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Nitroblue tetrazolium blocks BK channels in cerebrovascular smooth muscle cell membranes

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1 The effects of *p*-nitroblue tetrazolium (NBT) on large conductance, calcium-activated potassium channels (BK channels) in enzymatically dispersed rat cerebrovascular smooth muscle cells (CVSMCs) were examined.

2 Patch clamp methods were employed to record single BK channel currents from inside-out patches of CVMC membrane maintained at 21–23°C.

3 When applied to the cytoplasmic face of inside-out membrane patches (internally applied NBT), micromolar concentrations of NBT reversible reduced the mean open time of BK channels, without changing channel conductance.

4 NBT altered the frequency distribution of BK channel open times from a two exponential to a single exponential form.

5 In the absence of NBT, mean channel open time increased on membrane depolarization. In the presence of internally applied NBT, mean channel open became essentially independent of membrane potential.

6 Internally applied NBT also reduced the mean closed time of BK channels when measured at membrane potentials in the range −80 mV to +20 mV.

7 The combined effects of internal NBT on mean open and closed times resulted in the suppression of BK channel open probability when measured at positive membrane potentials.

8 When applied to the external membrane face, micromolar concentrations of NBT reduced mean channel open time progressively as the membrane was hyperpolarized, and also reduced open probability at negative membrane potentials.

9 A model is proposed in which NBT alters channel gating by binding to a site at or near to the cytoplasmic membrane face.

10 Externally applied NBT suppressed BK channel open probability at concentrations which also inhibit nitric oxide synthase (NOS). Therefore, the potential role of potassium channel block in NBT actions previously attributed to NOS inhibition is discussed.

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Abbreviations: BH_4, (6R)-5,6,7,8-tetrahydrobiopterin; BK channel, large conductance, calcium-activated potassium channel; $[Ca^{2+}]_i$, free internal calcium concentration; cNOS, constitutive nitric oxide synthase; CVSMC, cerebrovascular smooth muscle cell; EGTA, ethylene glycol-bis(β-aminoethyl ether) N,N,N′-tetraacetic acid; eNOS, endothelial nitric oxide synthase; HEPES, (N-[hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]); iNOS, inducible nitric oxide synthase; kHz, kiloHerz; mM, millimoles/litre; MΩ, megOhms; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; NBT, *p*-nitroblue tetrazolium; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; P_o, channel open probability; pS, picoSiemen; PTZ, pentylenetetrazole; T_c, mean channel closed time; T_o, mean channel open time; V, membrane voltage

Introduction

The amphoteric electron acceptor *p*-nitroblue tetrazolium (3,3′-[3,3′ dimethoxy-(1,1′ biphenyl)-4,4′-diyl]bis[2-(4-nitrophenyl)-5-phenyl-2H-tetrazolium dichloride], NBT) is widely employed in basic and clinical research and in the diagnostic evaluation of patients (Ardati *et al*., 1997; Armstead, 1999; Sun *et al*., 1997; Suga *et al*., 1996). NBT is reduced to the insoluble blue dye formazan in the presence of electron donors, such as the free oxygen radicals generated by nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase).

During bacterial and fungal infections, the NADPH oxidase activity of neutrophils is elevated. Hence, the rate of formazan production from NBT can be used to screen for the presence of infection (Binder *et al*., 1975; Chomarat *et al*., 1997; Suga *et al*., 1996) and to monitor the competency of the non-specific immune system (Ardati *et al*., 1997). The NBT reduction test can be employed to detect genetic defects in NADPH oxidase function, as seen in chronic granulomatous disease (Zentilin *et al*., 1996; Bjorgvinsdottir *et al*., 1997). Reduction of NBT by mitochondrial succinate dehydrogenase occurs only in healthy cells, allowing NBT and related tetrazolium salts to be employed as sensitive indicators of cell viability (Hale & Wenzel, 1978; Sun *et al*., 1997; Janin *et al*., 1998).

