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Synergistic action of NS-004 and internal Ca^{2+} concentration in modulating pulmonary artery K^+ channels

Christophe Vandier, Pierre Bonnet *,[1]

Laboratoire de Physiologie des Cellules Cardiaques et Vasculaires, CNRS EP-21, Parc de Grandmont, 37200 Tours, France

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Abstract

Considering the singular vasomotor behavior of the pulmonary artery, we were interested to test NS-004 (1-(2'-hydroxy-5'-chlorophenyl)-5-trifluoromethyl-2(3*H*)-benzimidazolone) on pulmonary artery smooth muscle cells. Using the patch clamp technique, we identified a delayed rectifier K^+ current and a Ca^{2+}-activated K^+ current. With a low free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), 10–50 μM NS-004 activated a noisy outward current which was blocked by iberiotoxin. 50 μM NS-004 also inhibited a smooth inactivating outward current. Under these conditions, 10 μM NS-004 induced no change in the resting membrane potential. With a higher free $[Ca^{2+}]_i$, 10 μM NS-004 was 3.5 times more efficacious in increasing the noisy current and it induced a hyperpolarization. We concluded that increasing free $[Ca^{2+}]_i$ induced potentiation of the NS-004-induced activation of high conductance Ca^{2+}-sensitive K^+ channels and of the NS-004-induced hyperpolarization of the cell. The delayed rectifier K^+ channel was inhibited by NS-004 as well as by an increased free $[Ca^{2+}]_i$.

Keywords: NS-004; $[Ca^{2+}]_i$, free; K^+ channel, Ca^{2+}-activated; K^+ channel, delayed rectifier; Whole cell patch clamp; Membrane potential; Pulmonary artery smooth muscle cell

1. Introduction

The most important distinctive property of the pulmonary circulation (compared to the systemic circulation) is the low blood pressure. This is explained by the low flow resistance of the pulmonary artery itself. This low pressure is essential because it allows the vessels to retain fluid that would otherwise flood the alveoli and prevent gas exchange. Another peculiarity of the pulmonary artery is that it constricts in response to hypoxia. Robertson et al. (1992) suspected that this hypoxic vasoconstriction is linked to the presence in the pulmonary artery cell membrane of specific K^+ channels. An understanding of the pharmacology of pulmonary artery K^+ channels could either have therapeutic implications or give some insight into the mechanism that underlies the singular behavior of the pulmonary artery tree.

Pulmonary artery K^+ channels are specific because they possess some features of both high conductance-activated K^+ channels (BK_{Ca}) and delayed rectifier K^+ channels (K_{dr}). Like K_{dr} they are more sensitive to 4-aminopyridine than to tetraethylammonium, which inactivate (Okabe et al., 1987; Clapp and Gurney, 1991), and they are activated by an elevation of intracellular ATP (Evans et al., 1994; Albarwani et al., 1994). Also, they possess a unitary conductance (in symmetrical K^+) within the range reported for BK_{Ca} channels, they are sensitive to Ca^{2+} influx (Clapp and Gurney, 1991) and they are activated by an elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Robertson et al., 1992).

So it is not easy to classify these channels as either K_{dr} or BK_{Ca}. However, in a different study Gelband et al. (1993) have clearly identified in canine pulmonary artery cells, two classical K^+ channels. The first one is the BK_{Ca}, which was blocked by tetraethylammonium

* Corresponding author. Laboratoire de Physiologie des Cellules Cardiaques et Vasculaires, CNRS EP-21, Parc de Grandmont, 37200 Tours, France. Tel.: 47-36-70-12; fax: 47-36-71-12.

[1] Present address: Service de Cardiologie B, Centre Hospitalier Universitaire, 37250 Tours, France.

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(1 mM) and activated by intracellular Ca^{2+}. The second one is the K_{dr} which was blocked by 1 mM 4-aminopyridine.

Since K^+ channels regulate cell excitability and could control the tone of the pulmonary vasculature, the identification of drugs that open these specific channels could be useful in the treatment of pulmonary hypertension.

Recently, a new class of Ca^{2+}-activated K^+-channel activator, chemically derived from benzimidazole, was studied: 1-(2′-hydroxy-5′-trifluoromethylphenyl)-5-trifluoromethyl-2(3*H*)-benzimidazolone (NS-1619) and 1-(2′-hydroxy-5′-chlorophenyl)-5-trifluoromethyl-2(3*H*)-benzimidazolone (NS-004). In systemic smooth muscle cells, these agents have been reported to stimulate the opening of BK_{Ca} and to inhibit K_{dr}, ATP-sensitive K^+ channels (K_{ATP}) and L-type Ca^{2+} channels (Olesen et al., 1993; Xu et al., 1994; Edwards et al., 1994). We thought that these new compouds could be a useful tool for the identification of the properties of the K^+ channels of the pulmonary artery.

