British Journal of Pharmacology (1998) 123, 5–12

Internally applied endotoxin and the activation of BK channels in cerebral artery smooth muscle via a nitric oxide-like pathway

L.M. Hoang & [1]D.A. Mathers

Department of Physiology, University of British Columbia, 2146, Health Sciences Mall, Vancouver, B.C. V6T 1Z3, Canada

1 In this study the role of nitric oxide synthase (NOS) in the acute activation of large conductance, Ca^{2+}-activated K^+ channels (BK channels) by internally applied *E. coli* lipopolysaccharide (LPS, endotoxin) was examined in vascular smooth muscle cells.

2 Cerebrovascular smooth muscle cells (CVSMCs) were enzymatically dispersed from the middle, posterior communicating and posterior cerebral arteries of adult Wistar rats and maintained at 4°C for 2–4 days before recording with standard patch-clamp techniques.

3 Acute application of LPS (100 μg ml^{-1}) to inside-out patches of CVSMC membrane isolated in a cell-free environment rapidly and reversibly increased the open probability, P_o of BK channels in these patches by 3.3 ± 0.30 fold.

4 Acute application of the nitric oxide (NO) donor sodium nitroprusside (SNP, 100 μM) to inside-out patches of CVSMC membrane, studied in the presence of intact cells, also reversibly increased P_o, by some 1.8 ± 0.2 fold over control.

5 Kinetic analysis showed that both LPS and SNP increased P_o by accelerating the rate of BK channel reopening, rather than by retarding the closure of open channels.

6 Neither LPS nor SNP altered the reversal potential or conductance of BK channels.

7 The NOS substrate L-arginine (1 μM) potentiated the acute activation of BK channels by LPS, while the synthetic enantiomer D-arginine (1 μM) inhibited the action of LPS on BK channels.

8 The acute activation of BK channels by LPS was suppressed by pre-incubation of cells with N^{ω}-nitro-L-arginine (50 μM) or N^{ω}-nitro-L-arginine methyl ester (1 mM), two competitive antagonists of nitric oxide synthases. N^{ω}-nitro-D-arginine (50 μM), a poor inhibitor of NOS in *in vitro* assays, had no effect on BK channel activation by LPS.

9 These results indicate that excised, inside-out patches of CVSMC membrane exhibit a NOS-like activity which is acutely activated when LPS is present at the cytoplasmic membrane surface. Possible relationships between this novel mechanism and the properties of known isoforms of nitric oxide synthase are discussed.

Keywords: Lipopolysaccharide; calcium-activated potassium channel; nitric oxide; nitric oxide synthase; meningitis; vasodilatation

Introduction

An important common factor in meningitis caused by Gram-negative bacteria is the presence of lipopolysaccharide (LPS) or endotoxin, an outer membrane component released after lysis of the bacterial cells (Friedland *et al*., 1993; Rietschel *et al*., 1994). LPS triggers the secretion of vasoactive cytokines from activated neutrophils in the subarachnoid space (Saez-Llorens *et al*., 1990; Townsend & Scheld, 1993). In part, these cytokines may mediate the marked cerebrovascular changes seen in infected patients, which include impaired autoregulation, spasm and abnormal vasodilatation (Igarashi *et al*., 1984; Yamashima *et al*., 1985; Beasley & Eldrige, 1994).

However, LPS also relaxes isolated cerebral and systemic vessels studied *in vitro* (Marczin *et al*., 1993; Ueno & Lee, 1993). This effect is apparently mediated by the L-arginine-nitric oxide guanosine 3′:5′-cyclic monophosphate (cyclic GMP) pathway, in which nitric oxide (NO) is synthesized from L-arginine by nitric oxide synthases (NOSs) (Bredt & Snyder, 1990; Knowles & Moncada, 1994). Subsequent binding of NO to cyclic GMP-dependent protein kinase (cyclic GMP-PK) (Ignarro, 1990; Schmidt *et al*., 1993) results in the phosphorylation and activation of large conductance, Ca^{2+}-dependent K^+ channels (BK channels) in vascular smooth muscle cells (VSMCs) (Robertson *et al*., 1993; Taniguchi *et al*., 1993; Bolotina *et al*., 1994; Archer *et al*., 1994). These channels are believed to control myogenic tone in blood vessels, an important mechanism in the autoregulation of blood flow in the brain and other organs (Brayden & Nelson, 1992; Asano *et al*., 1993; Nelson & Quayle, 1995).

Under normal conditions, basal levels of NO are generated in the vascular system mainly by the Ca^{2+}/calmodulin-dependent constitutive NOS (eNOS) of endothelial cells (Faraci & Brian, 1994; Huang *et al*., 1995). However, bath application of LPS for several hours leads to the expression of an inducible NOS (iNOS) in both vascular endothelia and smooth muscle cells (Fleming *et al*, 1991; Miyoshi & Nakaya, 1994). It has been suggested that over-production of NO by iNOS results in excessive activation of BK channels in cerebrovascular smooth muscle cells (CVSMCs), leading to the abnormal dilatation of cerebral vessels seen in meningitis patients (Marczin *et al*., 1993; Schmidt *et al*., 1993).

However, we have recently shown (Hoang & Mathers, 1996) that high concentrations of LPS (10–100 μg ml^{-1}) applied to the cytoplasmic face of the CVSMC membrane activates BK channels within one minute, a time scale which is apparently much too rapid to permit iNOS induction. Since the only known link between LPS and BK channel function is NO synthesis, we have now examined the effects of NOS substrates and inhibitors on the acute activation of BK channels by internally applied LPS. We have also compared the effects of

[1] Author for correspondence.

LPS on BK channel gating with those of the nitric oxide donor, sodium nitroprusside (SNP) (Smith & Kruszyna, 1974; Ivankovich *et al*., 1978). Preliminary accounts of some of these results have appeared (Chen & Mathers, 1995; Hoang & Mathers, 1996).

Methods

Cell separation

CVSMCs were enzymatically dissociated from the middle, posterior communicating and posterior cerebral arteries of adult Wistar rats (250–300 g) by use of 0.1% trypsin and 0.3% collagenase (Wang & Mathers, 1993). Dispersed cells were washed and resuspended in minimum essential medium (Gibco, Grand Island, New York) containing 15% horse serum (heat inactivated) and 10 mM HEPES (Gibco). Cells were plated onto glass cover-slips pre-coated with poly-D-lysine and laminin, and maintained for 2–4 days at 4°C before recording. The positions of individual cells used for recording were noted by their spatial relationship to surrounding cells in the microscope field of view. These cells were subsequently identified as CVSMCs by use of a monoclonal antibody specific for smooth muscle α-actin (Wang *et al*., 1991).

Single-cell electrophysiology

Patch-clamp recordings took place at 21–23°C, within 1–5 h of removal of cells from the incubator. Inside-out membrane patches were excised from CVSMCs by use of published methods (Hamill *et al*., 1981) and voltage-clamped to a membrane potential, $V = -50$ mV or $V = +20$ mV. During experiments with LPS, excised, inside-out patches of CVSMC membrane were completely isolated from all cells in the culture dish by translocating the patch electrode to the second, cell-free compartment of a dual-well recording chamber. This prevented effects on BK channels by vasoactive substances released from neutrophils and endothelia in response to LPS application (Saez-Llorens *et al*., 1990; Townsend & Scheld, 1993). In experiments in which sodium nitroprusside was employed, excised, inside-out membrane patches were studied in the culture dish of their origin, since the presence of cells promotes the rapid liberation of NO from this molecule (Ivankovich *et al*., 1978). In view of the short half-life of NO in solution (Bolotina *et al*., 1994), SNP-containing salines were kept sealed from room air until immediately before use.