A further agent capable of reducing NBT is nitric oxide synthase (NOS), a homodimeric enzyme which catalyses the synthesis of nitric oxide (NO) from L-arginine. NOS exists predominantly in two constitutively expressed (cNOS) and one inducible isoform (iNOS) in mammalian tissues (Schmidt *et al*., 1993; Knowles & Moncada, 1994). cNOS is calcium/

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calmodulin-dependent enzyme expressed in neurones, skeletal muscle and pancreatic islet cells as neuronal NOS (nNOS) (Hope *et al*., 1991), and in vascular endothelial cells as endothelial NOS (eNOS) (Faraci & Brian, 1994; Föstermann, 1994; Busse & Fleming, 1995). iNOS is a calmodulin-containing enzyme which is active at trace Ca^{2+} levels and can be induced by tissue injury, proinflammatory cytokines and bacterial endotoxins in many cell types, including macrophages, vascular endothelia and smooth muscle cells (Nathan & Xie, 1994; Wileman *et al*., 1995; Clark *et al*., 1996).

NBT non-competitively inhibits NOS by providing an alternative substrate for the NADPH-diaphorase activity common to all NOS isoforms. This activity normally converts the intermediate NOS product N^{ω}-OH-L-arginine to NO and L-citrulline, and utilizes NADPH and (6R)-5,6,7,8-tetrahydrobiopterin (BH_4) as cofactors. In the presence of NBT, however, the NADPH-diaphorase reaction yields formazan (Schmidt *et al*., 1993; Nathan & Xie, 1994). This had lead to the use of NBT in a histochemical test for the presence of NOS in cells and tissues (Hope *et al*., 1991; Schmidt *et al*., 1992; Gabbott & Bacon, 1993). In addition, NBT inhibits lower oesophageal sphincter relaxation in the opossum, suggesting that this relaxation is normally mediated by NOS activity (Conklin *et al*., 1995).

Previously, NBT has been characterized as lacking non-specific neurotoxic or myotoxic effects (Conklin *et al*., 1995). However, the molecule does possess two positively charged tetrazolium rings. This structure, in its uncharged form, is a feature of the convulsant pentylenetetrazole (PTZ), a known blocker of both calcium-dependent and voltage-dependent K^+ channels (Ewald & Eckert, 1983; Oyama, 1987; Sugaya *et al*., 1989; Madeja *et al*., 1994). We now report that NBT significantly reduces the open probability of large conductance, calcium-activated K^+ channels (BK channels) in the membrane of rat cerebrovascular smooth muscle cells (CVSMCs). Furthermore, this effect is seen at low micromolar concentrations of NBT, comparable to the doses employed for NOS inhibition (Hope *et al*., 1991; Conklin *et al*., 1995).

Methods

Cell dispersal

CVSMCs were enzymatically dissociated from the middle, posterior communicating and posterior cerebral arteries of adult Wistar rats (250–300 g). Cerebral arteries were removed aseptically and incubated for 30 min at 37°C in 0.06% protease (Type XXIV, Sigma, U.S.A.), 0.05% collagenase (Type 1A, Sigma) and 0.04% trypsin inhibitor (Type II-S, Sigma). Enzymes were dissolved in Ca^{2+}-free Tyrode's solution of composition (mM): NaCl 138, KCl 4.5, $MgCl_2$ 0.5, Na_2HPO_4 0.33, HEPES 10, (N-[hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]), 5.5 glucose, pH 7.4. Cells were then washed in a holding solution of composition (mM): KOH 70, KCl 70, L-glutamic acid 50, taurine 20, $MgCl_2$ 0.5, K_2HPO_4 1, EGTA 0.5 (ethylene glycol-bis(β-aminoethyl ether) N,N,N′-tetraacetic acid) HEPES 10, creatine 5, pyruvic acid 5 and Na_2ATP 5, pH 7.4. Single smooth muscle cells were isolated by trituration and plated onto glass coverslips. Cells were maintained at 4°C in the above holding solution for up to 48 h prior to use. CVSMCs were identified as smooth muscle cells by indirect fluorescence using a monoclonal antibody against murine smooth muscle alpha-actin (A2547, Sigma, 1 : 400 dilution).