Since Ca^{2+} sensitivity is a major criterion for the classification of K^+ channels, we were interested to test the role of $[Ca^{2+}]_i$ elevation on the modulation of pulmonary artery K^+ channels induced by NS-004. We thought that this would cast light upon the singular vasomotor behavior of the pulmonary artery. In this paper we show a synergistic action of NS-004 and free $[Ca^{2+}]_i$ on K^+ channels in rabbit pulmonary artery cells. Increasing free $[Ca^{2+}]_i$ potentiated the NS-004-induced activation of BK_{Ca} and the hyperpolarization of the cell.

2. Materials and methods

2.1. Pulmonary artery cell isolation

Rabbits (2–3 kg) were killed by cervical dislocation. Proximate pulmonary arteries were carefully dissected. Pulmonary artery rings were dissected and placed in physiological salt solution (PSS). The connective tissue and adventitia were carefully removed and the cleaned vessel was opened along its longitudinal axis. The endothelium was removed by rubbing the intimal surface of the pulmonary artery with a small steel wire. Vessels were first incubated in Ca^{2+}-free PSS at 37°C for 5 min. They were then cut into small pieces ($\sim 2 \times 3$ mm) and placed in 5 ml PSS, without Ca^{2+}, but containing 191 u/ml collagenase (CLS 2, Worthington Biochemical Corporation), 0.22 u/ml pronase E (Sigma), 3 mM dithiothreitol (Sigma) and 1 mg/ml bovine albumin (Sigma) at 37°C for 15–20 min on an tridimensional agitator.

Vessel pieces were then removed and placed in PSS without Ca^{2+} and gently agitated for approximately 40 min. Cells were kept at 4°C in PSS and used between 2 and 10 h after isolation.

2.2. Electrophysiological measurements

Using the patch clamp technique (Hamill et al., 1981) in the whole cell configuration, membrane potentials and currents were recorded with a List EPC7 Patch Clamp amplifier in current clamp ($I = 0$) or voltage-clamp mode, respectively. Patch pipettes were pulled from borosilicate glass capillaries and had a tip resistance of 3–5 MΩ when filled with the pipette solution.

The junction potentials between the electrode and the bath were cancelled by using the voltage pipette offset control of the amplifier. The electrode capacitances were electronically compensated (3–4 pF).

Series resistance values ($Rs = 5.8 \pm 0.9$ MΩ, $n = 15$) were calculated from the time constant of the decay of the transient capacitive current recorded in response to a 10 mV pulse applied from -50 to -60 mV and from the measurement of cell capacitance ($Cm = 25.0 \pm 0.7$ pF, $n = 15$). During the experiments, cell capacitances were not compensated. To take into account differences in cell volume, results were expressed in current density (pA/pF) calculated by dividing, for each cell, the current magnitude by the membrane capacitance.

All measurements were made with superfused cells to avoid stretch or mechanical disturbances. Since the bath volume was 1 ml and the flow rate 4 ml/min, final drug concentrations were reached after a delay of 1.5 min after switching from PSS (control) to drug reservoirs.

Outward currents were elicited by applying, at 2 s intervals, 300 ms depolarizing pulse steps (in 10 mV increments channel) from a negative holding potential of -60 mV. Average current amplitudes for the last 50 ms of the pulse were measured. Any deviations from this voltage protocol are mentioned in the text. For on line data collection onto an IBM PC/AT, current signals were usually filtered at 3 kHz (-3dB) and digitized at 2–6 kHz with a labmaster TL1-125 interface (Scientific solutions, Ohio, USA), using the pClamp software package (Axon Instruments). All recordings were carried out at room temperature (20–24°C).

2.3. Statistics

Data are expressed as means $\pm$ S.E.M and differences between means were tested with Student's *t*-test and were accepted as significant if $P < 0.05$.

2.4. Solutions and drugs

The PSS used in the bath had the following composition (in mM): NaCl, 138.6; KCl, 5.4; $CaCl_2$, 1.8;

$MgCl_2$, 1.2; NaH_2PO4, 0.33; Hepes, 10; glucose, 11. The pH was adjusted to 7.4 using NaOH. When the ionic composition of this solution was altered, the osmolarity (280 mosmol) was maintained by adjusting the NaCl concentration.

The pipette solution for recording K^+ currents had the following composition (in mM): potassium aspartate, 110; KCl, 20; Hepes, 5; EGTA, 2; Na_2-creatine phosphate, 2; Na_2ATP, 1; $MgCl_2$, 1. The pH was adjusted to 7.2 with KOH. Estimated free Ca^{2+} was 1 nM. Appropriate amounts of EGTA, $MgCl_2$ and $CaCl_2$ were added to the pipette solution to obtain the desired concentrations of free Ca^{2+}. For a free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of about 100 nM, EGTA was 5 mM, $CaCl_2$ 3 mM and $MgCl_2$ 1.15 mM. The free $[Ca^{2+}]_i$ was calculated by a computer program developed by Godt and Lindley (1982).

