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Bacterial endotoxin alters kinetics of BK channels in rat cerebrovascular smooth muscle cells

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Abstract

Patch-clamp recordings were used to study the effects of *Escherichia coli* bacterial endotoxin (lipopolysaccharide, LPS) on the properties of large-conductance, Ca^{2+}-dependent K^+ channels (BK channels) in the membrane of enzymatically dispersed rat cerebrovascular smooth muscle cells (CVSMCs). LPS had negligible effects on the kinetic and conductance properties of BK channels when applied to the extracellular domain of these channels. However, acute application of LPS (10–100 μg/ml) to inside-out patches of CVSMC membrane isolated in a cell-free environment rapidly and reversibly increased the open probability of BK channels, leaving the conductance of these channels unaltered. The magnitude of this effect decreased as the concentration of free Ca^{2+} at the cytoplasmic membrane face was lowered, but was little affected by changes in membrane potential. Kinetic analysis showed that LPS accelerated reopening of BK channels while having little effect on mean channel open time. Detoxified *E. coli* LPS, from which the fatty acid chains of Lipid A were partially removed, showed slightly reduced activity when compared to the parent endotoxin molecule. A purified *E. coli* Lipid A had negligible effects on BK channel function. These results indicate that LPS activates BK channels in cerebrovascular smooth muscle cells when present at the cytoplasmic membrane face. This novel mechanism may provide insights into the regulation of BK channels by intracellular, membrane-associated elements. © 1998 Elsevier Science B.V.

Keywords: Lipopolysaccharide; Ca^{2+}-dependent K^+ channel; Cerebrovascular smooth muscle cell; Meningitis; Lipid A; Tetraethylammonium

1. Introduction

The contractile state of cerebrovascular smooth muscle cells (CVSMCs) in arteries and arterioles regulates cerebral blood flow and is partially determined by the balance between inward and outward currents flowing across the CVSMC membrane [1,2]. Inward currents flow in voltage-operated calcium channels (VOCCs) [3,4], non-selective cation channels [5] and chloride channels [6]. Outward currents utilize several types of potassium selective channels [7]. These include large conductance, Ca^{2+}-dependent K^+ channels (BK channels) which open in response to membrane depolarization and to a rise in the concentration of free intracellular calcium concentration, $[Ca^{2+}]_i$ of CVSMCs [4,7–9]. These properties allow BK channels to exert negative feedback during stretch-induced depolarization and contraction in cerebral artery smooth muscle. BK channels may, therefore, play an important role in the control of

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myogenic tone, a key element in the autoregulation of cerebral blood flow [4,7,10].

The gating of BK channels between open and closed states is altered by application of certain fatty acids and their derivatives. These agents include myristic [11], 2-decenoic [12], and arachidonic acids [13], as well as the epoxyeicosatrienoic acids (EETs) which are eicosanoid derivatives of arachidonate [13]. These compounds all increase the open probability, P_0 of BK channels without altering the flow of potassium ions through open channels. Myristic acid, 2-decenoic acid and arachidonic acid increase P_0 when applied to either the external or to the cytoplasmic membrane surface [11–13]. Both myristic acid and the EETs dilate blood vessels and may help to regulate blood flow in both normal and pathological states [13–15].

Lipopolysaccharide (LPS), the outer membrane endotoxin of gram-negative bacteria [16], also exerts potent vasodilatory effects on cerebral [17,18] and systemic blood vessels [19]. These LPS-induced changes in vascular tone are believed to be very important in the pathogenesis of both bacterial meningitis [20,21] and of septic shock [22]. Interestingly, the toxic Lipid A moiety found in LPS contains several types of fatty-acid chain, including the 3-hydroxy derivative of myristic acid [23]. Partial removal of these fatty acids by alkaline hydrolysis spares the phosphorylated glucosamine-disaccharide backbone of Lipid A, as well as the O-antigen and core polysaccharide components of endotoxin. The resulting molecule, 'detoxified' LPS (dLPS), exhibits one-thousand-fold less activity in the *Limulus* amoebocyte lysate test for endotoxicity [24,25].

In the light of this comparison, therefore, it seemed possible that LPS might itself influence the gating of BK channels in vascular smooth muscle, in a manner dependent on the presence of fatty acid chains in the endotoxin molecule. We have tested this hypothesis by applying normal LPS, detoxified LPS and purified Lipid A to the cytoplasmic surface of rat CVSMC membranes isolated in vitro.

2. Materials and methods

CVSMCs were enzymatically dissociated from the middle, posterior communicating and posterior cere-