The solution bathing the exposed cytoplasmic face of membrane patches could be replaced within 30 s by a saline containing test agents. The identity of BK channels in our membrane patches was confirmed by their large conductance (>200 pS) and block by 10 mM tetraethylammonium ions (TEA^+) or by 5 nM free Ca^{2+} saline applied to the cytoplasmic membrane surface (Brayden & Nelson, 1992; Wang & Mathers, 1993). This low calcium solution had the following composition, in mM: KCl 140, $CaCl_2$ 0.267, EGTA 3, HEPES 10; pH 7.4.

Patch current (bandwidth dc-2 kHz, - 3 dB Bessel) and voltage were analysed by use of commercial software (Instrutech Corporation, U.S.A.). Simplex maximization of likelihood was employed to fit exponential components to the observed open and closed time distributions. In order to increase the efficiency of curve fitting, these distributions were plotted as the square root of the number of observations (ordinates) against the logarithm of the open or closed time (abscissae). Frequency distributions of current amplitudes were fitted by Simplex methods to computer-generated Gaussian functions.

The probability, P_o of finding a single BK channel in the open state during a recording of total duration, T_{tot} was calculated from the relation $P_o = (T_1 + 2.T_2 + ... + N.T_N)/N.T_{tot}$. Here, N is the total number of functional BK channels in the patch, estimated under conditions which strongly favour channel opening. T_1, T_2, ...T_N were the times when at least 1,2 ... N channels were open.

Drugs and solutions

Patch electrodes (10–15 MΩ resistance) were filled with solution A of composition (in mM): KCl 140, $CaCl_2$ 1.48, HEPES 10 and EGTA 3; pH 7.4 (free calcium concentration 50 nM). The cytoplasmic face of inside-out membrane patches was normally exposed to solution B of composition (in mM): KCl 140, $CaCl_2$ 2.86, HEPES 10 and EGTA 3; pH 7.4 (free calcium concentration 1 μM).

LPS (*E. coli* serotype 0127:B8, Sigma, U.S.A.) was suspended in solution B by vortexing immediately before its application. Sodium nitroprusside (SNP), L-arginine (L-Arg), D-arginine (D-Arg), N^{ω}-nitro-L-arginine methyl ester (L-NAME), N^{ω}-nitro-L-arginine (L-NOARG) and N^{ω}-nitro-D-arginine (D-NOARG) were also obtained from Sigma and dissolved in solution B before use. Cells were pre-incubated for 1–2 h (L-NAME) or 15–20 min (D-NOARG, L-NOARG) at 21°C in the presence of the inhibitor, dissolved in solution B. The same concentration of inhibitor was also present during recordings made with inside-out membrane patches excised from these cells.

Statistical analysis

Results are expressed as mean ± s.e.mean. Parametric ANOVA statistics were used to evaluate differences between experimental groups, except in the case of normalized data, in which the Kruskal-Wallis multiple comparison test was substituted. A P value of less than 0.05 was held to be significant. For normalized data, the effects of agents on P_o were expressed as a percentage change from the corresponding control value taken from the same membrane patch, i.e. % change in $P_o = [(P_{o(Drug)} - P_{o(Control)})/P_{o(Control)}] \times 100$.

Results

SNP mimics the effects of LPS on the kinetics of BK channel gating and both agents leave channel conductance unchanged

As an initial test for the involvement of NO in the action of LPS, the effects of endotoxin on the kinetics and conductance of BK channels were compared with those of the NO donor, SNP. LPS (100 μg ml^{-1}) was applied to inside-out patches of CVSMC membrane completely isolated in a cell-free environment, while SNP (100 μM) was applied to membrane patches studied in the culture dish of origin. Both agents rapidly and reversibly increased P_o in BK channels (Figure 1, Table 1). Amplitude distributions for single channel currents seen in the presence of LPS or SNP remained well described by single Gaussian terms with modal values typical of BK channels in the CVSMC membrane (not shown). Neither LPS nor SNP altered the reversal potential or conductance of BK channels (see Table 1).

Open time distributions of BK channel currents studied in control patches were well described by the sum of two exponential functions, that is $y = A_{of}.e^{-t/\tau_{of}} + A_{os}.e^{-t/\tau_{os}}$. Here, the fast and slow time constants τ_{of} and τ_{os} governed the amplitude terms A_{of} and A_{os} respectively. In the presence of 100 μM SNP or of 100 μg ml^{-1} LPS, open time distributions remained of this form (Figure 2). Similarly, the mean open time of BK channels, calculated from the relation $\tau_{open} = A_{of}/(A_{of}+A_{os}).\tau_{of} + A_{os}/(A_{of}+A_{os}).\tau_{os}$ was unchanged on exposure to SNP or LPS (Table 1).

Kinetic analysis was also performed for closed time distributions of BK channel currents. In both the absence and presence of SNP or of LPS, these distributions were well described by the sum of two exponentials (Figure 3). Exposure to SNP or to LPS had no effect on the time constant governing the short duration channel closures, τ_{cf}. However, the time constant which governed long duration closures, τ_{cs} was significantly reduced by application of SNP or of LPS, as was the mean closed time of BK channels, τ_{closed} (Table 2). These results indicated that SNP and LPS exert qualitatively similar effects on the kinetic properties of single BK channels in the CVSMC membrane. Both agents increased P_o by accelerating the rate of channel reopening, rather than by retarding the closure of open channels.

The NOS substrate L-Arg potentiates the acute activation of BK channels by LPS, while D-Arg inhibits LPS action

Application of the nitric oxide synthase substrate L-Arg potentiates NOS-mediated signalling in vascular tissues (Miyoshi & Nakaya, 1994). Known NOS isoforms bind the amino acid with affinities in the range of 2–4 μM (Schmidt *et al.*, 1993). The effect of micromolar concentrations of L-Arg on BK channel activation by LPS was therefore examined.

When applied by itself (Figure 4a), L-Arg (1 μM) produced a small, reversible increase in the open probability of BK channels, averaging 68 ± 19% over control values ($n = 6$ patches, $P < 0.05$). In addition, application of 100 μg ml^{-1} LPS in the presence of

Table 1 Effects of SNP (100 μM) or of LPS (100 μg ml^{-1}) on the open probability, P_0, conductance and mean open time of BK channels

Drug	*Control*	*Drug*	*Wash*	P
Open probability				
LPS	0.04 ± 0.01	0.13 ± 0.04	0.03 ± 0.01	<0.05
SNP	0.09 ± 0.02	0.16 ± 0.05	0.13 ± 0.05	<0.05
Conductance (pS)				
LPS	219 ± 12	223 ± 10	218 ± 11	NS
SNP	225 ± 13	239 ± 10	230 ± 11	NS
Mean open time (ms)				
LPS	8.9 ± 1.2	10.2 ± 1.8	8.2 ± 2.1	NS
SNP	10.2 ± 1.9	10.8 ± 2.4	10.4 ± 2.0	NS

The BK channels were studied in isolated, inside-out patches of CVSMC membrane voltage clamped to a potential V = −50 mV with $[Ca^{2+}]_i = 1$ μM. Both agents were applied to the exposed, cytoplasmic face of the isolated membrane patches by bath perfusion. Values given represent the mean ± s.e.mean for 6 patches studied in the LPS group and 10 patches studied with SNP. $P < 0.05$ indicates significantly different from the corresponding control value (ANOVA) while NS indicates not significantly different from control.