1 mM Na_2ATP added in the pipette solution inhibited $I_{K(ATP)}$ (Xu and Lee, 1994).

The novel benzimidazolone 1-(2′-hydroxy-5′-chlorophenyl)-5-trifluoromethyl-2(3*H*)-benzimidazolone (NS-004; Bristol-Myers, Squibb) was prepared as a 0.1 M stock solution in dimethyl sulfoxide (maximum 0.1%) and diluted to the desired concentrations in the bath solution. The chloride channel blocker anthracene-9-carboxylic acid was dissolved in dimethyl sulfoxide (0.05%).

4-Aminopyridine, tetraethylammonium chloride and iberiotoxin (Alomone laboratories, Jerusalem, Israel) were directly dissolved in the external solution. Except where specified, drugs were obtained from Sigma.

3. Results

3.1. Main characteristics of outward K^+ current on pulmonary artery smooth muscle cells: separation of delayed rectifier K^+ current $I_{K(dr)}$ and Ca^{2+}-activated K^+ current $I_{BK(Ca)}$

Different types of K^+ currents have been shown to exist in pulmonary artery cells (Clapp and Gurney, 1991; Okabe et al., 1987; Robertson et al., 1992; Gelband et al., 1993). However, these specific current possess features of $I_{K(dr)}$ and $I_{BK(Ca)}$. Therefore, we isolated the different current components according to their electrophysiological and pharmacological properties.

Fig. 1Aa,Ba show typical current traces recorded in response to depolarizing voltage pulses applied from a holding potential of −60 mV to −40 mV to +80 mV in 10 mV steps. At the more hyperpolarized voltages, this protocol induced a smooth voltage-activated outward current with an activation threshold near −30 mV. At 0 mV, this current reached its steady state in about 15 ms and inactivated very slowly with a time

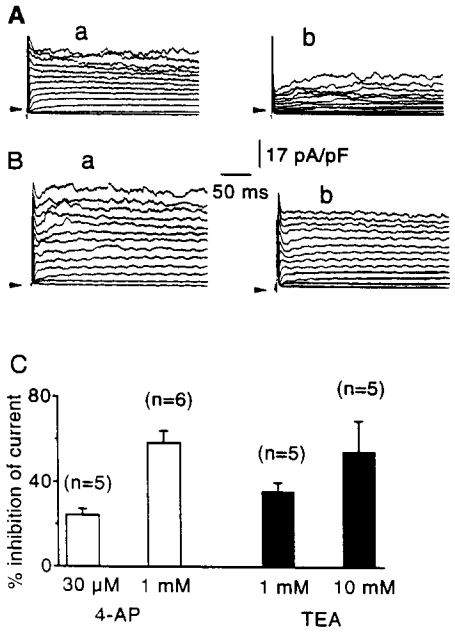

Fig. 1. Effects of 1 mM 4-aminopyridine and of 1 mM tetraethylammonium on the outward currents recorded in isolated smooth muscle cells of rabbit pulmonary artery. The membrane potential was held at −60 mV and only current traces for depolarizing pulses from −40 mV up to 80 mV are shown. The capacitive transients have been omitted for clarity. (Aa) Series of outward current records in control conditions (PSS). (Ab) Same cell and same voltage command protocol as in (Aa) but in the presence of 1 mM 4-aminopyridine. In a different cell, using the same pulse protocol as in (A), recordings of control currents before (Ba) and after application of 1 mM tetraethylammonium (Bb). (C) Inhibition of the current expressed as a percentage of the control value, measured from a holding potential of −60 mV at a +20 mV depolarizing pulse to test the 4-aminopyridine effect (open columns) and measured at +80 mV for the tetraethylammonium effect (black columns). The results represent the mean (histogram) and S.E.M. (vertical bar) effect for the number of cells shown in parentheses. Compared to control values all differences were statistically significant ($P < 0.05$, paired *t*-test). The arrowheads in (A) and (B) indicate the zero current level.

constant of 11 s (data not shown). For larger depolarizations above +20 mV, we observed a transient component which activated and inactivated rapidly, followed by a steady-state and noisy current component that was sustained over 50 ms (Fig. 1Ba). 4-Aminopyridine, at the concentration of 1 mM, markedly decreased the smooth currents developing in response to low voltage depolarizating pulses (less than +20 mV) and almost totally blocked the transient current component (Fig. 1Ab). However, the noisy current that developed at potentials more positive than +40 mV did not seem to be affected (Fig. 1A). As seen in Fig. 1C, the inhibitory effect of 4-aminopyridine on the steady-state outward current observed at +20 mV amounted to 58 ± 5% for a concentration of 1 mM. At this concentration, 4-aminopyridine also depolarized the cell by 16.0 ± 0.5 mV. In contrast to 4-aminopyri-

dine, tetraethylammonium at the concentration of 1 mM did not seem to affect the smooth current, nor the transient current component (Fig. 1Bb), but decreased the noisy current component observed for depolarizations more positive than +40 mV (Fig. 1B). The inhibitory effect of tetraethylammonium on the noisy current measured at +80 mV amounted to 36 ± 4% at 1 mM (Fig. 1C). 1 mM tetraethylammonium did not alter the cell resting potential. All these effects were fully reversible upon drug washout (data not shown).