Single-cell electrophysiology

All patch clamp recordings were made at 21–23°C using a List EPC-7 amplifier and microelectrodes of 10–15 MΩ resistance. Standard techniques were used to isolate inside-out membrane patches from relaxed CVSMCs (Hamill *et al*., 1981; Wang & Mathers, 1993). For these recordings, patch electrodes contained solution A of composition (in mM): KCl 140; $CaCl_2$ 1.48; HEPES 10; EGTA 3, pH 7.4 (free calcium concentration 50 nM). The cytoplasmic face of inside-out membrane patches was bathed in solution B of composition (mM): KCl 140; $CaCl_2$ 2.86; HEPES 10; EGTA 3, pH 7.4 (free internal calcium concentration, $[Ca^{2+}]_i = 1\ \mu M$). NBT (Calbiochem, La Jolla, CA, U.S.A.) was applied to the cytoplasmic membrane face of these patches by dissolving the agent in solution B. To determine the effects of NBT when applied at the extracellular membrane face, NBT was dissolved in solution A and injected into patch electrodes.

Single BK channel currents were recorded on videotape at a bandwidth of DC-2 kHz and analysed using commercial software (Instrutech, NY, U.S.A.). Maximization of likelihood was used to fit exponential or Gaussian terms to the observed open time, closed time or amplitude distributions of single BK channel currents. The open probability, P_o of single BK channels was calculated from the relation $P_o = (T_1 + 2.T_2 + ... N.T_N)/N.T_{tot}$, where N was the number of BK channels in the patch, T_{tot} the total record duration, and T_1, T_2 ...T_N the times when at least 1,2 ...N channels were open. BK channels were identified on the basis of their large conductance (>200 pS) and susceptibility to block by 5 nM free Ca^{2+} saline applied to the cytoplasmic membrane surface (Asano *et al*., 1993; Wang & Mathers, 1993; Nelson & Quayle, 1995; Hoang & Mathers, 1998a). Results were expressed as mean ± standard error of the mean (s.e.mean).

Results

NBT reduces the open time of BK channels when applied to the cytoplasmic face of the CVSMC membrane

NBT exerts its known biological actions in the intracellular compartment. Hence we investigated the electrophysiological effects of NBT when applied to the cytoplasmic face of inside-out patches of CVSMC membrane. Figure 1 shows the influence of 7.5 μM NBT on the properties of BK channels studied in one such membrane patch, voltage-clamped to a membrane potential, V = +60 mV. It can be seen that the predominant effect of NBT under these conditions was to reduce the average time BK channels remain in the open state. This effect was readily reversible on wash-out of the drug (Figure 1).

Inspection of traces such as those shown in Figure 1 suggested that NBT had little effect on the amplitude of currents flowing in single BK channels. This impression was confirmed by measuring the conductance and reversal potential associated with single BK channel activity. Single channel conductance was 254 ± 13 pS in Control solution and 255 ± 11 pS in the presence of 7.5 μM NBT (not significantly different, ANOVA, $n = 7$ patches). Reversal potentials calculated from linear regression analysis of current-voltage relations were found to be −0.07 and 0.17 mV for Control and NBT data respectively (not significantly different from each other or from the expected value of 0 mV). When tested at the higher concentrations of 12 μM ($n = 4$ patches) and 37 μM ($n = 4$ patches), NBT remained without effect on the reversal potential or conductance of BK channels.