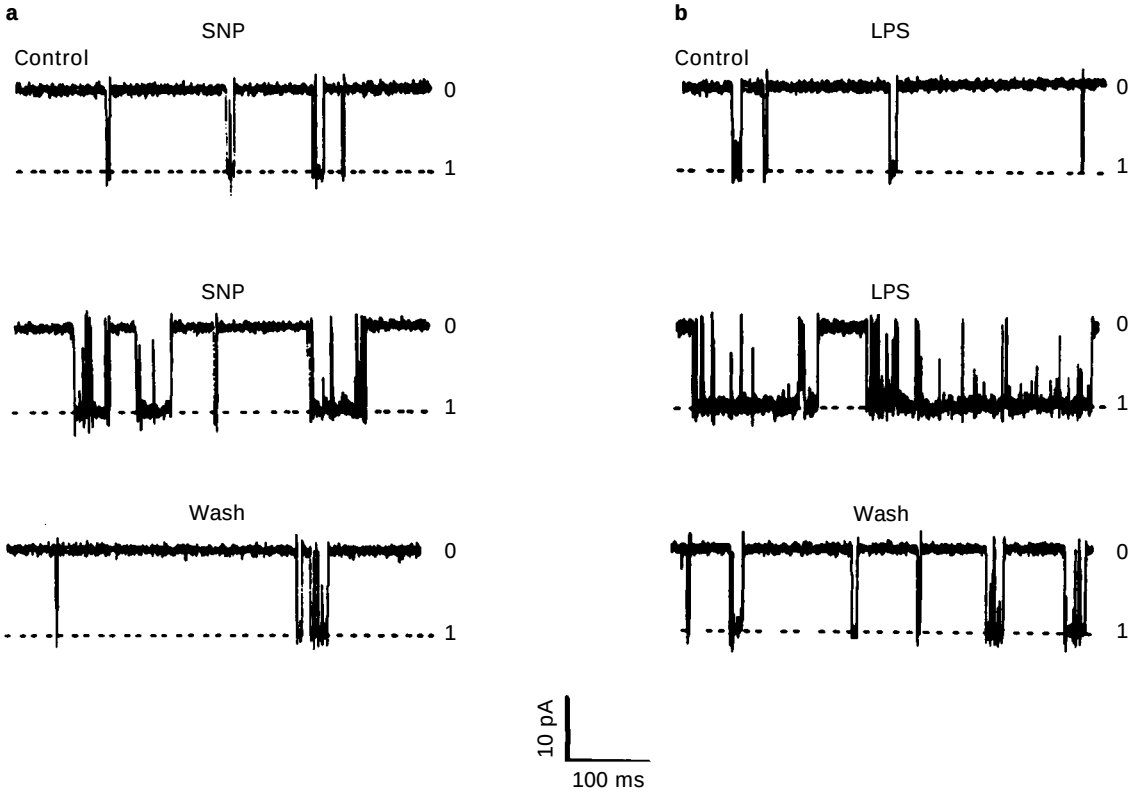

Figure 1 Reversible activation of BK channels in two different isolated, inside-out patches of CVSMC membrane by application of (a) 100 μM SNP or (b) 100 μg ml^{-1} LPS. These patches were voltage-clamped at a membrane potential, V = −50 mV and each patch contained a single BK channel. 0 and 1 denote channel closed and channel open states, respectively. The dashed lines indicate mean channel current in the open state. Mean channel currents and open probabilities for records from which these sample traces were obtained were as follows. Control −11.6 pA, 0.072; SNP −11.8 pA, 0.206; wash −11.8 pA, 0.051. Control −11.8 pA, 0.064; LPS −11.5 pA, 0.412; wash −11.7 pA, 0.105. Bandwidth of recordings dc-1 kHz. $[Ca^{2+}]_i = 1$ μM.

1 μM L-Arg produced a larger potentiation in P_o than that seen in the absence of the amino acid (Figures 4b and 5).

Nitric oxide synthases exhibit stereospecificity in substrate selection, and strongly favour the naturally occurring isomer L-Arg over the synthetic enantiomer D-Arg (Knowles & Moncada, 1994). The effects of D-Arg on BK channel activation by LPS were therefore examined. Applied in the absence of LPS, 1 μM D-Arg did not itself alter the open probability of BK channels (mean increase over control, $0.8 \pm 31\%$, $P > 0.05$). However, when co-applied with 100 μg ml^{-1} LPS, 1 μM D-Arg significantly inhibited the effect of LPS on BK channel activation (Figure 5).

The acute activation of BK channels by LPS is blocked by the competitive NOS inhibitors L-NAME and L-NOARG, but not by D-NOARG

The role of NO in LPS-induced enhancement of BK channel opening was further studied with L-NAME, a competitive, reversible antagonist of L-arginine binding to NO synthases (Schmidt *et al*., 1993; Knowles & Moncada, 1994). Cells were

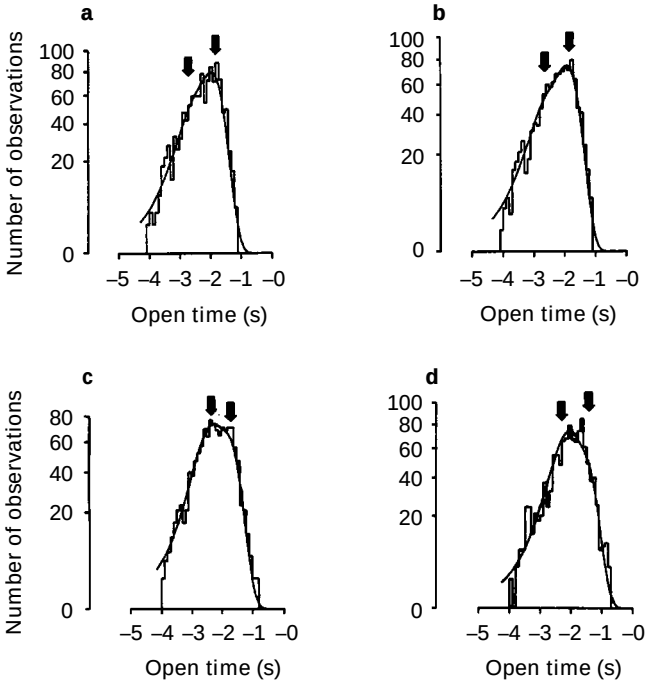

Figure 2 Effects of 100 μM SNP (b) or 100 μg ml^{-1} LPS (d) on the open time distributions of BK channels in two inside-out patches of CVSMC membrane voltage-clamped to $V = -50$ mV with $[Ca^{2+}]_i = 1$ μM. These open time distributions were plotted as the square root of the number of observations (ordinates) against the logarithm of the open time (abscissae). (a and b) Open time distributions obtained from the first membrane patch in (a) control (550 channel openings) and (b) SNP-containing salines (561 channel openings). Both distributions were well described by the sum of two exponential terms (smooth curves) with the following fit parameters defined in the text. Control: $\tau_{of} = 2.1$ ms; $\tau_{os} = 13.4$ ms. SNP: $\tau_{of} = 2.3$ ms; $\tau_{os} = 13.3$ ms. Mean channel open times, τ_{open} calculated as defined in the text, were 11.2 ms and 10.8 ms, respectively. The arrows indicate the positions of τ_{of} and τ_{os} on the time axis. (c and d) Open time distributions obtained from the second membrane patch bathed in (c) control (613 channel openings) and (d) LPS-containing salines (682 channel openings). Each distribution was well described by the sum of two exponential terms (smooth curves) with the following fit parameters defined in the text. Control: $\tau_{of} = 3.2$ ms; $\tau_{os} = 17.3$ ms. LPS: $\tau_{of} = 6.1$ ms; $\tau_{os} = 27.8$ ms. Mean channel open times, τ_{open} were 11.9 ms and 18.0 ms for control and LPS data, respectively.