Decreasing the holding potential from −60 mV to 0 mV (a maneuver known to inactivate the transient current in a number of preparations) markedly affected the current recorded in response to depolarizing pulses, as well as its sensitivity towards 4-aminopyridine and tetraethylammonium (compare Figs. 1 and 2). Indeed, with a holding potential of 0 mV, the current measured at the end of a 300 ms pulse to +20 mV was decreased by about 73% and the transient current elicited by voltage pulses more positive than +20 mV (Fig. 1Aa) was fully inactivated. However, the noisy current component observed for larger depolarizations was still present (Fig. 2Aa,Ba). Under these conditions, 4-aminopyridine (1 mM) had no effect, except for decreasing the holding current (Fig. 2Ab). In contrast, the inhibitory effect of 1 mM tetraethylammonium on the noisy current was more pronounced than it was with a holding potential of −60 mV and amounted to 58 ± 4% (Fig. 2C). No supplemental effect was observed with 10 mM tetraethylammonium compared to 1 mM tetraethylammonium.

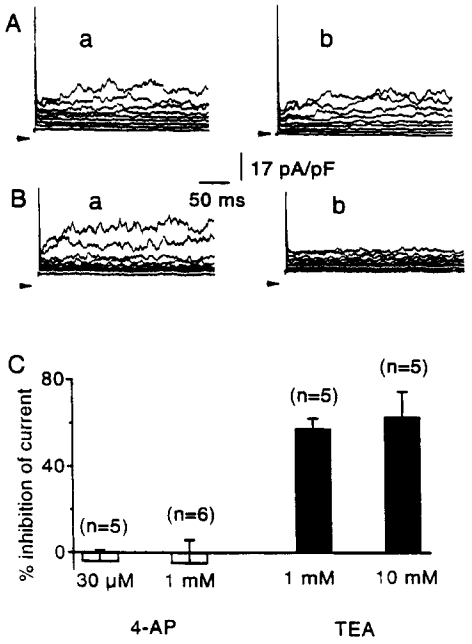

Fig. 2. Same type of experiments as those described in Fig. 1 but with a holding membrane potential of 0 mV. Only current traces for depolarizing pulses from 0 mV up to 80 mV are shown. In (A) the control currents were unchanged after application of 1 mM 4-aminopyridine. 1 mM tetraethylammonium (Bb) almost totally abolished the noisy voltage-activated currents which developed at strong depolarizations (Ba). (C) As in Fig. 1 the histogram represents the mean inhibition (vertical bar, S.E.M.) of the current, expressed as a percentage of the control value measured from a holding potential of 0 mV at a +20 mV. The effect of 4-aminopyridine was not significant (open columns) whereas tetraethylammonium (black columns) reduced significantly the current measured at +80 mV ($P < 0.05$, paired t-test). The arrowheads in (A) and (B) indicate the zero current level.

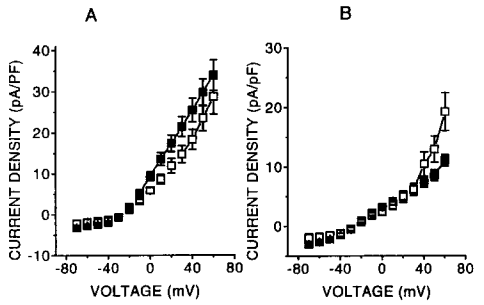

Fig. 3. Effect of free $[Ca^{2+}]_i$ on the mean current-voltage relationships obtained in cells held at −60 mV (A) or 0 mV (B). Data points in (A) and (B) represent the mean values of 20 control cells with 1 nM free $[Ca^{2+}]_i$ (■) and nine cells with 100 nM free $[Ca^{2+}]_i$ (□). The vertical bars represent ±S.E.M.

Elevating the free $[Ca^{2+}]_i$ from 1 nM to 100 nM decreased the steady-state outward K^+ current at all potentials when the holding potential was −60 mV (Fig. 3A) but increased the K^+ current only for potentials more positive than +30 mV when the holding potential was 0 mV (Fig. 3B).

We can conclude from our results that beside a transient K^+ current component, the K^+ current of pulmonary artery cells is composed of (i) a smooth, slowly inactivating, Ca^{2+}- and 4-aminopyridine-sensitive current component with a low threshold for activation (~ −30 mV) and which is inactivated at 0 mV; (ii) a noisy, non-inactivating, Ca^{2+}- and tetraethylammonium-sensitive component with a high threshold for activation (more positive than +30 mV). From its characteristics and pharmacological properties, the smooth component corresponds to the previously described delayed rectifier K^+ current ($I_{K(dr)}$) and the noisy component to the high conductance, Ca^{2+}-activated K^+ current ($I_{BK(Ca)}$).