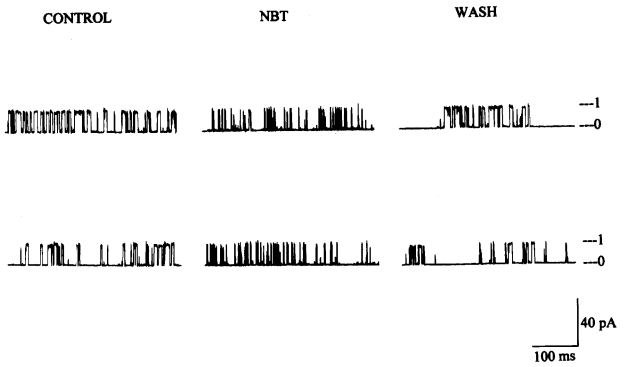

Figure 1 Reversible effect of 7.5 μM NBT on the gating of a single BK channel studied in an inside-out patch of CVSMC membrane. This patch was voltage clamped at a membrane potential of V = +60 mV with $[Ca^{2+}]_i = 1$ μM. NBT was applied by perfusion to the cytoplasmic membrane face. 0 and 1 denoted channel closed and channel open states, respectively. Bandwidth of recordings dc-1 kHz.

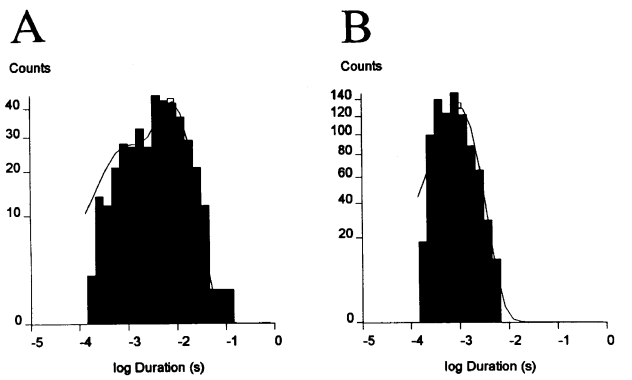

Figure 2 Effect of NBT on the open time distribution of a single BK channel in an inside-out membrane patch, voltage clamped to V = −20 mV with $[Ca^{2+}]_i = 1$ μM. These distributions were plotted as the square root of the number of observations (ordinates) against the logarithm of the open time (abscissae). (A) Open time distribution obtained in Control medium. This distribution contained 396 channel openings and was well described by the sum of two exponential terms (smooth curve) using the following fit parameters, defined in the text. $\tau_{of} = 0.59$ ms; $\tau_{os} = 7.8$ ms, $A_{of}/(A_{of} + A_{os}) = 0.288$. This yielded a mean open time, $T_o = 5.7$ ms. (B) Open time distribution after application of 7.5 μM NBT to the cytoplasmic membrane face. This distribution comprised 840 channel openings. It was well described by a single exponential term (smooth curve) with a time constant, equivalent to T_o, of 0.99 ms.

NBT alters the kinetic parameters governing the open times of BK channels

Kinetic analysis was undertaken to further investigate the effect of NBT on the open times of BK channels. In the absence of NBT, frequency distributions of channel open times were well described by the sum of two exponential terms at all membrane voltages studied, as expected from previous reports (Wang & Mathers, 1993; Hoang & Mathers, 1998a,b; Pfründer & Kreye, 1991). These distributions (see Figure 2) were therefore well fit by an equation of form

$$y = A_{of} \cdot e^{-t}/\tau_{of} + A_{os} \cdot e^{-t}/\tau_{os}. \quad (1)$$

The voltage-dependent fast and slow time constants τ_{of} and τ_{os} governed the amplitude terms A_{of} and A_{os} respectively. The mean channel open time, T_o was calculated from the relation $T_o = A_{of}/(A_{of} + A_{os}) \cdot \tau_{of} + A_{os}/(A_{of} + A_{os}) \cdot \tau_{os}$. At a membrane potential V = +60 mV, mean values of $\tau_{of} = 3.2 \pm 0.5$ ms, $\tau_{os} = 13.1 \pm 0.54$ ms and $A_{of}/(A_{of} + A_{os}) = 0.754$) were obtained, yielding a value of $T_o = 5.6 \pm 0.28$ ms ($n = 5$ patches).

When 7.5 μM NBT was applied at the cytoplasmic membrane face, open time distributions of BK channels were altered to a single exponential term (see Figure 2), characterized by a rapid time constant of 1.4 ± 0.02 ms at V = +60 mV ($n = 5$ patches). Hence a novel pathway causing channel closure or occlusion became dominant in the presence of NBT.