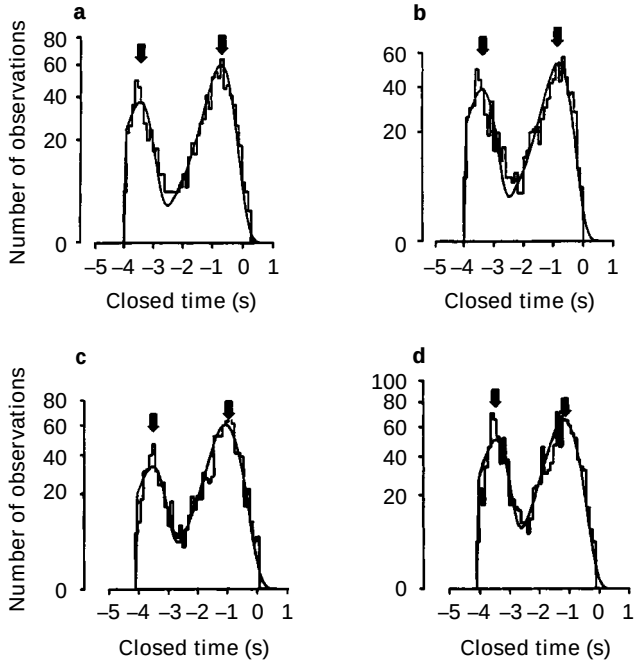

Figure 3 Effects of 100 μM SNP (b) or 100 μg ml^{-1} LPS (d) on the closed time distributions of BK channel currents in two inside-out patches of CVSMC membrane voltage-clamped to $V = -50$ mV with $[Ca^{2+}]_i = 1$ μM. These closed time distributions were plotted as the square root of the number of observations (ordinates) against the logarithm of the closed time (abscissae). (a and b) Closed time distributions obtained from the first of these patches exposed to (a) control saline (526 closings) or (b) SNP saline (500 closings). These distributions were well described by the sum of two exponentials (smooth curves) with the following parameters. Control: $\tau_{cf} = 0.4$ ms; $\tau_{cs} = 235$ ms. SNP: $\tau_{cf} = 0.5$ ms; $t_{cs} = 177$ ms. Mean channel closed times, τ_{closed} were calculated as 146 ms and 103 ms for control and SNP data, respectively. The arrows indicate the positions of τ_{cf} and τ_{cs} on the time axis. (c and d) Closed time distributions obtained from the second patch exposed to (c) control saline (556 closings) or (d) LPS saline (573 closings). These distributions were well described by the sum of two exponentials (smooth curves) with the following parameters. Control: $\tau_{cf} = 0.4$ ms; $\tau_{cs} = 172$ ms. LPS: $\tau_{cf} = 0.42$ ms; $\tau_{cs} = 103$ ms. Mean channel closed times, τ_{closed} were calculated as 124 ms and 61 ms for control and LPS data, respectively.

Table 2 Effect of LPS (100 μg ml^{-1}) and SNP (100 μM) on the time constants (ms) describing brief BK channel closings (τ_{cf}) and long channel closings (τ_{cs})

	Control	*Drug*	*Wash*	P
LPS				
τ_{cf}	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	NS
τ_{cs}	284 ± 46	122 ± 22	267 ± 61	< 0.05
τ_{closed}	151 ± 32	46 ± 12	160 ± 46	< 0.05
SNP				
τ_{cf}	0.6 ± 0.1	0.7 ± 0.1	0.5 ± 0.2	NS
τ_{cs}	313 ± 26	188 ± 15	302 ± 21	< 0.05
τ_{closed}	120 ± 32	71 ± 18	101 ± 28	< 0.05

Also shown is the effect of these agents on the mean closed time (ms) of BK channels (τ_{closed}), as calculated from the equation $\tau_{closed} = A_{cf}/(A_{cf} + A_{cs}).\tau_{cf} + A_{cs}/(A_{cf} + A_{cs}).\tau_{cs}$. Here, A_{cf} and A_{cs} were the relative areas of the two exponential terms fitted to observed closed time distributions. Data were obtained as in Table 1 from 6 patches studied with LPS and 10 patches studied with SNP. NS, not significantly different from control. $P < 0.05$, significantly different than value seen in corresponding control (ANOVA).

pre-incubated for 1–2 h in the presence of 1 mM L-NAME before the membrane patch excision. L-NAME (1 mM) was also present during recording from these patches. Under these conditions, P_o values measured in the presence of 100 μg ml^{-1} LPS were not significantly different from values obtained before the application of endotoxin (Figure 6).

Alkyl ester analogues of arginine are known (Buxton *et al.*, 1993) to act as muscarinic receptor antagonists at concentrations of $\geqslant$0.1 mM, which are also required to inhibit effectively some NOS-mediated pathways (Quian & Jones, 1995). Accordingly, we repeated this experiment with the more potent NOS inhibitor L-NOARG which blocks NOS activity with an IC_{50} of 10–20 μM and lacks anti-muscarinic effects (Dawson *et al.*, 1991; Michel *et al.*, 1993). Cells were preincubated for 15–20 min in 50 μM L-NOARG, and the drug was also present at this concentration during recording from excised membrane patches. Under these conditions, 50 μM L-NOARG abolished the enhancement in P_o normally seen on application of 100 μg ml^{-1} LPS (Figure 6).

As a control of possible non-specific effects of L-NOARG on BK channels, cells were pre-incubated in 50 μM D-NOARG for 15–20 min at room temperature, before membrane patch excision. This agent was also present at the same concentration during recording from these excised membrane patches. D-NOARG has been shown to be considerably less active than L-NOARG as an inhibitor of NOS in *in vitro* assays of enzyme function (Wang *et al.*, 1993). As shown in Figure 6, D-NOARG had no effect on the acute activation of BK channels by LPS.

We next omitted the cell pre-incubation step from this experimental protocol, in order to determine the acute effects of L-NAME and L-NOARG on BK channel gating in the

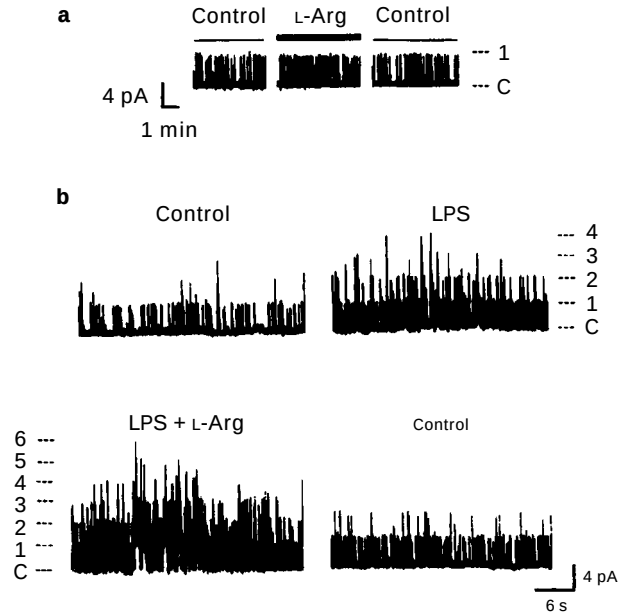

Figure 4 (a) Effect of 1 μM L-arginine (L-Arg) on the open probability, P_o of BK channels in an isolated, inside-out patch of CVSMC membrane voltage-clamped to V = +20 mV with $[Ca^{2+}]_i$ = 1 μM. The vertical scale bar indicates current levels when zero (C) or 1 BK channel was open. In control saline, P_o was 0.004, and this increased to 0.006 in the presence of L-Arg. On return to control saline, P_o declined to 0.003. (b) Potentiation of LPS activation of BK channels in the presence of L-arginine (L-Arg). Traces were obtained from a second, inside-out patch, voltage-clamped at V = +20 mV with $[Ca^{2+}]_i$ = 1 μM. The vertical scale bars indicate current levels when zero (C) or 1–6 BK channels were open in this membrane patch. Replacement of control saline by a solution containing 100 μg ml^{-1} LPS increased P_o from 0.019 to 0.082. When 1 μM L-Arg was co-applied with the LPS, P_o increased further to 0.184. On return to control saline, P_o recovered to a value of 0.035.

