

Expression of Kv1.5 K⁺ Channels in Activated Microglia In Vivo

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KEY WORDS microglia; K⁺ channels; activation; LPS; iNOS

ABSTRACT We examined the expression of outward rectifier K⁺ channels in activated microglia in vivo. For this purpose, lipopolysaccharide (LPS, 2 µg) was injected into the cortex near the hippocampal region of rat brains, and K⁺ channel expression was examined using antibodies against shaker-type K⁺ channels, Kv1.5 and Kv1.3. OX-42-positive microglia were found around the injection sites from 8 h after the LPS injection and remained there for 3 days. The OX-42-positive microglia expressed Kv1.5 immunoreactivity, and the time course of Kv1.5 expression was closely correlated with that of OX-42. In saline-injected brains, OX-42-positive cells also expressed Kv1.5 immunoreactivity even though far fewer OX-42-positive cells were found. Increase of Kv1.5 expression after LPS injection was also demonstrated by immunoblot analysis. On the other hand, Kv1.3 immunoreactivity was barely detected in OX-42-positive cells over the entire experimental period. The expression of Kv1.5 preceded that of inducible nitric oxide synthase (iNOS), which is a prominent indication of microglial activation. iNOS was not detectable until 12 h, and thereafter it was maintained for 3 days together with Kv1.5 and OX-42. These results suggest that in vivo as well as in vitro activated microglia expressed outward K⁺ channels and that some of the channels at least are Kv1.5. *GLIA* 24:408–414, 1998. © 1998 Wiley-Liss, Inc.

INTRODUCTION

Microglia, the main immune effector cells of the brain, become activated in brain injury and various neurodegenerative diseases such as Alzheimer's disease and multiple sclerosis (Woodroffe et al., 1986; McGeer et al., 1988; Meda et al., 1995; McRae et al., 1997). The activated microglia have been suggested to aggravate neuronal injury (Chao et al., 1992; Giulian et al., 1996; Khoury et al., 1996). They differ from inactive resting microglia. Morphologically, activated microglia are round whereas inactive ones are ramified (Giulian and Baker, 1986; Streit et al., 1988). Functionally, activated microglia enhance phagocytosis and produce several substances, such as nitric oxide (NO), prostaglandins (PGs), and tumor necrosis factor, that are involved in inflammation (Zielasek et al., 1992; Lee et al., 1993; Minghetti and Levi, 1995; Kitamura et al., 1996). Electrophysiologically, activated microglia in culture express outward rectifier K⁺ channels, which are absent in resting microglia (Kettenmann et al.,

1990; Korotzer and Cotman, 1992; Nörenberg et al., 1992). The function of K⁺ channels in microglia is not yet known. However, the K⁺ channels could be involved in microglial activation as the expression of K⁺ channels preceded that of iNOS; K⁺ currents were detected within 6 h after lipopolysaccharide (LPS) treatment whereas iNOS expression was detected 12 h after the treatment. Furthermore, 4-aminopyridine, a blocker of the outward K⁺ channels, significantly reduced NO release (Pyo et al., 1997).

The subtypes of K⁺ channels expressed in activated microglia have not yet been characterized. Nörenberg et al. suggested Kv1.3 as a major type of K⁺ channel expressed in activated microglia, as electrophysiological properties of microglial K⁺ currents were similar to

Contract grant sponsor: KOSEF; Contract grant number: 971-0704-073-2; Contract grant sponsor: BM; Contract grant number: 97-108.

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Received 5 August 1997; Accepted 6 April 1998

those of Kv1.3 (Nörenberg et al., 1992) and Kv1.3 mRNA was detected in polymerase chain reaction (PCR) analysis (Nörenberg et al., 1993). However, it is still possible that K⁺ channels other than Kv1.3 were expressed in activated microglia. Indeed, cultured microglia were stained with both Kv1.3 (Pyo et al., unpublished observation) and Kv1.5 antibodies after LPS treatment (Pyo et al., 1997). Despite numerous studies on outward rectifier K⁺ channels in cultured microglia, it has not been clearly elucidated whether the K⁺ channels are expressed in activated microglia *in vivo*. This is the first report to examine whether outward rectifier K⁺ channels are expressed in activated microglia *in vivo* and, if so, which types of K⁺ channels are expressed by injection of LPS into rat brains.

