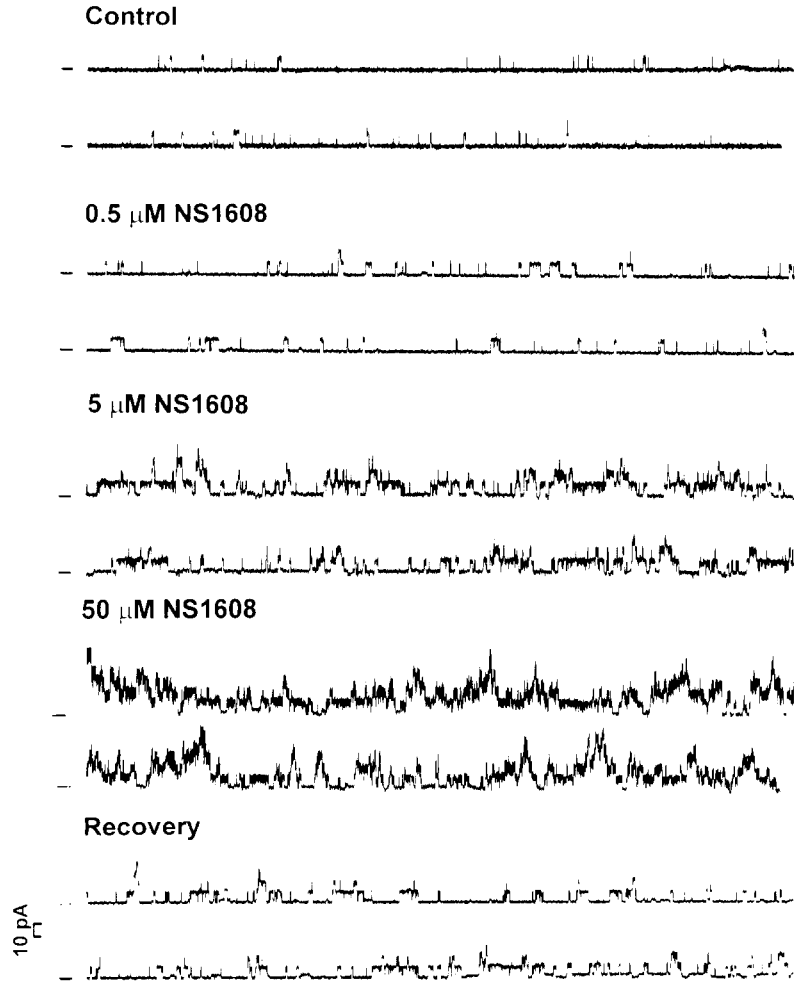

Fig. 5. Effects of NS1608 on single BK_{Ca} channels. Representative single BK_{Ca} channel recordings in control (top), during sequential exposure to NS1608 at 0.5 (second), 5 (third), and 50 μM (fourth) and 10 min after washout of NS1608 (bottom). Recordings were made from an inside-out patch held at +40 mV and exposed to symmetrical 140 mM K^+ solutions. NS1608 was applied in the bath solution with a free Ca^{2+} concentration adjusted to 10^{-8} M. In all panels, two current traces are consecutive recordings with a total duration of 20 s. Upward deflections are the channel opening events. The closed state of the channels is indicated by the short lines at the left of the traces. Current was filtered at 2 kHz.

the presence of 50 μM NS1608 fit with the description of a drug acting in an 'intermediate' time scale, that is in the range of millisecond. With this time scale, a molecule of NS1608 would repeatedly block and unblock a BK_{Ca} channel which has a typical mean open dwell time of around 10 ms. Given the fact that NS1608 induced flickers only in the open-state but not the closed-state, the blockade required open channels.

Since the whole-cell I_{BK} is a time average current through all single BK_{Ca} channels in the entire cell membrane, the observed bell-shaped concentration-response curve of the action of NS1608 on I_{BK} manifested the counterbalance between the stimulatory (lengthening the open time) and the inhibitory (blocking the open state) effects on single BK_{Ca} channels. When the latter overplayed the former as the concentration of NS1608 molecules became higher, an attenuation of the I_{BK} amplitude and a retardation of current activation kinetics were demonstrated (Fig. 4).