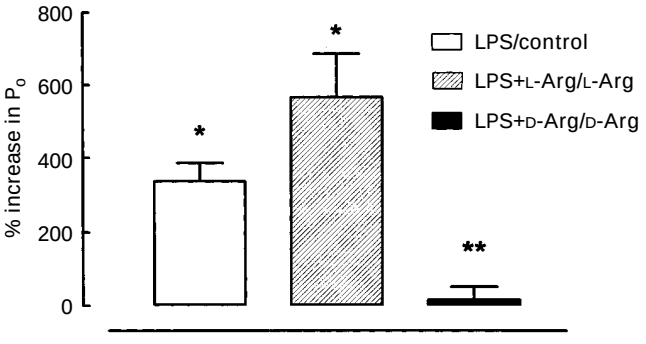

Figure 5 Effects of 1 μM L-arginine (L-Arg) or of 1 μM D-arginine (D-Arg) on the increase in BK channel open probability, P_o caused by application of 100 μg ml^{-1} LPS to isolated inside-out patches of CVSMC membrane. All patches were voltage-clamped to V = 20 mV with $[Ca^{2+}]_i$ = 1 μM. Results were expressed as the mean percentage increase in P_o seen on replacement of control by LPS solution (n = 7 patches), of L-Arg saline by saline containing both LPS and L-Arg (n = 4), or of D-Arg saline by solution containing both LPS and D-Arg (n = 4). The Newman-Keuls test indicated that the increase in P_o seen on replacing L-Arg with LPS + L-Arg saline was significantly larger than that seen on replacing control saline with LPS solution (*P < 0.05). In contrast, the increase in P_o seen on replacing D-Arg saline with LPS + D-Arg solution was significantly less than that seen on replacing control saline with LPS solution (**P < 0.05).

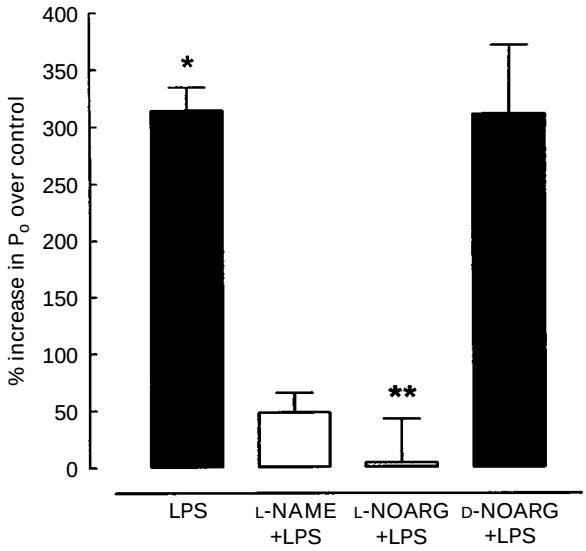

Figure 6 Inhibition of the potentiating effect of 100 μg ml^{-1} LPS on BK channel opening by the NOS inhibitors L-NAME (1 mM) and L-NOARG (50 μM) but not by D-NOARG (50 μM). All patches were voltage-clamped to V = 20 mV with $[Ca^{2+}]_i$ = 1 μM. In experiments with L-NAME, CVSMCs were pre-incubated with this agent for 1–2 h before the membrane patch excision. In experiments with L-NOARG or D-NOARG, CVSMCs were pre-incubated with one of these agents for 15–20 min before the patch excision. Results are expressed as the mean percentage increase in P_o seen on replacing control saline with LPS saline (n = 33 patches), L-NOARG saline with solution containing LPS + L-NOARG (n = 5), L-NAME saline with solution containing LPS + L-NAME (n = 5) or D-NOARG solution with saline containing LPS + D-NOARG (n = 4). The Newman Keuls test indicated that the mean increases in P_o seen in the presence of L-NOARG or L-NAME were significantly smaller than that seen on replacing control with LPS saline (*P < 0.05). In addition, the increase in P_o seen on replacing L-NOARG saline with LPS + L-NOARG solution was not significantly different from zero increase (**). On replacing D-NOARG saline with solution containing LPS + D-NOARG, the increase in P_o was not significantly different from that seen on replacing control saline with LPS solution.

absence and presence of LPS. Under these conditions, inconsistent results were obtained, since only some patches showed diminished LPS responses in the presence of acutely applied NOS inhibitors. This indicated that the blocking action of these agents on LPS responses required a period of time for its development. The time-dependent block of a putative NOS-mediated pathway by L-NOARG has previously been observed during the endothelium-dependent relaxation of rat aortic rings studied *in vitro* (Wang *et al*., 1993).

The acute application of 1 mM L-NAME to isolated inside-out patches was found to have no significant effects on either the open probability or conductance of BK channels in these patches (control $P_o = 0.04 \pm 0.01$, L-NAME, 0.03 ± 0.01; control conductance, 219 ± 12 pS, L-NAME, 218 ± 22 pS, $n = 6$, $P > 0.05$, ANOVA). Similarly, acute application of 50 μM L-NOARG also had no effects on these parameters (control $P_o = 0.02 \pm 0.01$, L-NOARG, 0.03 ± 0.01; control conductance, 246 ± 5 pS, L-NOARG, 246 ± 6 pS, $n = 4$, $P > 0.05$, ANOVA).

Discussion

Acute application of LPS or of the known NO releaser SNP rapidly and reversibly increased the open probability of BK channels in inside-out patches of CVSMC membrane. Both agents accelerated the kinetics of BK channel opening, leaving the closing kinetics and conductance of BK channels unaltered. The effects of SNP on BK channel function were as noted in a previous study performed on rat aortic smooth muscle (Bolotina *et al*., 1994). No evidence was found for activation of small conductance, Ca^{2+}-dependent K^+ channels by SNP, as shown in rat mesenteric artery smooth muscle (Tang & Zhang, 1993), since the amplitude distributions of the single channel currents remained well described by single terms typical of BK channels.

The activation of BK channels by LPS was suppressed by the competitive NOS inhibitors L-NAME and L-NOARG, but not by the less active inhibitor D-NOARG, and was potentiated in a stereospecific manner by the NOS substrate L-arginine. These results are consistent with the view that LPS action was mediated by a NOS-like enzyme present in isolated, inside-out patches of CVSMC membrane. These observations also decrease the likelihood that LPS activates BK channels by a simple, direct interaction with the channel protein.