3.2. Effect of NS-004 on $I_{K(dr)}$

The effects of NS-004 were examined on the membrane K^+ current of pulmonary artery cells. With a low free $[Ca^{2+}]_i$, and a membrane potential held at −60 mV, voltage command pulses were applied from −70 to +20 mV, conditions which mainly activate $I_{K(dr)}$. NS-004 had no effect on $I_{K(dr)}$ (Fig. 4Ba) at 10 μM, but

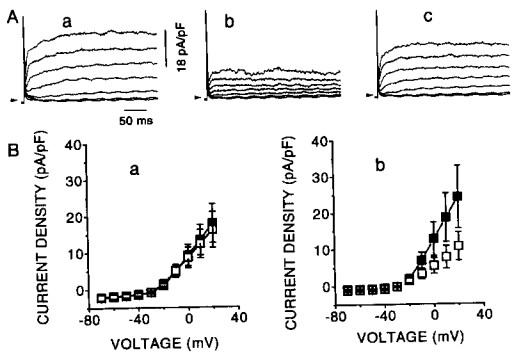

Fig. 4. Reversible inhibition of the delayed rectifier current by NS-004. (A) Examples of the delayed rectifier current induced by depolarizing pulses applied between −40 mV and 20 mV (10 mV step), membrane potential held at −60 mV in control (a), in 50 μM NS-004 (b), or after removal of the drug (c). (B) Current-voltage relationships calculated for cells with a membrane potential held at −60 mV. In eight cells, compared to control condition (a,b,■), NS-004 10 μM (a,□) induced no significant effect but at 50 μM NS-004 (b,□) a significant decrease was observed. $P < 0.05$, paired t-test. The arrowheads in (A) indicate the zero current level and the vertical bars represent ±S.E.M.

significantly reduced $I_{K(dr)}$ at 50 μM (Fig. 4A,Bb). These effects of NS-004 were reversible upon washout of the drug, as shown in Fig. 4A.

3.3. Effects of NS-004 on $I_{BK(Ca)}$

With a low free Ca^{2+} concentration in the pipette and a membrane potential held at 0 mV, strong depolarizations up to +60 mV were applied (Fig. 5). NS-004

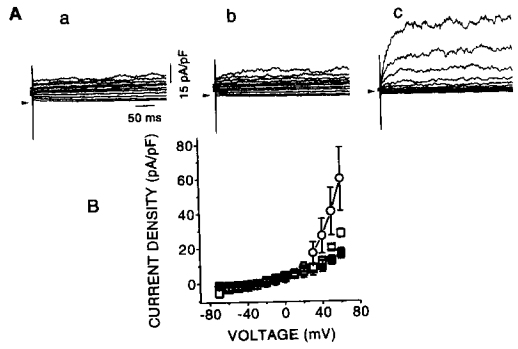

Fig. 5. Activation of the Ca^{2+}-activated K^+ current by NS-004 and by low free $[Ca^{2+}]_i$ (1 nM). (A) Example of Ca^{2+}-activated K^+ current induced by depolarizing pulses applied between −40 mV and +60 mV (10 mV steps) from a membrane potential held at 0 mV in control (a), with 10 μM NS-004 (b), with 50 μM of drug (c). (B) Current-voltage relationships calculated for cells with a membrane potential held at 0 mV. In five cells compared to control conditions (■), NS-004 at 10 μM (□) induced a significant increase that was still observed in three cells treated with 50 μM NS-004 (○). The arrowheads in (A) indicate the zero current level and the vertical bars represent ±S.E.M.