When applied at the cytoplasmic membrane face, NBT renders the mean channel open time voltage-independent

The influence of membrane potential on the ability of NBT to decrease the mean open time of BK channels was now studied. In the absence of NBT the mean open time, calculated as indicated in the previous paragraph, increased on membrane depolarization (Figure 3). This was in agreement with previous reports (Wang & Mathers, 1993; Pfründer & Kreye, 1991). T_o increased from a mean of 1.6 ± 0.18 ms at V = −80 mV to 5.6 ± 0.28 ms at V = +60 mV ($P < 0.05$, ANOVA, $n = 5$ patches). However, when 7.5 μM NBT was applied to the cytoplasmic membrane face, T_o became essentially independent of membrane voltage, adopting a value of about 1 ms (Figure 3). At all membrane potentials tested, T_o was significantly reduced in the presence of NBT ($P < 0.05$, ANOVA, Figure 3).

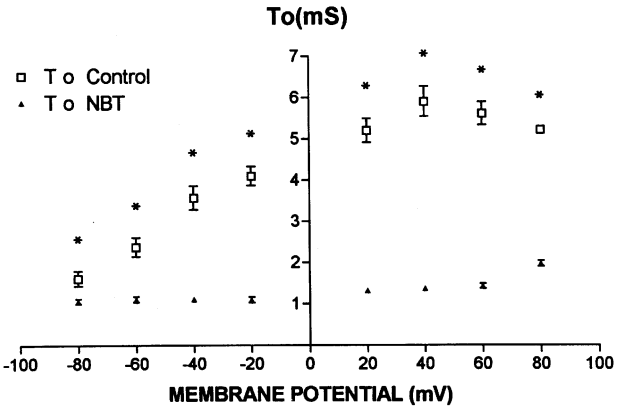

Figure 3 Influence of NBT on the voltage-dependence of mean channel open time, T_o. The graph shows the relationship between T_o and membrane potential, V in Control medium and in the presence of 7.5 μM NBT, applied to the cytoplasmic face of inside-out membrane patches. Data represent means ± s.e.mean from five patches. Asterisks indicate membrane voltages at which mean values of T_o were significantly different in the absence and presence of NBT ($P < 0.05$, ANOVA). $[Ca^{2+}]_i = 1$ μM.

NBT also reduces the mean closed time of BK channels at negative membrane potentials

The effect of adding NBT to the cytoplasmic face of the membrane on the mean closed time, T_c of BK channels was now examined. In the absence of NBT, T_c decreased strongly on membrane depolarization, exhibiting values of 2.5 ± 0.10 s at V = −80 mV and 13.8 ± 2.4 ms at V = +80 mV ($P < 0.05$, ANOVA, $n = 8$ patches, Figure 4).

In the presence of 7.5 μM NBT, T_c remained strongly voltage-dependent (Figure 4) while T_c was reduced relative to control values when measured at membrane potentials in the range of −80 mV to +20 mV ($P < 0.05$, ANOVA, Figure 4).

At more positive potentials, in the range of +40 mV to +80 mV, NBT produce no significant change in T_c (Figure 4).

The combined effects of NBT on T_o and T_c result in a depression of BK channel open probability at positive membrane potentials

Since channel open probability, P_o is a function of both T_o and T_c, the influence of NBT on P_o and the dependence of this influence on membrane potential were next examined. As shown Figure 5, P_o increased on membrane depolarization, in agreement with previous studies (Wang & Mathers, 1993; Hoang & Mathers, 1998a). When 7.5 μM NBT was applied to the cytoplasmic membrane face, P_o remained voltage-dependent. Over the potential range V = −80 mV to V = +20 mV, no change was seen in the value of P_o relative to Control (Figure 5). However, NBT significantly reduced P_o at membrane potentials in the range V = +40 mV to +80 mV (Figure 5). The effect of NBT on P_o was dose-dependent. At V = +60 mV, 7.5, 12 and 37 μM NBT reduced P_o by an average of 31±5, 80±10 and 92±13% respectively (n = 6 patches at each NBT concentration tested).