NO synthases exist in three known isoforms in vascular tissues, namely nNOS and eNOS, constitutively expressed in perivascular neurones and in endothelial cells, respectively, and iNOS, induced by exposure to LPS and/or cytokines in smooth muscle cells, endothelia and macrophages (Schmidt *et al*., 1992; 1993; Knowles & Moncada, 1994). Following pre-incubation with externally applied LPS for several hours, smooth muscle cells of porcine coronary arteries generate NO when supplied with exogenous L-arginine in the bathing solution, presumably as a result of iNOS induction. Furthermore, the NO so produced rapidly activates BK channels in cell-attached patches of smooth muscle membrane (Miyoshi & Nakaya, 1994; Taguchi *et al*., 1996). However, induction of iNOS requires 1–4 h exposure of cells to endotoxin and involves protein synthesis, tyrosine kinase activity and transcription of the TNF-α gene (Julou-Schaeffer *et al*., 1991; Salter *et al*., 1991; Szabo *et al*., 1993). Given the very short latency period seen in the present response to LPS, and its occurrence in an isolated membrane preparation, it is unlikely that our results were due to the acute induction of iNOS by LPS.

Attempts to detect the presence of constitutive NOS in vascular smooth muscle by use of cytochemical methods have to date yielded conflicting results. Vascular smooth muscle cells from normal adult rats generally fail to react to anti-sera raised against eNOS (Föstermann *et al*., 1991; Loesch *et al*., 1994) or iNOS (Buttery *et al*., 1994). However, eNOS-like immunoreactivity has been observed in arterial smooth muscle of newborn Wistar rats (Loesch & Burnstock, 1995). In addition, nicotinamide-adenine-dinucleotide phosphate (NADPH)-dependent diaphorase activity, which frequently colocalizes with NO synthase in other tissues (Schmidt *et al*., 1992; Gabbott & Bacon, 1993), is found in the smooth muscle cells of large-diameter cerebral arteries of adult rats (Gabbott & Bacon, 1993), the vessels employed in the present study.

A number of functional studies have failed to detect constitutive NOS enzymatic activity in vascular smooth muscle cells (Bredt & Snyder, 1990; Fleming *et al*., 1991; Nozaki *et al*., 1993). However, the marked facilitatory effects of NOS inhibitors on contractions in endothelium-denuded strips of aorta and carotid artery suggest that some VSMCs do indeed possess a constitutive form of NOS (Charpie & Webb, 1993; Yeh *et al*., 1996). Additionally, the NO produced by this Ca^{2+}-calmodulin dependent enzyme may serve as an autocrine inhibitor of contraction in vascular smooth muscle (Wood *et al*., 1990). It is conceivable, therefore, that rat cerebrovascular smooth muscle cells may also possess a NOS-like enzyme which is activated when LPS is present at the cytoplasmic membrane face.

All known NOS isoforms are complex enzymes, which required the presence of flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and (6**R**)-5,6,7,8-tetrahydrobiopterin (BH_4) as cofactors for NO synthesis, in addition to the cosubstrates NADPH and O_2, the substrate L-arginine and the calcium-binding protein calmodulin (CaM) (Schmidt *et al*., 1993; Knowles & Moncada, 1994). Of these compounds, FAD, FMN, BH_4 and CaM exhibit high affinity binding to at least one of the known NOS isoforms, and in these cases can remain tightly bound, even after purification of the enzyme to homogeneity (Cho *et al*., 1992b; Nathan & Xie, 1994; Sennequier & Stuehr, 1996).

However, all known NOS isoforms bind both L-arginine and NADPH with relatively low affinity ($K_d \sim 0.1-20$ μM) (Föstermann, 1994; Knowles & Moncada, 1994). In addition, only iNOS exhibits high affinity binding for the CaM molecule, which is essential for the activity of all NOS isoforms (Schmidt *et al*., 1993).

Therefore, it might be expected that rapid dialysis of these molecules would occur in an excised, inside-out patch of cell membrane. However, responsiveness to LPS was clearly present in membrane patches which had been completely excised from their parent cells.

In some vascular smooth muscle preparations (Fujino *et al*., 1991), though not in others (Robertson *et al*., 1993), excised patches of VSMC membrane retain the cyclic GMP-dependent protein kinase of vascular smooth muscle in a functional state. However, cyclic GMP is generated in VSMCs via an NO-sensitive, soluble guanylate cyclase (Beasley, 1990; Moro *et al*., 1996), and hence this pathway step is also potentially vulnerable to membrane patch excision.

Based on the properties of known NOS isoforms, therefore, there are difficulties in accepting the hypothesis that our results reflected the acute activation of a NOS-like enzyme by LPS. One possible resolution of this paradox is to postulate that the CVSMC membrane contains a truncated, NOS-like molecule which retains the L-arginine binding domain of NOS, but lacks the domains needed for cofactor binding. In principle, this

molecule could interact directly with the BK channel protein, eliminating the need for NO synthesis.

Alternatively, a functionally complete NOS may exist in CVSMCs as part of a submembranous signalling complex, as recently suggested for the NOSs of neuronal (Brenman *et al*., 1996) and skeletal muscle membranes (Kobzik *et al*., 1994). These complexes are stabilized through PDZ domains (Cho *et al*., 1992a; Adams *et al*., 1995), may incorporate other enzymes, cytoskeletal proteins and ion channels (Brenman *et al*., 1995; Kornau *et al*., 1995) and could conceivably be retained in membrane patches which have been completely excised from their parent cells (Horber *et al*., 1995).

It was possible that the present results reflected iNOS pre-induction or de-differentiation of CVSMCs during their 2–4 day period *in vitro*. However attempts to test these hypotheses proved unsuccessful, due to the poor attachment of freshly isolated CVSMCs to the substrates employed. Although these possibilities remain as viable hypotheses, we feel that the low temperature employed in our study would have kept such changes to a minimum. Whatever the mechanism which underlies the action of endotoxin on BK channels, it seems likely that LPS represents a useful probe for the future study of channel modulation by sub-membranous elements.

This work was supported by a grant from the Heart and Stroke Foundation of British Columbia and the Yukon.

References

ADAMS, M.E., DWYER, T.M., DOWLER, L.L., WHITE, R.A. & FROEHNER, S.C. (1995). Mouse α1- and β2-syntrophin gene structure, chromosome localization, and homology with a discs large domain. *J. Biol. Chem.*, **270,** 25859–25865.

ARCHER, S.L., HUANG, J.M.C., HAMPL, V., NELSON, D.P., SHULTZ, P.-J. & WEIR, E.K. (1994). Nitric oxide and cGMP cause vasorelaxation by activation of a charybdotoxin-sensitive K channel by cGMP-dependent protein kinase. *Proc. Natl. Acad. Sci. U.S.A.*, **91,** 7583–7587.

ASANO, M., MASUZAWA-ITO, K., MATSUDA, T., SUZUKI, Y., OYAMA, H., SHIBUYA, M. & SUGITA, K. (1993). Increased Ca^{2+} influx in the resting state maintains the myogenic tone and activates charybdotoxin-sensitive K^+ channels in dog basilar artery. *J. Cerebr. Blood Flow Metab.*, **13,** 969–977.

BEASLEY, D. (1990). Interleukin 1 and endotoxin activate soluble guanylate cyclase in vascular smooth muscle. *Am. J. Physiol.*, **259,** R38–44.

BEASLEY, D. & ELDRIDGE, M. (1994). Interleukin-1β and tumor necrosis factor-alpha synergistically induce NO synthase in rat vascular smooth muscle. *Am. J. Physiol.*, **266,** R1197–R1203.

BOLOTINA, V.M., NAJIBI, S., PALACINO, J.J., PAGANO, P.J. & COHEN, R.A. (1994). Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature*, **368,** 850–853.