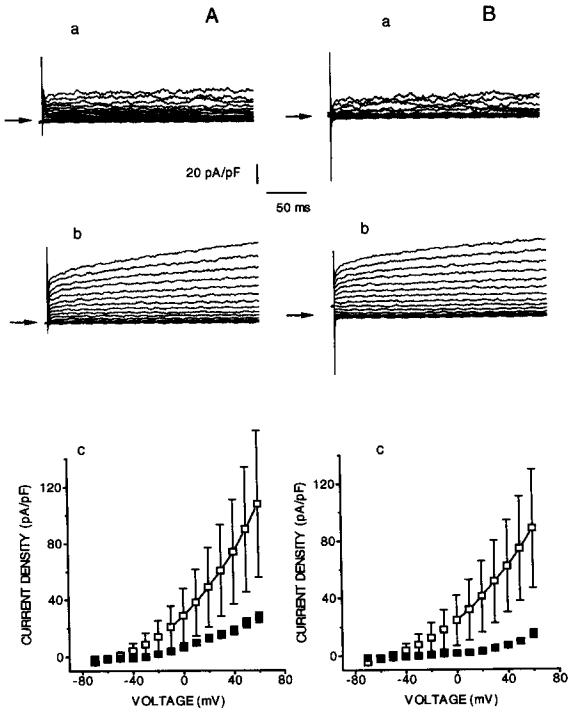

Fig. 6. Effect of NS-004 on outward currents in the presence of high free $[Ca^{2+}]_i$ (100 nM). (A) Typical current recordings for depolarizing pulses from −70 mV to 60 mV (10 mV steps) with a holding potential of −60 mV in control conditions (a), and after application of 10 μM NS-004 (b). The currents strongly increased. (B) Similar results were observed in the cell held at 0 mV. Compared to control conditions (Ba), 10 μM NS-004 (Bb) increased the Ca^{2+}-activated K^+ current. Mean current-voltage relationships calculated for cells with a membrane potential held at −60 mV (Ac) and at 0 mV (Bc). In each case, compared to control conditions (■), 10 μM NS-004 (□) increased the Ca^{2+}-activated K^+ current ($n = 5$). The arrowheads in (A) and (B) indicate the zero current level. In (c), the vertical bars represent ±S.E.M.

at 10 μM induced a significant increase in the current only for depolarizing pulses above +40 mV (Fig. 5B). This effect seemed to be dose-dependent (Fig. 5A) since 10 μM and 50 μM of NS-004 increased $I_{BK(Ca)}$ by 45 ± 18% and 252 ± 60% at +60 mV, respectively (Fig. 5B). 4-Aminopyridine and NS-004 both reduced the holding current (compare Fig. 2Ab and Fig. 5Ac). Morover, the effect of NS-004 on $I_{BK(Ca)}$ was potentiated by an elevation of the free $[Ca^{2+}]_i$ (Fig. 6). Indeed, the increase in the current at +60 mV observed with 10 μM NS-004 was 3.5 times larger with 100 nM free $[Ca^{2+}]_i$ than with 1 nM free $[Ca^{2+}]_i$. Iberiotoxin, a potent blocker of the high conductance Ca^{2+}-activated K^+channel, at 10 nM inhibited by 70% the effect of 10 μM NS-004 (data not shown), strongly suggesting that NS-004 did increase $I_{BK(Ca)}$. As seen in Fig. 6c, in the presence of 100 nM free $[Ca^{2+}]_i$, NS-004 at 10 μM increased the K^+ current at all activating potentials, whatever the holding potential. Thus, elevating the free $[Ca^{2+}]_i$ completely blunted the NS-004-induced

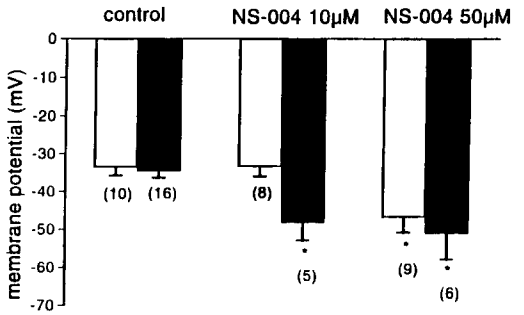

Fig. 7. Effect of NS-004 on the membrane potential measured at 1 nM (open columns) and 100 nM (black columns) free $[Ca^{2+}]_i$ (mean (histogram) ± S.E.M (vertical bars)). * Significant versus control ($P < 0.05$, unpaired t-test).

inhibition of $I_{K(dr)}$ seen for voltage pulses less positive than +20 mV and for a holding potential of −60 mV.

3.4. Effects of NS-004 on the cell resting potential

With a low free $[Ca^{2+}]_i$ (1 nM), 10 μM NS-004 had no effect on the cell resting potential but at 50 μM NS-004 hyperpolarized the cell by 13.8 ± 5 mV (Fig. 7). When $I_{BK(Ca)}$ was further activated by a high free $[Ca^{2+}]_i$ (100 nM), NS-004 (at 10 μM and 50 μM) induced greater membrane hyperpolarizations than those observed with a low free $[Ca^{2+}]_i$ (Fig. 7). Nevertheless, in control conditions, increasing the free $[Ca^{2+}]_i$ did not change the membrane potential.