Externally applied NBT also influences the gating, but not the conductance of BK channels

The effects of externally applied NBT on BK channels were examined in inside-out patches by including the agent in the patch electrode solution. As shown in Figure 6, externally applied NBT (7.5 μM) had no significant effect on the mean open time of BK channels when examined at strongly positive membrane potentials in the range +60 mV to +80 mV. However, NBT reduced T_o in a progressive manner as the membrane was hyperpolarized from +40 mM to −80 mV. Externally applied NBT (7.5 μM) had no significant effects on either the conductance or reversal potential of BK channels (Control conductance 255±14 pS, reversal potential −0.2 mV; NBT conductance 259±16 pS, reversal potential −2.4 mV, P > 0.05, ANOVA for both parameters, n = 8 patches).

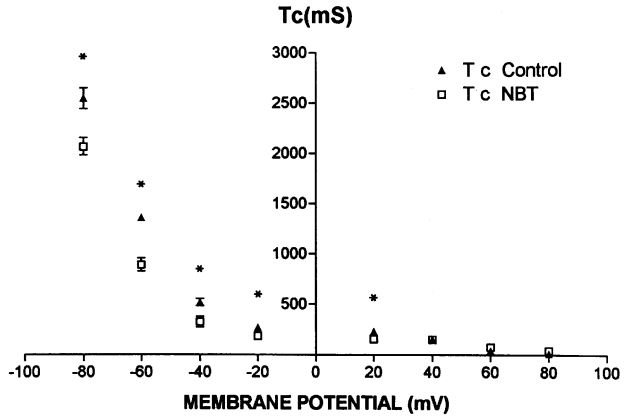

Figure 4 The influence of NBT on the relationship between membrane voltage and the mean closed time, T_c of BK channels. NBT (7.5 μM) was applied to the cytoplasmic face of inside-out membrane patches. Data represent means±s.e.mean from eight patches. Asterisks indicate membrane voltages at which mean values of T_c were significantly different in the absence (Control) and presence of NBT (P < 0.05, ANOVA). $[Ca^{2+}]_i$ = 1 μM.

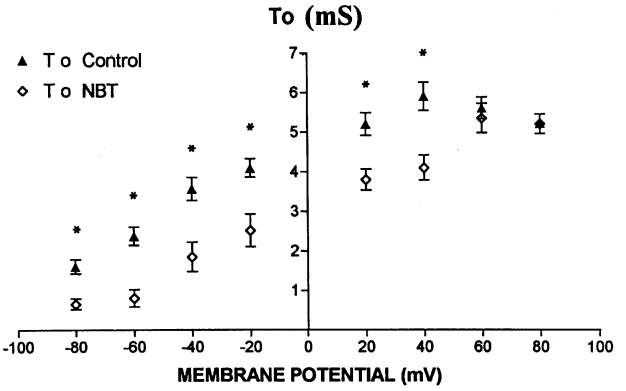

Figure 6 The influence externally applied NBT on the relationship between membrane voltage and the mean open time, T_o of BK channels. NBT (7.5 μM) was applied to the external face of inside-out membrane patches. Data represent means±s.e.mean from eight patches. Asterisks indicate membrane voltages at which mean values of T_o were significantly different in the absence (Control) and presence of NBT (P < 0.05, ANOVA). $[Ca^{2+}]_i$ = 1 μM.