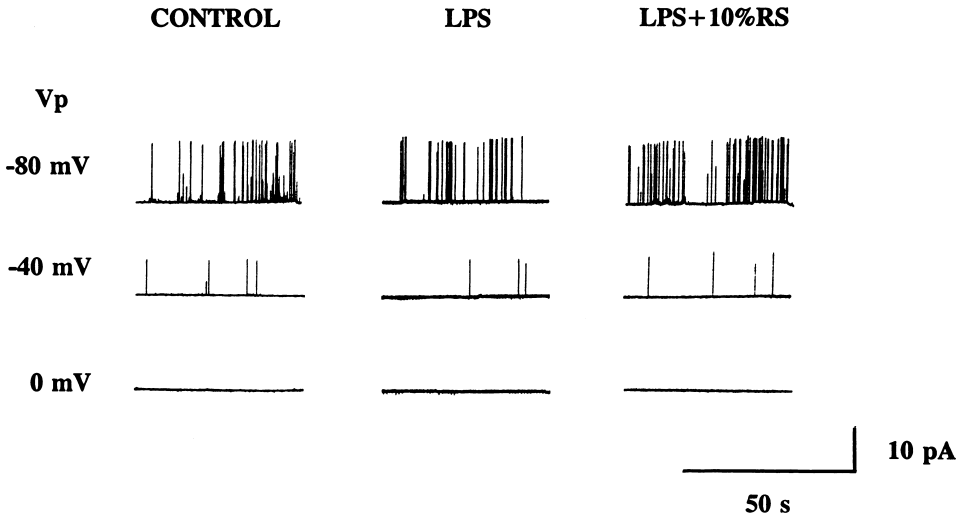

Fig. 1. LPS applied to the external face of the CVSMC membrane via the patch electrode does not acutely activate BK channels. Shown here are representative, cell-attached recordings made from three CVSMCs bathed in *solution D*. Patch electrodes contained *solution C* alone (Control), with 100 μg/ml LPS, or 100 μg/ml LPS with 10% normal rat serum. Patches were voltage-clamped to the pipette potentials, V_p, indicated at left of the traces. In all three membrane patches, a single BK channel was present, as determined by later patch excision and exposure of the cytoplasmic membrane face to 1.6 mM Ca^{2+}. In all three recordings, no spontaneous openings of BK channels were observed at $V_p = 0$ mV. Depolarization of these membrane patches by application of negative pipette voltages ($V_p = -40$ mV or -80 mV) increased the frequency of single BK channel currents as well as the magnitude of outward current flowing in open channels (upward deflections from baseline). No appreciable changes were noted in the behavior of BK channels exposed to LPS or to LPS with normal rat serum.

bral arteries of adult Wistar rats (250–300 g) using 0.1% trypsin and 0.3% collagenase, as previously described [8]. 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 prior to recording. Individual cells used for recording were later identified as CVSMCs using a monoclonal antibody (Sigma, St. Louis, MO) specific for smooth muscle α-actin, which is absent from endothelia and fibroblasts of cerebrovascular origin [8]. Use of this antibody, visualized by fluorescein isothiocyanate labelling, and the Trypan Blue dye exclusion test for viability showed that 4-day old cultures typically contained > 75% CVSMCs, and that more than 80% of these cells remained viable at this time.

Inside-out membrane patches were excised from CVSMCs using standard techniques [26]. Patch clamp recordings took place at 21–23°C and were obtained 1–5 h after removal of cells from the incubator. Patch electrodes (10–15 MΩ resistance) normally contained *solution A* of the following composition (in mM): 140 KCl; 1.48 $CaCl_2$; 10 HEPES, 3 EGTA, pH 7.4 (free calcium concentration 50 nM). The very low calcium concentration in the patch pipette solution prevented activation of BK channels by inward Ca^{2+} currents during depolarization of excised membrane patches. The cytoplasmic face of inside-out membrane patches was normally exposed to *solution B* of composition (in mM): 140 KCl; 2.86 $CaCl_2$, 10 HEPES; 3 EGTA, pH 7.4 (free calcium concentration 1 μM). Excised inside-out patches of CVSMC membrane were completely isolated from all cells by translocating the patch electrode to the second, cell-free compartment of a dual-well recording chamber. This eliminated the possible effects on BK channel gating of factors released from neutrophils and endothelia in response to LPS application [21,27]. LPS, *E. coli* serotype 0127:B8, its detoxified analog (dLPS) and *E. coli* serotype F-583 diphosphoryl Lipid A were obtained from Sigma and suspended in *solution B* by vortexing immediately prior to use. The solution bathing the exposed cytoplasmic face of membrane patches could be completely replaced by a new test saline within 30 s.

For determination of the effects of externally applied LPS on BK channels, in the absence and presence of serum, cell-attached recordings were made