4.3. Ca^{2+} requirement for the actions of NS004 and NS1608

A consensus on the Ca^{2+} requirement of the actions of NS004 and NS1608 has thus far not been reached. Several earlier studies reported that internal Ca^{2+} was necessary for the activation of the BK_{Ca} channel by NS004 in bovine aortic cells (Olesen et al., 1994c) or by its analog NS1619 in rat cortical neurons (Lee et al., 1995), rat cerebral arterial cells (Holland et al., 1996), and rabbit pulmonary arterial cells (Vandier and Bonnet, 1996). In addition, the stimulation was potentiated as the Ca^{2+} concentration increased. In contrast, McKay et al. (1994) showed that in GH_3 cells, a rat clonal pituitary cell line, the efficacy of NS004 was reciprocally related to the initial state of single channel activity determined by the intracellular Ca^{2+} concentration, namely the degree of activation was greater at lower Ca^{2+} concentration. Another distinct observation was that NS1619 activated the BK_{Ca} channels in the absence of Ca^{2+} in canine coronary arterial cell (Xu et al., 1994) or in a Ca^{2+}-independent manner in rat portal vein cells (Edwards et al., 1994). Attempt was made in this study on bladder cells to assess the Ca^{2+} dependence of the actions of NS004. We found that 5 μM NS004 administered in a nominally Ca^{2+}-free bath solution supplemented with 2 mM EGTA (free $[Ca^{2+}]_i < 10^{-10}$ M) activated the single BK_{Ca} channels in inside-out patches by elevating NP_o to 3.94-fold of the control, that was not different from 4.05-fold in a bath solution with 0.1 mM Ca^{2+} buffered with 0.6 mM EGTA (free $[Ca^{2+}]_i = 10^{-8}$ M, results in Fig. 3). In addition, we observed that whole-cell I_{BK} was augmented robustly by NS004 when the cells were bathed in a solution containing zero Ca^{2+} and 2 mM EGTA. Our observations strongly suggest that the effects of NS004 is independent of Ca^{2+}. The cause for the discrepancy is yet unclear, possibly due to tissue or animal species difference in the Ca^{2+} sensitivity of the BK_{Ca} channels.

4.4. Possible site of action

NS004 and NS1608 stimulated BK_{Ca} channels when applied to the cytosolic side of the inside-out patches, in which the Ca^{2+} concentration was well clamped and all cellular signaling pathways were absent, indicating that these compounds directly act on the channel gating component rather than indirectly stimulate the production of biochemical second messengers followed by Ca^{2+} release from the intracellular store. This conclusion is in agreement with the results in some earlier studies (Olesen et al., 1994c; Holland et al., 1996).

The location of the sites of action in relation to the membrane surface can not be decided at present because (1) the high lipophilicity of NS004 and NS1608 allowed the drug molecules to pass through the lipid bilayer readily; (2) the activation of BK_{Ca} channels by NS004 and NS1608 was seen by applying the drugs either to the intracellular side of inside-out patches or to the extracellular side of intact whole cells; (3) the onset of actions of NS004 in these two patch configurations were comparable and rapid (< 2 min). These results did not seem to fit the assumption proposed by Holland et al. (1996) of an internal site of action of NS1619, an analog of NS004. Analogous to some lipid-soluble Na channel gating modifiers whose molecules can not freely enter or leave a site within aqueous pore (Hille, 1992), we speculate that the site of action lies in a strongly hydrophobic region such as at the boundary between membrane lipids and the membrane-crossing peptide chains of the channels. An interesting finding with 50 μM NS1608 was that in inside-out patches the stimulatory (lengthening of the open dwell time) and inhibitory (flickery blockade of the channel) effects were seen almost simultaneously with relatively fast onset, whereas in whole-cell recordings the stimulatory effect consistently preceded the blocking effect by several minutes (see Section 3.2). It is, therefore, possible that the site of action is buried in the membrane with relatively shorter distance to channel inner mouth. The binding of the openers to the site must induce protein conformational change leading to distortion of channel inactivation, shift of voltage dependence and etc. Given the structural feature of NS1608, the carbocyclic ring with the trifluoromethyl group that is more lipophilic may sit on the site of action within the membrane while the other end of the molecule may reach out to block and unblock stochastically the inner mouth of the channel.

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