BRAYDEN, J.E. & NELSON, M.T. (1992). Regulation of arterial tone by activation of calcium-dependent potassium channels. *Science*, **256,** 532–535.

BREDT, D.S. & SNYDER, S.H. (1990). Isolation of NO synthase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. U.S.A.*, **87,** 682–685.

BRENMAN, J.E., CHAO, D.S., XIA, H., ALDAPE, K. & BREDT, D.S. (1995). Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy. *Cell*, **82,** 743–752.

BRENMAN, J.E., CHAO, D.S., GEE, S.H., MCGEE, A.W., CRAVEN, S.E., SANTILLANO, D.R., WU, Z., HUANG, F., XIA, H., PETERS, M.F., FROEHNER, S.C. & BREDT, D.S. (1996). Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains. *Cell,* **84,** 757–767.

BUTTERY, L.D.K., EVANS, T.J., SPRINGALL, D.R., CARPENTER, A., COHEN, J. & POLAK, J.M. (1994). Immunocytochemical localization of inducible nitric oxide synthase in endotoxin-treated rats. *Lab. Invest.*, **71,** 755–764.

BUXTON, I.L., CHEEK, D.J., ECKMAN, D., WESTFALL, D.P., SANDERS, K.M. & KEEF, K.D. (1993). N^G-nitro L-arginine methyl ester and other alkyl esters of arginine are muscarinic receptor antagonists. *Circ. Res.*, **72,** 387–395.

CHARPIE, J.R. & WEBB, R.C. (1993). Vascular myocyte-derived nitric oxide is an autocrine that limits vasoconstriction. *Biochem. Biophys. Res. Commun.*, **194,** 763–768.

CHEN, C. & MATHERS, D.A. (1995). Nitric oxide activates calcium-dependent potassium channels in cerebrovascular smooth muscle cells. *Soc. Neurosci. Abstracts*, **21,** 1831.

CHO, K.-O., HUNT, C.A. & KENNEDY, M.B. (1992a). The rat brain postsynaptic density fraction contains a homolog of the Drosophila discs-large tumor suppressor protein. *Neuron*, **9,** 929–942.

CHO, H.J., XIE, Q.-W., CALAYCAY, J., MUMFORD, R.A., SWIDEREK, K.M., LEE, T.D. & NATHAN, C. (1992b). Calmodulin is a subunit of nitric oxide synthase from macrophages. *J. Exp. Med.*, **176,** 599–604.

DAWSON, V.L., DAWSON, T.M., LONDON, E.D., BREDT, D.S. & SNYDER, S.H. (1991). Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proc. Natl. Acad. Sci. U.S.A.*, **88,** 6368–6371.

FARACI, F.M. & BRIAN, J.E. (1994). Nitric oxide and the cerebral circulation. *Stroke*, **25,** 692–703.

FLEMING, I., GRAY, G.A., SCHOTT, C. & STOCLET, J.-C. (1991). Inducible but not constitutive production of nitric oxide by vascular smooth muscle cells. *Eur. J. Pharmacol.*, **200,** 375–376.

FÖSTERMANN, U. (1994). Biochemistry and molecular biology of nitric oxide synthases. *Drug Res.*, **44,** 402–407.

FÖRSTERMANN, U., SCHMIDT, H.H.H.W., POLLOCK, J.S., SHENG, H., MITCHELL, J.A., WARNER, T.D., NAKANE, M. & MURAD, F. (1991). Isoforms of nitric oxide synthase. Characterisation and purification from different cell types. *Biochem. Pharmacol.*, **42,** 1849–1857.

FRIEDLAND, I.R., JAFARI, H., EHRETT, S., RINDERKNECHT, S., PARIS, M., COULTHARD, M., SAXEN, H., OLSEN, K. & MCCRACKEN, G.H. (1993). Comparison of endotoxin release by different antimicrobial agents and the effect of inflammation in experimental *Escherichia coli* meningitis. *J. Infect. Diseases*, **168,** 657–662.

FUJINO, K., NAKAYA, S., WAKATSUKI, T., MIYOSHI, Y., NAKAYA, Y., MORI, H. & INOUE, I. (1991). Effects of nitroglycerin on ATP-induced Ca^{2+}-mobilization, Ca^{2+}-activated K channels and contraction of cultured smooth muscle cells of porcine coronary artery. *J. Pharmacol. Exp. Ther.*, **256,** 371–377.

GABBOTT, P.L.A. & BACON, S.J. (1993). Histochemical localization of NADPH-dependent diaphorase (nitric oxide synthase) activity in vascular endothelial cells in the rat brain. *Neuroscience*, **57,** 79–95.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv.*, **391,** 85–100.

HOANG, L.M. & MATHERS, D.A. (1996). LPS rapidly increases BK channel opening in inside-out patches of rat cerebrovascular smooth muscle cells. *Soc. Neurosci. Abstracts*, **22,** 1253.

HORBER, J.K., MOSBACHER, J., HABERLE, W., RUPPERSBERG, J.P. & SAKMANN, B. (1995). A look at membrane patches with a scanning force microscope. *Biophys. J.*, **68,** 1687–1693.

HUANG, P.L., HUANG, Z., MASHIMO, H., BLOCH, K.D., MOSKOWITZ, M.A., BEVAN, J.A. & FISHMAN, M.C. (1995). Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature*, **377,** 239–242.

IGARASHI, M., GILMARTIN, R.C., GERALD, B., WILBURN, F. & JABBOUR, J.T. (1984). Cerebral arteritis and bacterial meningitis. *Arch. Neurol.*, **41,** 531–535.

IGNARRO, L.J. (1990). Biosynthesis and metabolism of endothelium-derived nitric oxide. *Ann. Rev. Pharmacol. Toxicol.*, **30,** 535–560.

IVANKOVICH, A.D., MILETICH, D.J. & TINKER, J.H. (1978). Sodium nitroprusside: metabolism and general considerations. *Int. Anesthesiol. Clin.*, **16,** 1–29.

JULOU-SCHAEFFER, G., GRAY, G.A., FLEMING, I., SCHOTT, C., PARRATT, J.R. & STOCLET, J.-C. (1991). Activation of L-arginine-nitric oxide pathway is involved in vascular hyporeactivity induced by endotoxin. *J. Cardiovasc. Pharmacol.*, **17,** S207–S212.

KNOWLES, R.G. & MONCADA, S. (1994). Nitric oxide synthase in mammals. *Biochem. J.*, **298,** 249–258.

KOBZIK, L., REID, M.B., BREDT, D.S. & STAMLER, J.S. (1994). Nitric oxide in skeletal muscle. *Nature*, **372,** 546–548.

KORNAU, H.-C., SCHENKER, L.T., KENNEDY, M.B. & SEEBURG, P.H. (1995). Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. *Science*, **269,** 1737–1740.

LOESCH, A. & BURNSTOCK, G. (1995). Ultrastructural localization of nitric oxide synthase and endothelin in coronary and pulmonary arteries of newborn rats. *Cell. Tiss. Res.*, **279,** 475–483.

LOESCH, A., BELAI, A. & BURNSTOCK, G. (1994). An ultrastructural study of NADPH-diaphorase and nitric oxide synthase in the perivascular nerves and vascular endothelium of rat basilar artery. *J. Neurocytol.*, **23,** 49–59.

MARCZIN, N., PAPAPETROPOULOS, A. & CATRAVAS, J.D. (1993). Tyrosine hydroxylase inhibitors suppress endotoxin- and IL-1 beta-induced NO synthesis in aortic smooth muscle cells. *Am. J. Physiol.*, **265,** H1014–H1018.