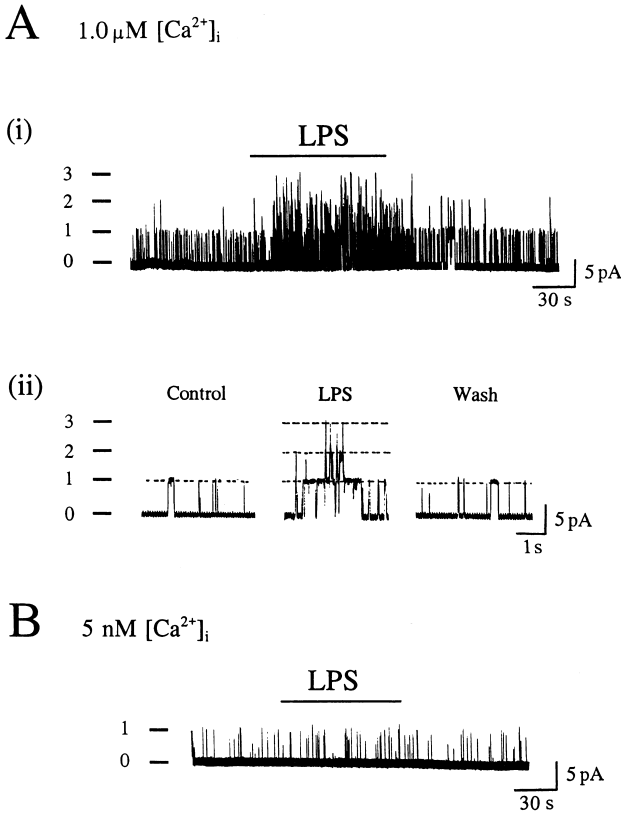

Fig. 2. A – (i): Rapid and reversible activation of ion channels by LPS in an isolated, inside-out patch of CVSMC membrane. LPS (100 μg/ml) was applied to the cytoplasmic face of this membrane patch for the duration indicated by the horizontal bar. This patch contained three channels and the scale at left indicates mean current levels when 0,1,2 and 3 channels were open. Outward membrane current is represented by an upward deflection from baseline. The patch was voltage-clamped to a membrane potential, $V = +20$ mV and $[Ca^{2+}]_i = 1$ μM. Bandwidth of recording DC – 200 Hz. (ii): Representative single channel currents taken from the experiment shown in (i) but displayed at higher time resolution (bandwidth DC – 2 kHz). These traces were obtained during the control, LPS and wash phases of the experiment, as indicated. The scale at left and the dashed lines indicate the mean current levels when 0,1,2 and 3 channels were open. B – Spontaneously active and LPS-activated ion channels in the same patch of CVSMC membrane seen in A above, during perfusion of the cytoplasmic membrane face with saline containing $[Ca^{2+}]_i = 5$ nM. Application of 100 μg/ml LPS in this saline (horizontal bar) now resulted in little additional single channel activity. Membrane potential, $V = +20$ mV. Bar at left indicates number of open channels. Bandwidth of recording DC – 200 Hz.

using patch electrodes filled with *solution C* of composition (mM): 135 NaCl; 4 KCl; 1.8 $CaCl_2$; 10 HEPES, pH 7.3. LPS was suspended in this saline by vortexing immediately prior to filling of the patch electrodes. In some experiments, 10% normal rat serum, prepared by exsanguination of adult Wistar rats, was added to *solution C*, alone, or in combination with LPS. During all cell-attached recordings, cells were bathed in *solution D* of composition (mM): 140 KCl; 1.6 $CaCl_2$; 10 HEPES; 5 glucose, pH 7.4.

For cell-attached recordings, cells were depolarized by equilibration in *solution D* containing 140 mM K^+. Patch electrodes contained *solution C* with 4 mM K^+, allowing large, outwardly directed currents to flow through open BK channels at patch pipette voltages, V_p in the 0–(−80) mV range. Following cell-attached recording, these membrane patches were excised, exposing the cytoplasmic membrane face to 1.6 mM Ca^{2+} and allowing the number of BK channels in each patch to be determined. Membrane patches containing the same number of channels were chosen when comparing channel activation between experimental groups.

Patch current (bandwidth DC-2 kHz, - 3 dB Bessel) and voltage were analyzed using commercial software (Instrutech, Great Neck, NY). Simplex maximization of likelihood was employed to fit exponential components to the observed open and closed time distributions, disregarding channel openings or closings briefer than 180 μs. Frequency distributions of current amplitudes were fitted using Simplex methods to computer-generated Gaussian functions. BK channels were identified in excised patches by their large conductance (> 200 pS) and block by 25 mM tetraethylammonium ions (TEA^+) or 5 nM free calcium saline applied to the cytoplasmic membrane surface [8]. The probability, P_0 of finding a single BK channel in the open state during a recording of total duration, T_{tot} was calculated from the relation $P_0 = (T_1 + 2T_2 + \cdots + NT_N)/NT_{tot}$. Here, N is the total number of functional BK channels in the patch, estimated under conditions which strongly favor

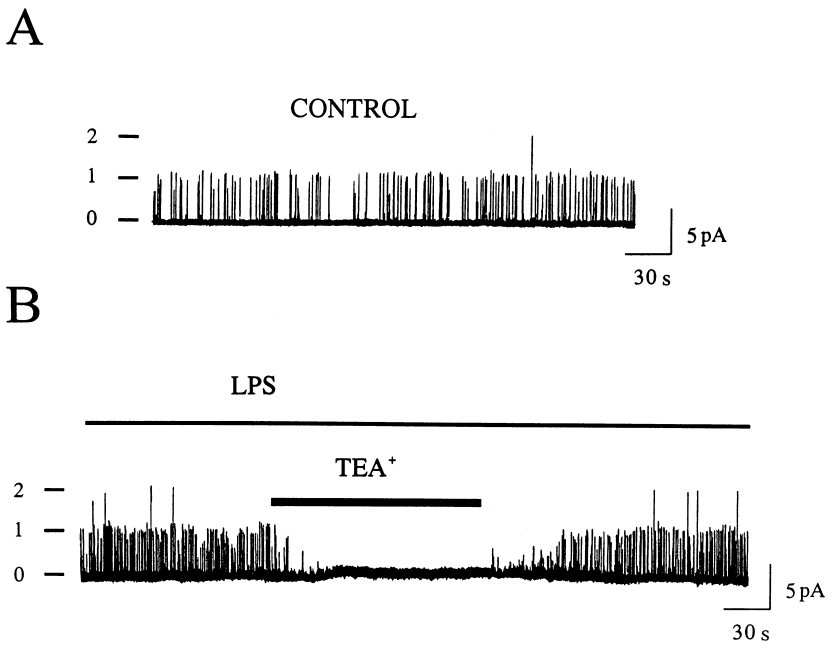

Fig. 3. Effects of TEA^+ on spontaneously active and LPS-activated ion channels in an isolated, inside-out patch of CVSMC membrane voltage clamped at $V = +20$ mV with $[Ca^{2+}]_i = 1$ μM. This patch contained two channels. The scales at left indicate mean current levels when 0,1 or 2 channels were open. A – Spontaneous channel activity in this patch recorded in the absence of LPS and TEA^+ (control). B – Application of 100 μg/ml LPS to this patch (long horizontal bar) increased the frequency of single-channel currents. Co-application of TEA^+ (10 mM) for the duration of the short, horizontal bar completely blocked both spontaneous and LPS-activated currents in a reversible fashion. Bandwidth of recording DC – 200 Hz.

channel opening. $T_1, T_2, \cdots, T_N$ were the times when at least 1,2, $\cdots$ N channels were open. Results were expressed as mean ± S.E.M. The Student's *t*-test and ANOVA were used to evaluate differences between experimental groups.