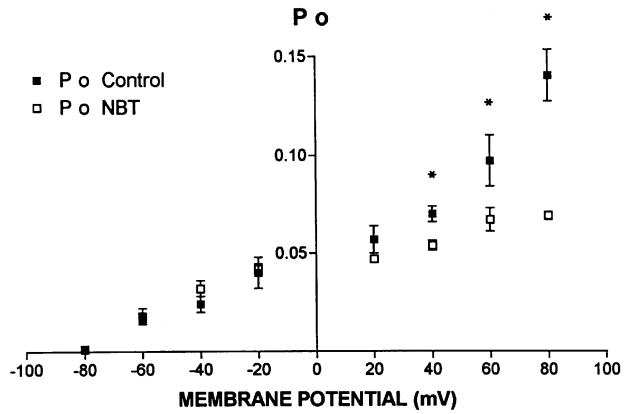

Figure 5 The effect of NBT on the relationship between membrane potential and open probability, P_o of BK channels. NBT (7.5 μM) was applied to the cytoplasmic face of inside-out membrane patches. Data represent means±s.e.mean from eight patches. Asterisks indicate membrane voltages at which mean values of P_o were significantly different in the absence (Control) and presence of NBT (P < 0.05, ANOVA). $[Ca^{2+}]_i$ = 1 μM.

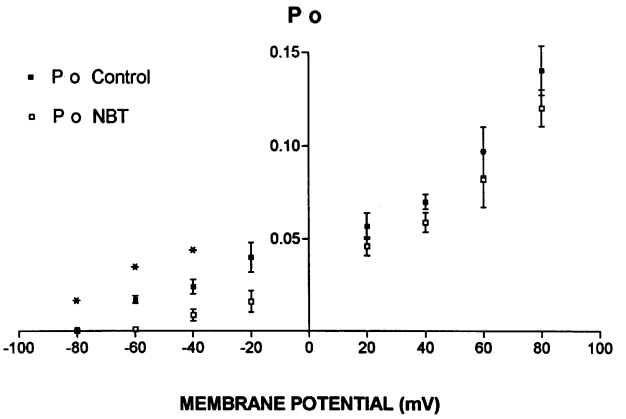

Figure 7 The effect of externally applied NBT on the relationship between membrane voltage and the open probability, P_o of BK channels. NBT (7.5 μM) was applied to the external face of inside-out membrane patches. Data represent means±s.e.mean from eight patches. Asterisks indicate membrane voltages at which mean values of P_o were significantly different in the absence (Control) and presence of NBT (P < 0.05, ANOVA). $[Ca^{2+}]_i$ = 1 μM.

Externally applied NBT reduces P_o at negative membrane potentials

The effect of externally applied NBT on the open probability of BK channels was now studied. As shown in Figure 7, NBT (7.5 μM) had no significant effect on P_o when examined at membrane potentials in the range of -20 mM to $+80$ mV. However, NBT reduced P_o at membrane potentials in the range of -80 mV to -40 mV, which encompasses the normal resting membrane potential in CVSMCs (Hirst *et al*., 1986; Wang & Mathers, 1993).

Discussion

The present study has revealed the ability of NBT to reversibly suppress the open probability of mammalian BK channels. NBT exerted this effect at micromolar concentrations and was active when applied at either the extracellular or the cytoplasmic face of the plasma membrane. Kinetic analysis showed that NBT acted primarily by reducing the mean open time, T_o of BK channels. The action of NBT on T_o was found to be largely independent of membrane voltage when NBT was applied at the cytoplasmic face. However, externally applied NBT reduced T_o more strongly as the membrane was hyperpolarized.

These observations can be explained by postulating that the site of NBT action is located at, or very close to, the cytoplasmic membrane face. Since internally applied NBT molecules need not traverse the membrane field to reach this site, their action is expected to be largely voltage-independent. However, externally applied NBT molecules must penetrate deeply through the membrane field. Hence their effectiveness should be lessened at positive membrane potentials, as was indeed seen experimentally.

The amphoteric NBT molecule penetrates cell membranes and may therefore interact with both the hydrophobic and charged domains of membrane proteins. This interaction could involve the direct binding of NBT to the BK channel protein, or to a closely associated regulatory molecule. NBT may enter the transiently block open BK channels, preventing channel closure until eventual dissociation of the drug. If NBT indeed acts in this manner, the observed reduction in T_o and unaltered conductance suggest that drug molecules reside in the channel for a few milliseconds. This is characteristic of the 'intermediate' class of open channel blocking agents (Neher & Steinbach, 1978; Hille, 1992; Pfründer & Kreye, 1991).