4. Discussion

4.1. Identification of the delayed rectifier K^+ current and of the Ca^{2+}-activated K^+ current

Most of the outward current recorded from isolated rabbit pulmonary arterial muscle cells appears to be carried by K^+ through K^+ channels. It is well established that the current is abolished when cesium replaces K^+ in the pipette solution; moreover, the current is inhibited by 4-aminopyridine and tetraethylammonium (Okabe et al., 1987; Clapp and Gurney, 1991; Bonnet et al., 1994). In the same preparation, we identified three types of outward currents as described by Okabe et al. (1987) and Clapp and Gurney (1991): the first one was a transient outward current, with rapid kinetics, which was blocked by 4-aminopyridine but not by tetraethylammonium. The second one was the delayed rectifier K^+ current, which plays an important role in maintaining the resting membrane potential (Smirnov et al., 1994) as 1 mM 4-aminopyridine depolarized the cells by about 15 mV. The third one was the Ca^{2+}-activated K^+ current, which activates in cells with a high free $[Ca^{2+}]_i$ and/or a depolarized membrane potential. In these conditions (like hypoxic conditions; Yuan et al., 1993), $I_{BK(Ca)}$ plays a role in the repolarization phase of the action potential. It could also serve as a negative feed-back mechanism in the regulation of the degree of membrane depolarization and hence in the vascular tone (Brayden and Nelson, 1992). Recently, Gelband et al. (1993) have shown that $I_{K(dr)}$ is inhibited by an elevation of intracellular Mg^{2+} and Ca^{2+}. Our results confirm a significant decrease in $I_{K(dr)}$ by an elevation of free $[Ca^{2+}]_i$ in rabbit pulmonary artery cells.

4.2. Activation of $I_{BK(Ca)}$ by NS-004

NS-004 has been reported to increase the whole-cell $I_{BK(Ca)}$ in calf cultured aortic smooth muscle cells (Olesen et al., 1993), in cerebellar granule cells (Olesen et al., 1994b) and in freshly isolated coronary artery smooth muscle cells (Xu et al., 1994) at concentrations up to 3 μM with 10 nM or 100 nM free $[Ca^{2+}]_i$. In our freshly isolated pulmonary artery cells, NS-004 induced the activation of a noisy outward current which was inhibited by 70% by 10 nM iberiotoxin, a specific blocker of high conductance Ca^{2+}-sensitive K^+ channels (Knaus et al., 1994). Since these results were observed at a holding potential of 0 mV and with strong depolarizations (electropysiological conditions which isolate $I_{BK(Ca)}$), they clearly indicate that NS-004 activated BK_{Ca}. We observed that 10 μM NS-004, with a low free $[Ca^{2+}]_i$, had little effect on $I_{BK(Ca)}$ and no effect on the membrane potential. However, at the same drug concentration, the effect on $I_{BK(Ca)}$ or on the membrane potential was considerably potentiated by increasing the free $[Ca^{2+}]_i$. Olesen et al. (1994a) also reported that 30 μM of NS-1619 (an analogue of NS-004) activated $I_{BK(Ca)}$ in aortic smooth muscle cells when the free $[Ca^{2+}]_i$ was 100 nM. This drug was without effect with a low free $[Ca^{2+}]_i$. In addition, in rat cortical neurons Lee et al. (1995) reported than the NS-1619-induced activation of BK_{Ca} required the presence of Ca^{2+} at the intracellular surface. The stimulation of BK_{Ca} by NS-1619 was increased by elevation of the free $[Ca^{2+}]_i$. Olesen et al., in aortic smooth muscle cells (Olesen et al., 1993) and in cerebellar granules cells (Olesen et al., 1994b), showed that NS-004 had a effect on BK_{Ca} kinetics similar to that induced by an increase in free $[Ca^{2+}]_i$ or by membrane depolarization. In addition, in coronary artery isolated cells with 10 nM free $[Ca^{2+}]_i$, NS-004 induced a shift of the current-voltage relationships to more negative potentials (Xu et al., 1994). This NS-004 effect was similar to that induced by an increase in free $[Ca^{2+}]_i$. All these observations suggest that NS-004 might change the Ca^{2+} sensitivity of BK_{Ca}. The underlying mechanism of this modification might be related to channel protein phosphorylation, which changes the protein configuration, thus modifying the affinity of sites for Ca^{2+}.

Such a hypothesis is supported by the findings of Gribkoff et al. (1994), in epithelial cells, that activation of the cystic fibrosis chloride channel by NS-004 was dependent on pretreatment with forskolin, an activator of protein kinase A. Kume et al. (1989) also reported that protein kinase A increases the opening probability of BK_{Ca}. A similar observation was made with aortic smooth muscle cells (Sadoshima et al., 1988). In addition, Robertson et al. (1992) demonstrated that magnesium-ATP increased the activity of a Ca^{2+}-sensitive K^+ channel. They suggested that this effect involved phosphorylation, which increased the protein channel sensitivity to Ca^{2+} and ultimately enhanced the voltage sensitivity (Albarwani et al., 1994). All these observations strongly suggest that NS-004 acts on a phosphorylated form of BK_{Ca} and might change its Ca^{2+} sensitivity.

Nevertheless, in coronary cells, NS-1619 increases the open probability of BK_{Ca} in inside-out patches, even in the absence of Ca^{2+} (Xu et al., 1994). In portal vein, NS-1619 also stimulates $I_{BK(Ca)}$ in a Ca^{2+}-independent manner (Edwards et al., 1994). These conflicting results could be explained by differences in the Ca^{2+} sensitivity of Ca^{2+}-activated K^+ channels in different tissues. The report by Sansom and Stockand (1994) of different sensitivities of BK_{Ca} isochannels in bovine mesenteric vascular smooth muscle may provide a further explanation.