MATERIALS AND METHODS

Intracerebral Microinjection of LPS and Tissue Preparation

Male Sprague-Dawley rats weighing approximately 200 g were anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg) and immobilized in a stereotaxic frame (Stoelting, Wheat Lane, IL). LPS (2 µg in 2 µl of saline) was injected into the brain following the coordinates relative to the bregma, 3.8 mm caudal, 3.0 mm right lateral, and 3.5 mm below the dura (Paxinos and Watson, 1986). Sham-operated rats were injected with 2 µl of saline. Rats were sacrificed at 6, 8, 12, and 24 h and 2, 3, 4, and 7 days after surgery. Brains were frozen in isopentane cooled by dry ice, and 16-µm coronal sections were obtained throughout the lesion site using a cryostat (Reichert-Jung, UK).

For immunoblot analysis, brain tissue (approximately 3 × 3 × 3 mm) around the injection sites was excised 24 h after LPS injection. Meninges were removed, and the tissue was minced and lysed in RIPA buffer (150 mM NaCl, 10 mM Na₂HPO₄, pH 7.2, 0.5% sodium deoxycholate, 1% Nonidet P-40) containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 100 µg/ml leupeptin, 2 mM EDTA, and 10 µg/ml pepstatin). Uninjected brain tissue was also prepared in the same way. Proteins (100 µg) were separated on 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose paper, incubated with either Kv1.5 antibodies (UBI, Lake Placid, NY and Alomone Labs, Israel; 1:200 for both antibodies) or Kv1.3 antibodies (Alomone Labs; 1:200) and visualized using enhanced chemiluminescence system (Amersham, Little Chalfont, UK). Kv1.5 antibodies were purchased from two companies to assure the specific immunoreactivity of antibodies, and these two antibody preparations gave the same results. The figures of Kv1.5 in this paper were prepared using antibodies from UBI.

Immunohistochemistry

Microglia and astrocytes were identified by antibodies against OX-42 (Serotec, UK) and glial fibrillary

acidic protein (GFAP; Sigma Chemical Co., St. Louis, MO), respectively. The expression of K⁺ channels was detected by antibodies against Kv1.5 (UBI and Alomone Labs) and Kv1.3 (Alomone Labs).

For immunohistochemistry, sections were fixed with 3.7% formaldehyde (or 3.7% formaldehyde containing 0.3% H₂O₂ for peroxidase staining) for 20 min at room temperature, washed with saline, and incubated with 1% goat serum to reduce nonspecific binding for 30 min. The sections were then incubated with primary antibodies (OX-42, 1:30; GFAP, 1:400; Kv1.3, 1:30; Kv1.5, 1:50 for both antibodies) overnight at 4°C and visualized with peroxidase- (Vector Laboratories, Burlingame, CA), rhodamine-, or fluorescein-conjugated secondary antibodies (Cappel, Durham, NC). Sections were examined under a microscope (Nikon Diaphot 300, Japan).

Confirmation of Kv1.5 and Kv1.3 Antibody Specificity

The specificity of Kv1.5 and Kv1.3 antibodies was tested in immunoblot analysis using a Kv1.5-rich microsomal fraction of cardiac muscles (Attali et al., 1993) and Kv1.3-rich lymphocytes (Spencer et al., 1993). To prepare the microsomal fraction of cardiac muscles, two rat hearts were homogenized using a Polytron (Heidolph DIAX 600, Germany) in 10 ml of 10 mM sodium phosphate buffer (pH 7.4) containing protease inhibitors. The homogenate was centrifuged at 10,000 × *g* for 30 min, and then the supernatant was centrifuged at 100,000 × *g* for 45 min using a Beckman 55 Ti rotor. The precipitated microsomal fraction was resuspended in 500 µl of the buffer used for homogenization. Lymphocytes were prepared from rat blood using a Ficoll gradient (Boyum, 1968) and lysed in RIPA buffer. Immunoblot analysis was carried out using 30 µg of protein from the microsomal fraction of cardiac muscles or lymphocyte lysates.

The specificity of Kv1.5 antibodies was also tested by immunohistochemistry using Kv1.5 antibodies (10 µg) preincubated with the microsomal fraction from cardiac muscles (10 µg) overnight at 4°C, and the disappearance of Kv1.5 immunoreactivity was then examined.