3. Results

Cell-attached recordings from CVSMCs were employed to study the effects of LPS when presented to the extracellular domain of BK channels. Since serum proteins facilitate high affinity binding of LPS to membrane surface receptors [28], the effect of normal rat serum on the response of BK channels to endotoxin was also studied.

At a pipette potential, $V_p = 0$ mV, corresponding to a membrane potential, $V \sim 0$ mV, cell-attached patches showed no spontaneous openings of the BK channel. This behaviour was observed in control recordings, as well as in recordings made using patch pipettes containing 100 μg/ml LPS with, or without 10% normal rat serum (Fig. 1).

On depolarization of cell-attached membrane patches by application of negative pipette voltages,

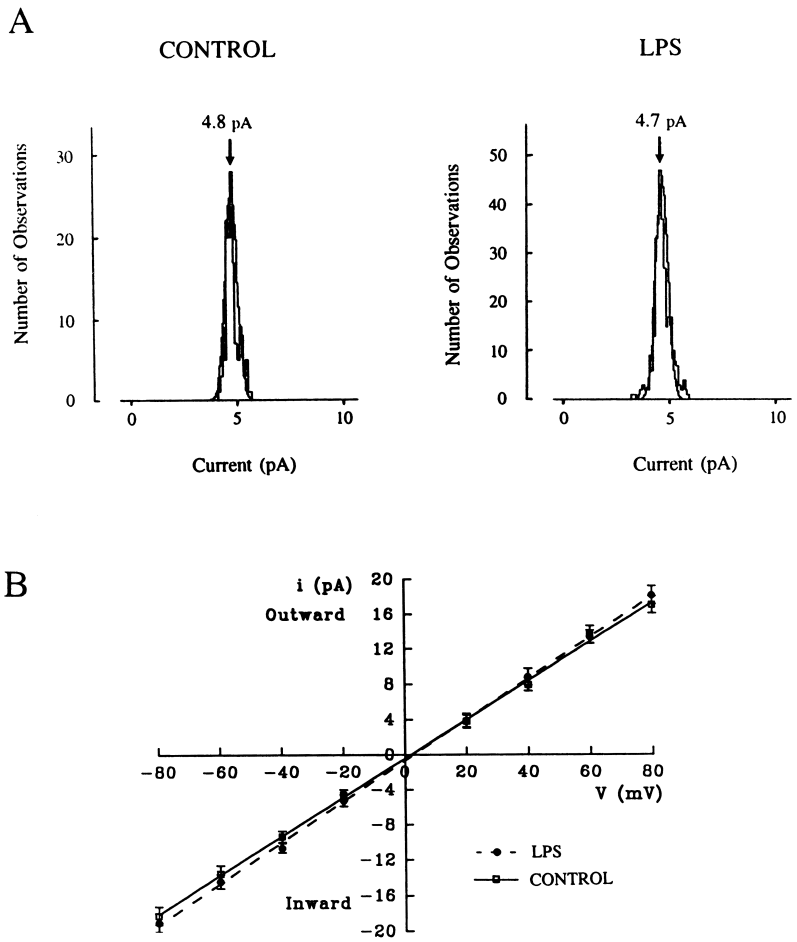

Fig. 4. Spontaneously active and LPS-activated membrane channels have the same conductance and reversal potential. A – Amplitude distributions for single-channel currents recorded from an inside-out patch of CVSMC membrane voltage clamped at $V = +20$ mV with $[Ca^{2+}]_i = 1$ μM. In both, the absence (control) and presence of 100 μg/ml LPS, these distributions were well-described by single Gaussian terms (smooth curves), with mean current amplitudes (arrows) located at 4.8 pA (control) and 4.7 pA (LPS). B – Current–voltage relationships for spontaneously active channels (control, □) and channels active in the presence of 100 μg/ml LPS (LPS, ●). Data points represent the mean ± S.E.M. values for six patches in each group. Data sets were fitted by linear regression by a solid line (control) or a dashed line (LPS), yielding single channel conductances of 219 and 223 pS, respectively. $[Ca^{2+}]_i = 1$ μM.

BK channel openings became more frequent, as expected for a voltage-dependent channel [7–9]. When measured over the $V_p = -20$ to -40 mV range, BK channels exhibited a chord conductance of 125–150 pS, consistent with previous reports for cell-attached recordings under a physiological concentration gradient for K^+ [11–15]. As shown in the representative traces of Fig. 1, BK channel activation did not appreciably alter when membrane patches were exposed to LPS or to LPS with serum applied *via* the patch electrode.

LPS was next applied to the cytoplasmic face of inside-out patches of CVSMC membrane containing 1 to 4 BK channels and voltage-clamped to a membrane potential of $V = +20$ mV, with $[Ca^{2+}]_i =$ 1 μM. Under these conditions, LPS (100 μg/ml) rapidly increased the activity of ion channels gating outward membrane currents in the 4–6 pA range (Fig. 2(A)). The induction of channel activity by LPS reached its full extent within ~30 s, a time scale comparable to that needed for complete solution exchange in the recording chamber. On wash-out of LPS, restoration of baseline levels of channel activity proceeded with a similar time course (Fig. 2(A)).