MICHEL, A.D., PHUL, R.K., STEWART, T.L. & HUMPHREY, P.P. (1993). Characterization of the binding of [3H]-L-NG-nitro-arginine in rat brain. *Br. J. Pharmacol.*, **109,** 287–288.

MIYOSHI, H. & NAKAYA, Y. (1994). Endotoxin-induced nonendothelial nitric oxide activates the Ca^{2+}-activated K^+ channel in cultured vascular smooth muscle cells. *J. Mol. Cell. Cardiol.*, **26,** 1487–1495.

MORO, M.A., RUSSELL, R.J., CELLECK, S., LIZASOAIN, I., SU, Y., DARLEY-USMAR, V.M., RADOMSKI, M.W. & MONCADA, S. (1996). cGMP mediates the vascular and platelet actions of nitric oxide: confirmation using an inhibitor of the soluble guanylyl cyclase. *Proc. Natl. Acad. Sci. U.S.A.*, **93,** 1480–1485.

NATHAN, C. & XIE, Q. (1994). Regulation of biosynthesis of nitric oxide. *J. Biol. Chem.*, **269,** 13725–13728.

NELSON, M.T. & QUAYLE, J.M. (1995). Physiological roles and properties of potassium channels in arterial smooth muscle. *Am. J. Physiol.*, **268,** C799–C822.

NOZAKI, K., MOSKOWITZ, M.A., MAYNARD, K.I., KOKETSU, N., DAWSON, T.M., BREDT, D.S. & SNYDER, S.H. (1993). Possible origins and distribution of immunoreactive nitric oxide-containing nerve fibers in cerebral arteries. *J. Cereb. Blood Flow Metab.*, **13,** 70–79.

QUIAN, Y.M. & JONES, R.L. (1995). Inhibition of rat colon contractility by prostacyclin (IP-) receptor agonists: involvement of NANC neurotransmission. *Br. J. Pharmacol.*, **115,** 163–171.

RIETSCHEL, E.T., KIRIKAE, T., SCHADE, F.U., MAMAT, U., SCHMIDT, G., LOPPNOW, H., ULMER, A.J., ZAHRINGER, U., SEYDEL, U., DI PADOVA, F., SCHREIER, M. & BRADE, H. (1994). Bacterial endotoxin: molecular relationships of structure to activity and function. *FASEB J.*, **8,** 217–225.

ROBERTSON, B.E., SCHUBERT, R., HESCHELER, J. & NELSON, M.T. (1993). cGMP-dependent protein kinase activates Ca-activated K channels in cerebral artery smooth muscle cells. *Am. J. Physiol.*, **265,** C299–C303.

SAEZ-LLORENS, X., RAMILLO, O., MUSTAFA, M.M., MERTSOLA, J. & MCCRACKEN, G.H. (1990). Molecular pathophysiology of bacterial meningitis: current concepts and therapeutic implications. *J. Pediatr.*, **116,** 671–684.

SALTER, M., KNOWLES, R.G. & MONCADA, S. (1991). Widespread tissue distribution, species distribution and changes in activity of calcium-dependent and calcium-independent nitric oxide synthases. *FEBS Lett.*, **291,** 145–149.

SCHMIDT, H.H.H.W., GAGNE, G.D., NAKANE, M., POLLOCK, J.S., MILLER, M.F. & MURAD, F. (1992). Mapping of neural nitric oxide synthase in the rat suggests frequent co-localization with NADPH diaphorase but not with soluble guanylyl cyclase, and novel paraneural functions for nitrinergic signal transduction. *J. Histochem. Cytochem.*, **40,** 1439–1456.

SCHMIDT, H.H.H.W., LOHMANN, S.M. & WALTER, U. (1993). The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. *Biochim. Biophys. Acta*, **1178,** 153–175.

SILVAGNO, F., XIA, H. & BREDT, D.S. (1996). Neuronal nitric-oxide synthase-mu, an alternatively spliced isoform expressed in differentiated skeletal muscle. *J. Biol. Chem.*, **271,** 11204–11208.

SMITH, R.P. & KRUSZYNA, H. (1974). Nitroprusside produces cyanide poisoning via a reaction with hemoglobin. *J. Pharmacol. Exp. Ther.*, **191,** 557–563.

SZABO, C., MITCHELL, J.A., THIEMERMANN, C. & VANE, J.R. (1993). Nitric oxide-mediated hyporeactivity to noradrenaline precedes the induction of nitric oxide synthase in endotoxic shock. *Br. J. Pharmacol.*, **108,** 786–792.

TAGUCHI, H., HEISTAD, D.D., CHU, Y., RIOS, C.D., OOBOSHI, H. & FARACI, F.M. (1996). Vascular expression of inducible nitric oxide synthase is associated with activation of Ca^{++}-dependent K^+ channels. *J. Pharmacol. Exp. Ther.*, **279,** 1514–1519.

TANG, Y.-G. & ZHANG, Y.-F. (1993). Endothelium-derived relaxing factor activates calcium-activated potassium channels of resistance vessel smooth muscle cells. *Science in China (Series B)*, **36,** 439–450.

TANIGUCHI, J., FURUKAWA, K.-I., SHIGEKAWA, M. (1993). Maxi K^+ channels are stimulated by cyclic guanosine monophosphate-dependent protein kinase in canine coronary artery smooth muscle cells. *Pflügers Archiv.*, **423,** 167–172.

TOWNSEND, G.C. & SCHELD, W.M. (1993). Adjunctive therapy for bacterial meningitis: rationale for use, current status and prospects for the future. *Clin. Infect. Dis.*, **17,** S537–S549.

UENO, M. & LEE, T.J.-F. (1993). Endotoxin decreases the contractile responses of the porcine basilar artery to vasoactive substances. *J. Cereb. Blood Flow Metab.*, **13,** 712–719.

WANG, Y. & MATHERS, D.A. (1993). Ca^{2+}-dependent K^+ channels of high conductance in smooth muscle cells isolated from rat cerebral arteries. *J. Physiol.*, **462,** 529–545.

WANG, Y., BAIMBRIDGE, K.G. & MATHERS, D.A. (1991). Effects of serotonin on intracellular free calcium of rat cerebrovascular smooth muscle cells in culture. *Can. J. Physiol. Pharmacol.*, **69,** 393–399.

WANG, Y.-X., POON, C.I. & PANG, C.Y. (1993). *In vitro* and *ex vitro* inhibitory effects of L- and D-enantiomers of N^G-nitro-arginine on endothelium-dependent relaxations of rat aorta. *J. Pharmacol. Exp. Ther.*, **265,** 112–119.

WOOD, K.S., BUGA, G.M., BYRNS, R.E. & IGNARRO, L.J. (1990). Vascular smooth muscle-derived relaxing factor (MDRF) and its close similarity to nitric oxide. *Biochem. Biophys. Res. Commun.*, **170,** 80–88.

YAMASHIMA, T., KASHIHARA, K., IKEDA, K., KUBOTA, T. & YAMAMOTO, S. (1985). Three phases of cerebral arteriopathy in meningitis: vasospasm and vasodilatation followed by organic stenosis. *Neurosurgery*, **16,** 546–553.

YEH, J.L., WHITNEY, E.G., LAMB, S. & BROPHY, C.M. (1996). Nitric oxide is an autocrine feedback inhibitor of vascular smooth muscle contraction. *Surgery*, **119,** 104–109.

(Received June 30, 1997
Revised September 10, 1997
Accepted September 23, 1997)