Results

In the cortical region of the uninjected brain, OX-42 antibodies barely detected microglia (Fig. 1A). However, 12 h after the LPS injection, microglia were found in the injection site (Fig. 1B, inset B1) and ipsilateral hippocampal regions (Fig. 1B, inset B2). In the saline-injected brain, microglia were also found but only in the injection sites (Fig. 1C, inset C1). In both LPS- and saline-injected brains, the OX-42-positive cells expressed iNOS (see below Fig. 5). A possible tissue damage due to LPS or saline injection was examined by hematoxylin/eosin staining and acid fuchsin staining for up to 7 days. In either case, tissue damage except

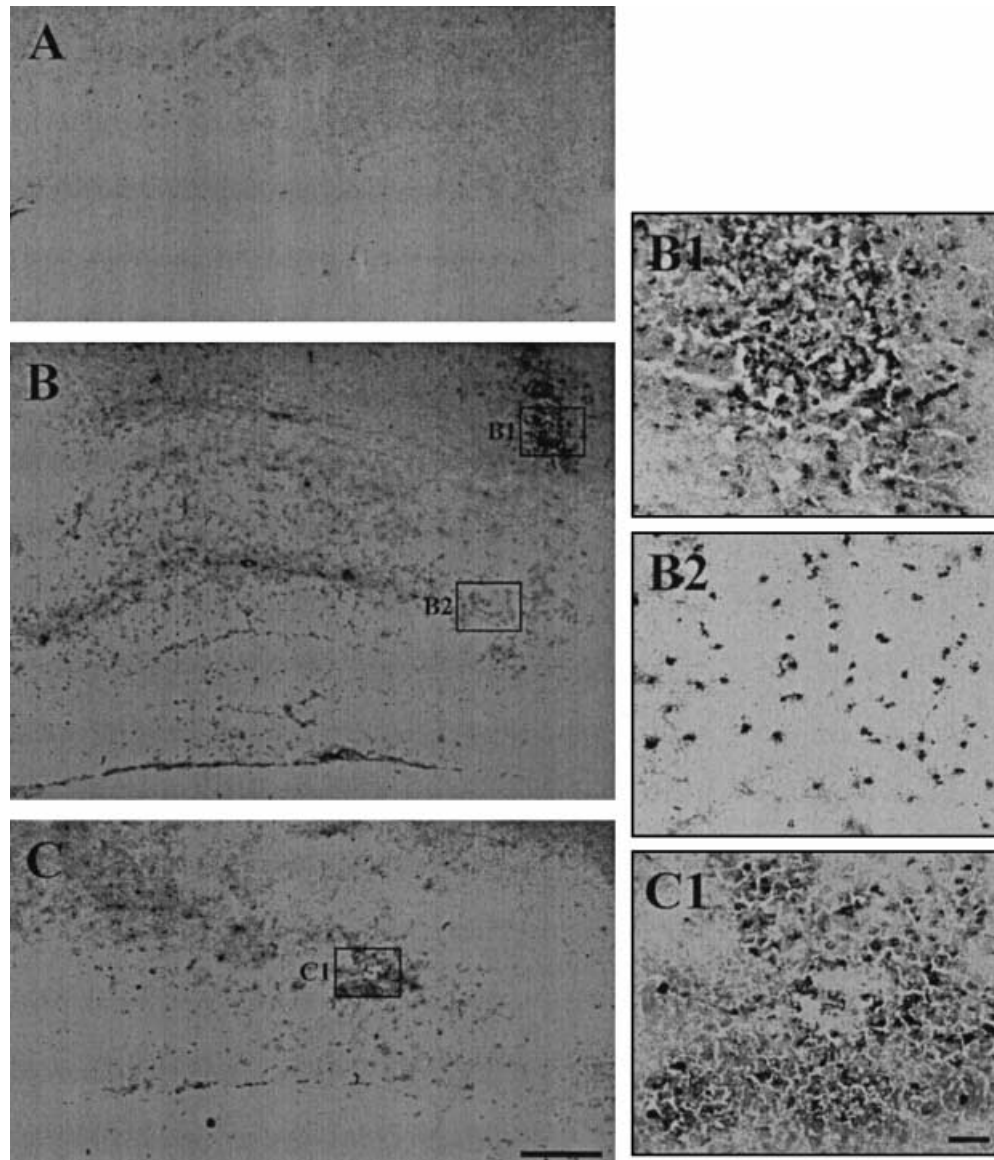


Fig. 1. Microglia were activated in both LPS- and saline-injected rat brains. Frozen sections from rats 12 h after the injection were labeled with antibodies against OX-42 and visualized with peroxidase-conjugated secondary antibodies. In the contralateral side of saline-injected brains, no OX-42-positive cells were detected (A). In LPS-

injected brains (B), OX-42-positive microglia appeared around the injection site (B1, comparable to the area shown in Figs. 2, 4, and 5) and hippocampal region (B2). In saline-injected brains (C), OX-42-positive cells were detected but only in the injection site (C1). Scale bar: 125 μ m; insets: 25 μ m.