The nature of these LPS-induced single channel events was investigated by reducing $[Ca^{2+}]_i$ and by co-application of the K^+ channel blocker tetraethylammonium (TEA^+) ions [29,30]. Both of these procedures strongly inhibit BK channel function [8,12,14]. When $[Ca^{2+}]_i$ was reduced to 5 nM,

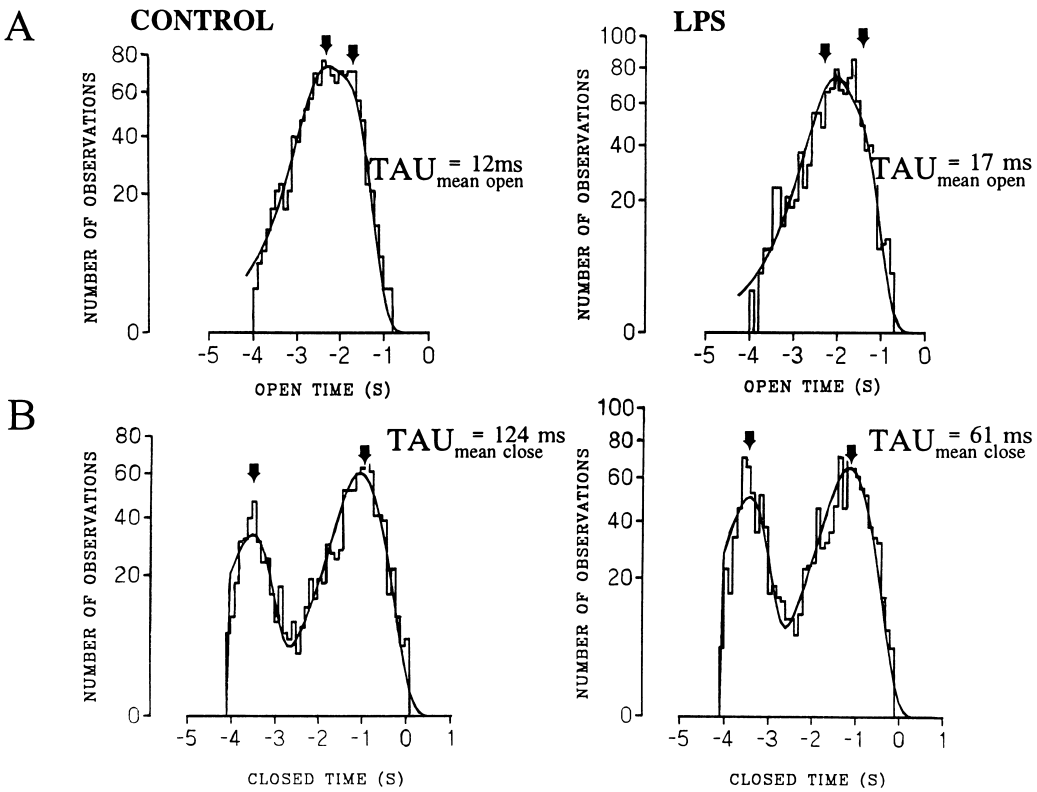

Fig. 5. Effects of 100 μg/ml LPS on the kinetics of BK channels in isolated, inside-out patches of CVSMC membrane voltage-clamped to $V = +20$ mV with $[Ca^{2+}]_i = 1$ μM. A – Open time distributions obtained from the same membrane patch in control (613 channel openings) and LPS-containing salines (682 channel openings). Each distribution was well-described by the sum of two exponential terms (smooth curves) using the following fit parameters defined in the text. Control: $\tau_{of} = 3.2$ ms; $\tau_{os} = 17$ ms. LPS: $\tau_{of} = 6.1$ ms; $\tau_{os} = 28$ ms. Arrows indicate positions of τ_{of} and τ_{os} on the time axis. Mean channel open times, $\tau_{mean\ open}$ were 12 and 17 ms for control and LPS data, respectively. B – Corresponding closed time distributions from same recordings as used in A. These distributions were also well-described by the sum of two exponentials (smooth curves) using the following parameters. Control (556 closings): $\tau_{cf} = 0.4$ ms; $\tau_{cs} = 172$ ms. LPS (573 closings): $\tau_{cf} = 0.42$ ms; $\tau_{cs} = 103$ ms. Arrows indicate positions of τ_{cf} and τ_{cs} on the time axis. Mean channel closed times, $\tau_{mean\ close}$ were calculated as 124 and 61 ms for control and LPS data, respectively.

Table 1
Effects of LPS (100 μg/ml) on the open probability, P_0 and gating kinetics of BK channels studied in isolated, inside-out patches of CVSMC membrane voltage-clamped to a potential $V = +20$ mV with $[Ca^{2+}]_i = 1$ μM. See text for explanation of symbols used. LPS was applied to the exposed, cytoplasmic face of the isolated membrane patches by bath perfusion. Values given represent the mean ± S.E.M. for six patches studied in each group

Parameter	Control	LPS	Wash
P_0	0.04 ± 0.01	0.13 ± 0.04 [a]	0.03 ± 0.01
τ_{of} ms	0.7 ± 0.3	1.8 ± 0.6	0.9 ± 0.2
τ_{os} ms	14.7 ± 2.2	18.2 ± 3.5	11.2 ± 2.3
$\tau_{mean\ open}$ ms	8.9 ± 1.2	10.2 ± 1.8	8.2 ± 2.1
τ_{cf} ms	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
τ_{cs} (ms)	284 ± 46	122 ± 22 [a]	267 ± 61
$\tau_{mean\ close}$ (ms)	151 ± 32	46 ± 12 [a]	160 ± 46

[a] Significantly different from corresponding control values ($P < 0.05$, ANOVA).

single-channel currents became very infrequent in both the absence and presence of LPS (Fig. 2(B)). This effect was reversed by reapplication of *solution B* containing 1 μM $[Ca^{2+}]_i$ to the cytoplasmic membrane face. Perfusion of this membrane face with *solution B* containing 10 mM TEA^+ reversibly abolished both spontaneous and LPS-induced single-channel currents (Fig. 3). During application and wash-out of TEA^+, an apparent reduction in the amplitude of single-channel currents was observed (Fig. 3). This is a well-established feature of BK channel blockade by TEA^+ and is believed to reflect the intermediate or flickery block of open channels by tetraethylammonium ions [8,30,31].