Gribkoff et al. (1994) have shown that NS-004 opens cystic fibrosis chloride channels and, in addition, that this activating effect of the drug occurs in a low free $[Ca^{2+}]_i$. They also suggested that NS-004 can directly activate this chloride channel since this drug was effective in excised patches. We tested the effect of NS-004 before and after application of the chloride channel inhibitor anthracene-9-carboxylic acid. No differences were observed in the NS-004-induced modification of the outward current (data not shown). Thus, this compound did not act through an anthracene-9-carboxylic acid-sensitive chloride current in pulmonary artery cells.

4.3. Inhibition of $I_{K(dr)}$ by NS-004

NS-004 and NS-1619 inhibit $I_{K(dr)}$ in coronary arteries (Xu et al., 1994) and in portal vein cells (Edwards et al., 1994), respectively. Our results demonstrated that, at low free $[Ca^{2+}]_i$, NS-004 inhibited $I_{K(dr)}$ in pulmonary artery cells at concentrations similar to those that activated $I_{BK(Ca)}$.

The comparative reducing effect of 4-aminopyridine (Fig. 2Ab) and NS-004 (Fig. 5Ac) on the holding current would suggest that (i) NS-004 inhibited a residual $I_{K(dr)}$ component which was not completely inactivated or (ii) NS-004 induced inhibition of another 4-aminopyridine-sensitive K^+ current which was not inactivated. The second hypothesis is supported by Evans et al. (1995) who found, in rabbit pulmonary artery cells, a 4-aminopyridine-sensitive K^+ conductance which do not inactivate.

4.4. Hyperpolarization of the cell membrane by NS-004

With 1 and 100 nM free $[Ca^{2+}]_i$, BK_{Ca} was activated only at very positive membrane potentials and 1 mM tetraethylammonium did not alter the membrane potential. Moreover at low free $[Ca^{2+}]_i$, 10 μM NS-004, which slightly increased $I_{BK(Ca)}$ at very depolarized potentials, had no effect on the membrane potential. This suggests that BK_{Ca} did not play an important role in regulating the resting membrane potential. When the free $[Ca^{2+}]_i$ was increased, 10 μM NS-004 induced a hyperpolarization of 13.6 ± 6 mV, and in these conditions the $I_{BK(Ca)}$ was the major current. This is consistent with the argument that BK_{Ca} may regulate the level of membrane potential in cells which have an increased $[Ca^{2+}]_i$ and depolarized membrane potential. $I_{BK(Ca)}$ serves, in these conditions, as a negative feed-back mechanism in the regulation of the degree of membrane depolarization (Brayden and Nelson, 1992). $I_{K(dr)}$, which plays an important role in maintaining the resting membrane potential (since 4-aminopyridine depolarizes the cells by about 15 mV), was inhibited by NS-004. However, we never observed a depolarization, suggesting that other mechanisms are effectively involved in the control of the cell resting potential.

Xu et al. (1994) have shown that NS-004 also inhibits an ATP-sensitive K^+ channel which modulates the membrane potential (Clapp and Gurney, 1992). Like Xu et al. (1994), we think that the effects of NS-004 on the resting membrane potential depend on the relative activities of the three types of K^+-channels and we suppose that NS-004 depolarizes the rabbit pulmonary artery smooth muscle cells when the inhibitory effects on K_{ATP} and K_{dr} were dominant.

Since the holding current was reduced by 4-aminopyridine (Fig. 2Ab) and NS-004 (Fig. 5Ac), we also suggest that NS-004 inhibits the novel K^+ conductance which determines the resting potential in rabbit pulmonary smooth muscle cells (Evans et al., 1995) and then induces depolarization.

4.5. Conclusions

This work clearly demonstrates that an elevation of free $[Ca^{2+}]_i$ induced a potentiation of NS-004-induced activation of high conductance Ca^{2+}-sensitive K^+ channels and of NS-004-induced hyperpolarization of the cell. Also, like an increased free $[Ca^{2+}]_i$, NS-004 inhibited the delayed rectifier K^+ channel.

The opposite Ca^{2+} and NS-004 sensitivity of the two types of K^+ current suggests that each of these chan-

nels plays a different role in controlling the ion flux through the pulmonary artery cell membrane. If this different role is confirmed, it could be an interesting explanation for the opposite effect of hypoxia reported for Ca^{2+}-sensitive K^+ channels in rat pulmonary artery cells (Post et al., 1992) and in rabbit pulmonary artery cells (Bonnet et al., 1994). We conclude that NS-004 could be a useful tool for investigation of the pharmacology of BK_{Ca} and for its characterization (Dworetzky et al., 1994). It could also be a potential therapeutic agent for pulmonary hypertension. NS-004, as a K^+ channel activator, could be a new class of therapeutic drug to modulate hypoxic vasoconstriction.

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