At negative membrane potentials, internally applied NBT produced blocked episodes which were brief compared to the average duration of the closed channel state. Hence NBT significantly decreased the mean channel closed time. At positive membrane potentials, however, the voltage-dependence of BK channel gating resulted in channel closed times comparable to the blocking time of NBT. Under these conditions, NBT had little effect on the average closed time of BK channels. This mechanism may account for the inability of internally applied NBT to reduce P_o when applied at negative membrane potentials, despite the marked effect of the drug on the mean open time of BK channels.

NBT (molecular weight 818 Daltons) is appreciably larger than the tetraethylammonium ion, an agent known to enter and block BK channels (Wang & Mathers, 1993; Carl *et al*., 1993). However, insertion of part of the NBT molecule, such as the charged tetrazolium ring, may be sufficient to occlude open BK channels. Alternatively, NBT may block by binding to an intramembranous domain of the channel protein which lies outside the pore of the BK channel. A binding site in this general location has been proposed to explain the effects of PTZ on the gating of cloned Kv1.1 potassium channels (Madeja *et al*., 1994). It should be noted, however, that NBT displayed much greater potency than PTZ as a K^+ channel blocker, being active at 1000 fold lower concentrations.

When applied to the external membrane face at a physiologically relevant membrane potential of -40 mV, NBT suppressed BK channel activity with an apparent K_i of about 7 μM. This is similar to values reported for non-competitive inhibition of NOS activity by NBT, namely $K_i = 3-11$ μM (Hope *et al*., 1991; Conklin *et al*., 1995). Unlike NBT, however, competitive NOS inhibitors such as N^{ω}-nitro-L-arginine methyl ester do not alter the open probability of BK channels in CVSMC membranes (Hoang & Mathers, 1998a). This implies that the effects of NBT on BK channel kinetics were probably not mediated by nitric oxide synthase.

NBT causes an abrupt increase in the tone of smooth muscle in the lower oesophageal sphincter of the opossum. Again, however, this effect is absent when competitive inhibitors of nitric oxide synthase are used (Conklin *et al*., 1995). The present results may explain this anomaly, since blockade of BK channels by NBT is likely to enhance contraction in smooth muscle fibres (Nelson & Quayle, 1995). Therefore, care should be exercised when employing NBT as a selective probe for NOS activation in excitable tissues.

Systemic injection of NBT results in varied and as yet unexplained changes in regional blood flow in the rat. Some vascular beds, such as the renal circulation, exhibit a marked increase in resistance, while others show a decreased resistance to blood flow (Davisson *et al*., 1993). BK channels open in response to membrane depolarization and to increases in intracellular free calcium levels. These channels are therefore important regulators of myogenic tone in vascular smooth muscle (Asano *et al*., 1993; Nelson & Quayle, 1995). Myogenic tone typically develops at resting membrane potentials in the range -40 to -20 mV (Nelson & Quayle, 1995). Hence the ability of externally applied NBT to block vascular BK channels in this potential range may contribute to the complex haemodynamic effects of NBT seen *in vivo*.

BK channels also exist on endothelial cells in a variety of vessels, including rabbit aorta (Hutcheson & Griffith, 1994), pig coronary artery (Baron *et al*., 1996), rat mesenteric artery (Plane *et al*., 1996), rabbit iliac artery (Cooke *et al*., 1991) and human umbilical vein (Haburcak *et al*., 1997). BK channels facilitate the release of nitric oxide from endothelial cells which have been stimulated by application of sheer stress to the cell membrane. Hence, pharmacological blockade of endothelial BK channels decreases NO release and suppresses the vasodilatation normally seen during high intraluminal flow (Cooke *et al*., 1991; Hutcheson & Griffith, 1994; Plane *et al*., 1996). If NBT blocks endothelial BK channels in a manner similar to its action on vascular channels, the drug may also disrupt regional blood flow *in vivo* by attenuating flow-induced vasodilatation in systemic vessels.

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