Taken together, these results suggested that the additional single channel currents seen in the presence of LPS were due to the activation of BK channels in the CVSMC membrane. This conclusion was further tested by quantitative analysis of the conductance and kinetic properties of these channels. In both, the absence and presence of LPS, the amplitude distributions of channel currents were well described by single Gaussian terms (Fig. 4(A)). In addition, neither the reversal potential for current flow, ($E_r \sim 0$ mV) nor the conductance, γ of single channels was altered by LPS (control saline, $\gamma = 219 \pm 12$ pS; in 100 μg/ml LPS, $\gamma = 223 \pm 10$ pS, $n = 6$ patches, $P > 0.05$, ANOVA, Fig. 4(B)).

As found in previous studies [8,31], open time distributions of BK channel currents studied in control patches were well described by the sum of two exponential functions, that is $y = A_{of}e^{-t/\tau_{of}} + A_{os}e^{-t/\tau_{os}}$ (Fig. 5(A)). Here, the fast and slow time constants τ_{of} and τ_{os} governed the amplitude terms A_{of} and A_{os}, respectively. When 100 μg/ml LPS was present at the cytoplasmic membrane face, open time distributions for observed single channel currents remained of this form (Fig. 5(A)). Similarly, values of τ_{of}, τ_{os} and of the mean open time of BK channels, calculated as $\tau_{mean\ open} = A_{of}/(A_{of} + A_{os})\tau_{of} + A_{os}/(A_{of} + A_{os})\tau_{os}$ were all unchanged on exposure to LPS (Table 1).

Kinetic analysis was also performed for closed time distributions of BK channel currents. In the absence as well as presence of 100 μg/ml LPS, these distributions were again well-described by the sum of two exponentials, the mean channel closed time being given by $\tau_{mean\ close} = A_{cf}/(A_{cf} + A_{cs})\tau_{cf} + A_{cs}/(A_{cf} + A_{cs})\tau_{cs}$ (Fig. 5(B)). Exposure 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 LPS, as was the mean channel closed time, $\tau_{mean\ close}$ (Table 1). These results provided further evidence that LPS acutely increased the open probability, P_0, of BK channels in the CVSMC membrane. In addition, they showed that LPS acted by accelerating the re-opening, rather than by retarding the closure of BK channels.

The effects of LPS were next re-examined in inside-out membrane patches voltage-clamped at $V = -50$ mV with $[Ca^{2+}]_i = 1$ μM. As shown in Fig. 6(A), LPS (100 μg/ml) also increased P_0 in membrane patches examined at $V = -50$ mV, and this increase was not significantly altered by the change in membrane voltage. However, the degree of potentiation, seen in P_0, did decrease when $[Ca^{2+}]_i$ was reduced from 1 μM to 0.1 μM, the latter value being typical of relaxed cerebrovascular smooth muscle cells [32]. No significant increase in P_0 was evident at $[Ca^{2+}]_i = 5$ nM (Fig. 6(B)). Therefore, the activation of BK channels by LPS was itself a Ca^{2+}-dependent process.

To determine whether the observed effects of LPS on BK channel gating required the presence of fatty acid chains, the relative potencies of parent LPS and of detoxified LPS were directly compared in the same series of inside-out patches voltage-clamped at

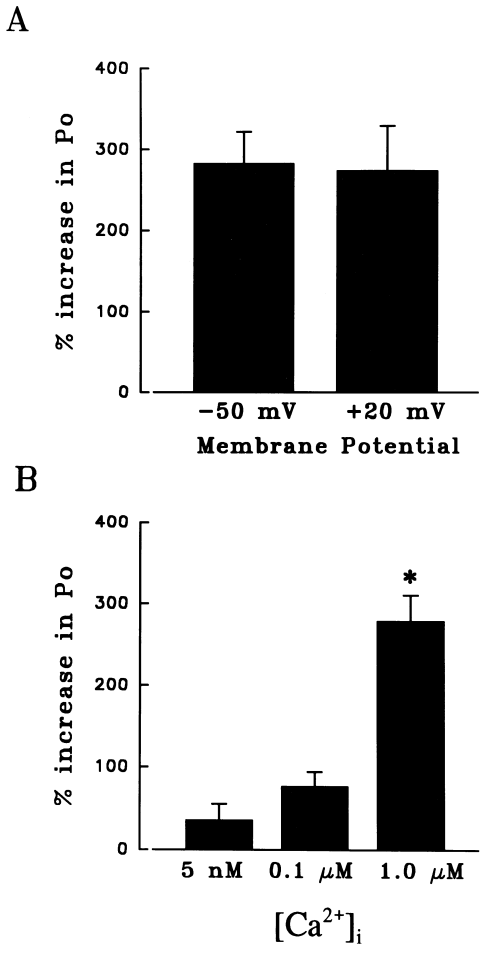

Fig. 6. A – Lack of effect of membrane potential on the activation of BK channels by 100 μg/ml LPS. Data were obtained from 10 inside-out membrane patches voltage-clamped to $V = -50$ mV and seven patches held at $V = +20$ mV with $[Ca^{2+}]_i = 1$ μM. The mean increases in P_0 seen at these two potentials were not significantly different ($P > 0.05$, ANOVA). B – Dependence of LPS-induced activation of BK channels on the concentration of free calcium ions at the cytoplasmic membrane face, $[Ca^{2+}]_i$. An 100 μg/ml LPS aliquot was applied to inside-out membrane patches voltage-clamped to $V = +20$ mV and bathed in saline containing 5 nM ($n = 4$ patches), 0.1 μM ($n = 7$) or 1 μM $[Ca^{2+}]_i$ ($n = 17$). At both, 0.1 μM and 1 μM, but not at 5 nM $[Ca^{2+}]_i$, LPS significantly potentiated P_0 over control values. The degree of potentiation seen at 1 μM $[Ca^{2+}]_i$ was significantly greater than that seen at 0.1 μM free calcium (asterisk, $P < 0.05$, ANOVA).

$V = +20$ mV with $[Ca^{2+}]_i = 1$ μM. Since the average molecular weight of LPS in aqueous solutions is not precisely known, LPS and dLPS were compared on an equal-weight basis.

Fig. 7 shows the effects of 10 and 100 μg/ml LPS and of 100 μg/ml dLPS on the open probability of BK channels. The threshold concentration for a significant effect of parent LPS on P_0 was 10 μg/ml. Both LPS and dLPS activated BK channels when applied at a concentration of 100 μg/ml. However, the degree of activation seen with dLPS was about half that obtained with the parent LPS (Fig. 7). These results indicated that the fatty acid chains of parent LPS, apparently, contribute to the action of this molecule on BK channel kinetics.

The gating of BK channels was now compared in the presence of parent LPS and of diphosphoryl Lipid A, the fatty-acid-bearing moiety of endotoxin [23]. In marked contrast to parent LPS, the purified Lipid A of *E. coli* serotype F-583 (2–20 μg/ml) was unable

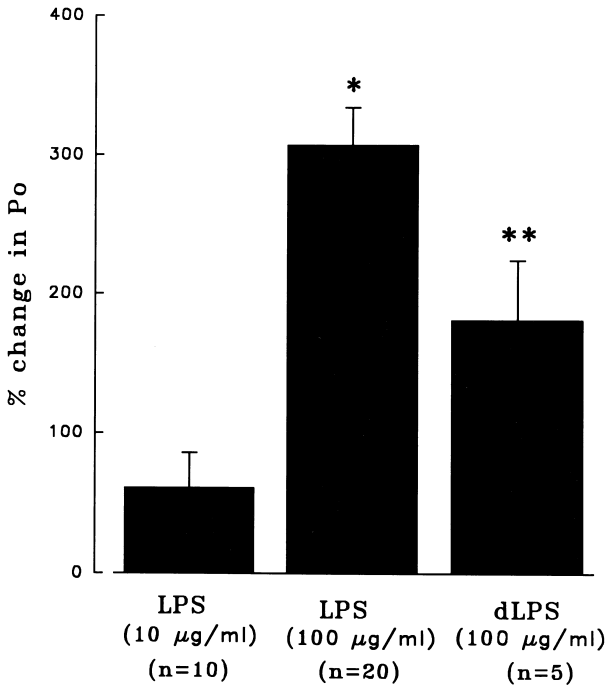

Fig. 7. Comparison of the increase in P_0 caused by application of 10 μg/ml or 100 μg/ml parent LPS with that produced by 100 μg/ml detoxified LPS (dLPS) to inside-out patches of CVSMC membrane voltage clamped to $V = +20$ mV with $[Ca^{2+}]_i = 1$ μM. Values shown are the mean ± S.E.M. for 10, 20 and 5 patches in these groups, respectively. All three treatments significantly increased P_0 over corresponding control levels. The increase seen with 100 μg/ml parent LPS was significantly larger than that obtained with 10 μg/ml parent LPS (single asterisk) or with 100 μg/ml dLPS (double asterisk, $P < 0.05$, ANOVA).

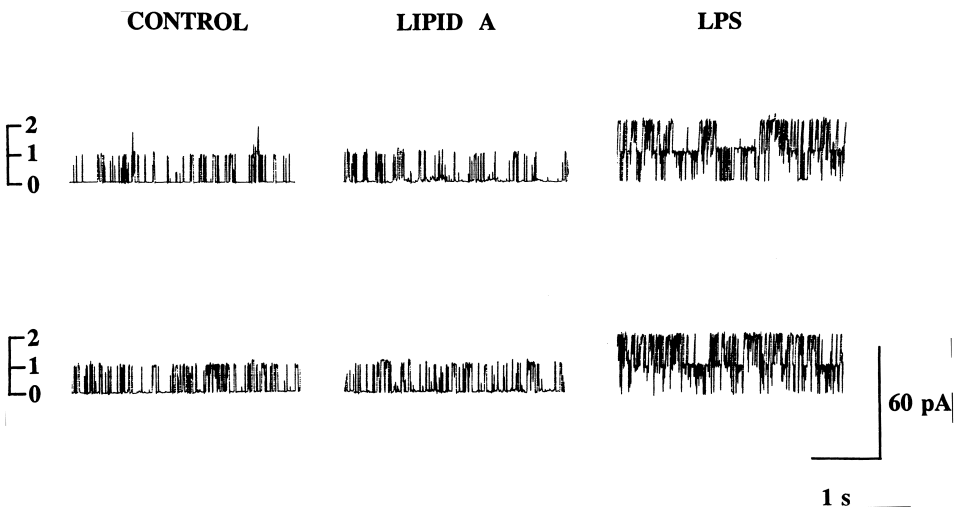

Fig. 8. In contrast to the whole LPS molecule, purified *E. coli* diphosphoryl Lipid A does not acutely activate BK channels when applied to inside-out patches of CVSMC membrane. This representative membrane patch was voltage-clamped to $V = +20$ mV with $[Ca^{2+}]_i = 1\,\mu M$. Lipid A (20 μg/ml) and LPS (100 μg/ml) were applied by bath perfusion. Numerals by scales at left indicate the number of open BK channels.

to acutely activate BK channels in inside-out patches of CVSMC membrane (Fig. 8).

4. Discussion

The present study has shown that LPS rapidly activates large conductance, Ca^{2+}-dependent potassium channels when applied to the cytoplasmic face of cerebrovascular smooth muscle cell membranes. This activation reversed readily on wash-out of LPS. In principle, LPS may activate a novel population of ion channels in the CVSMC membrane, or may simply promote the opening of pre-existing BK channels. The latter view is strongly favored for the following reasons. Like BK channels [11–14,29,30], LPS-activated channels were sensitive to block by low $[Ca^{2+}]_i$ saline, and current flow through open channels was suppressed in the presence of tetraethylammonium ions. In addition, the conductance, gating behavior and mean open time of channels active in the presence of LPS were all typical of spontaneously active BK channels in our preparation [8].

LPS-induced activation of BK channels was not significantly influenced by reversing the polarity of the electric field across the cell membrane. It seems unlikely, therefore, that electrostatic interactions between the polyanionic LPS molecules [33] and the membrane field played a major role in the mechanism of endotoxin action. Reversing the membrane field also inverted the electrochemical gradient for Ca^{2+} flow across the CVSMC membrane. Since LPS action was voltage-independent, it is unlikely that endotoxin stimulated BK channels indirectly by opening voltage-operated Ca^{2+} channels in the patch membrane [34].

Isolated *E. coli* outer membranes contain up to 70 nM Ca^{2+} per mg total dry weight, much of this calcium being bound to LPS molecules [35–37]. However, the LPS samples used in the present study were prepared using chelating agents and, therefore, contained only trace amounts of divalent cations. In addition, LPS was added to solutions in which the free calcium-ion concentration was closely buffered by the presence of EGTA. It is unlikely, therefore, that the present results simply reflected the release of Ca^{2+} from endotoxin molecules.

A variety of free fatty acids have been found to activate the BK channels of rabbit coronary artery smooth muscle cells. When applied to the cytoplasmic membrane face, long-chain molecules (linoleic acid) were more effective than the short-chain myristic acid, the degree of unsaturation being of negligible importance [11]. Since the Lipid A moiety of *E. coli* LPS contains a variety of fatty acid derivatives,

including 3-hydroxymyristic acid [16,23], it seemed possible that these residues were responsible for the action of endotoxin on BK channels. However, this hypothesis now seems unlikely for the following reasons.

Firstly, BK channels were strongly activated by myristic acid [15] and related substances [11,12,15], even when free intracellular Ca^{2+} was virtually absent, a condition which prevented channel activation by LPS. This observation suggests that LPS and free fatty acids activate BK channels by fundamentally different mechanisms, despite their structural similarities and a shared ability to increase the fluidity of membranes [38,39].

Secondly, partial removal of the fatty acid portions of Lipid A only slightly reduced the ability of the endotoxin molecule to activate BK channels. This implies that parts of the LPS molecule other than fatty acids play a major role in BK channel activation. Thirdly, purified *E. coli* Lipid A was unable to mimic the observed effects of LPS on BK channel function.

It is, therefore, likely that the hydrophobic fatty acid residues of Lipid A are not themselves responsible for BK channel activation. Rather, these residues may serve to stabilize the whole LPS molecule at a membrane site near the channel protein. An interaction between the polysaccharide residues of LPS and the BK channel then results in an increased probability of channel opening, possibly by altering the sensitivity of the channel to available calcium ions. The polysaccharide components of LPS are known to be required in several other endotoxin-mediated processes, including stimulation of nitric oxide synthesis from L-arginine in murine macrophages [40], induction of mitosis in mouse splenic cells [41] and activation of the host immune response [42,43].

Incubation of intact CVSMCs for several hours in the presence of extracellularly applied LPS results in expression of the inducible nitric oxide synthase gene, and in consequent nitric oxide (NO) production by these cells [44]. These events are followed by an NO-dependent activation of BK channels in the smooth muscle cell membrane [9]. The present results show that LPS also activates BK channels when applied to the cytoplasmic face of the CVSMC membrane. A comparison between these two mechanisms reveals that the intracellular action of LPS requires higher endotoxin concentrations, proceeds on a much more rapid time scale and persists even in the absence of intact cells or tissues. Whether the extracellular and intracellular events initiated by LPS share common features, or are completely distinct pathways, merits further detailed study.

LPS is known to accumulate in some mammalian cell types, either as a consequence of receptor-mediated endocytosis [45], or of intracellular replication by gram-negative bacteria [46]. Accumulation of endotoxin in cerebrovascular smooth muscle cells has, however, yet to be reported. Therefore, the relevance of the present results to the etiology of clinical meningitis remains unclear. Nonetheless, LPS clearly represents a useful probe with which to investigate the regulation of BK channel function by intracellular, membrane-associated elements in mammalian cells.

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