needle injection sites itself was not detected at the light microscopic level (data not shown). These results suggest that not only LPS but also needle injection itself activate microglia, as shown previously by others (Kitamura et al., 1996), and that the effect of LPS was not due to profound tissue damage. To examine the expression of outward rectifier K^+ channels in microglia in vivo, brain sections were double labeled with antibodies against OX-42 and Kv1.5 12 h after LPS or saline injection. Figure 2 shows that the immunoreactivity of Kv1.5 was co-localized with that of OX-42 in both LPS- (Fig. 2A,B) and saline-injected brains (Fig. 2C,D). The Kv1.5 antibodies clearly recognized Kv1.5 of cardiac muscles in immunoblot (Fig. 2E), and preincubation of

antibodies with the microsomal fraction obtained from cardiac muscles eliminated the immunoreactivity of Kv1.5 whereas that of OX-42 remained (Fig. 2F,G). The immunoreactivity of Kv1.5 was also detected in the choroid plexus (Fig. 3A, arrowhead) and in the cells inside of blood vessels (Fig. 3B, arrowhead) with similar intensities in either uninjected or injected brains. However, GFAP-positive astrocytes did not express Kv1.5 (Fig. 3C1,C2). This is in good agreement with a recent report that the expression level of Kv1.5 in astrocytes is quite low (Smart et al., 1997).

As electrophysiological study and PCR analysis have suggested Kv1.3 as a major type of K^+ channel in microglia activated by LPS in vitro (Nörenberg et al.,

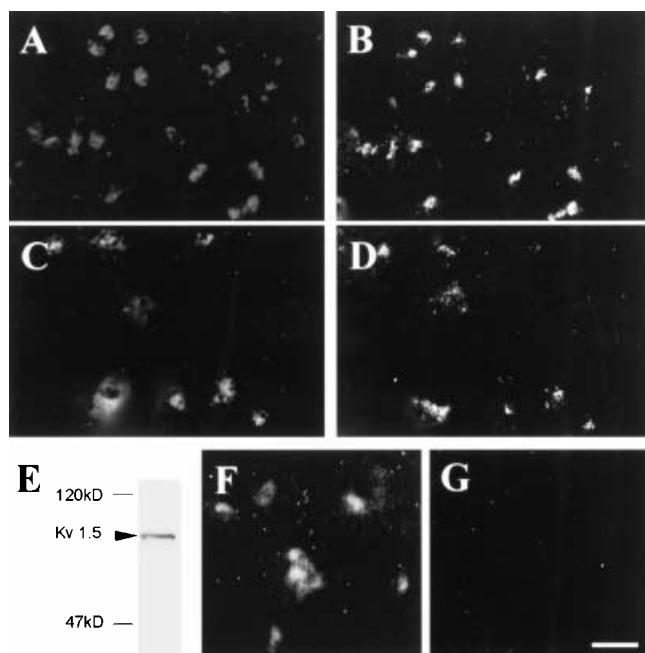


Fig. 2. Kv1.5 immunoreactivity was detected in OX-42-positive cells in both LPS- (A and B) and saline-injected brains (C and D). Kv1.5 antibodies recognized the channels in the microsomal fraction of rat cardiac muscles in immunoblots (E), and preincubation of the antibodies with the microsomal fraction eliminated Kv1.5 immunoreactivity from LPS-injected brains (G) whereas that of OX-42 remained (F). Brain sections obtained 12 h after LPS or saline injection were double labeled with antibodies against OX-42 (A, C, and F) and Kv1.5 (B, D, and G) and visualized with rhodamine- and fluorescein-conjugated secondary antibodies, respectively. Immunoblot analysis was performed as described in the Materials and Methods. Scale bar: 25 μ m.

1993), the expression of Kv1.3 in LPS-injected brain was also examined. Unexpectedly, Kv1.3 antibodies barely stained OX-42-positive cells (Fig. 4A,B) although the antibodies clearly detected Kv1.3 in lymphocytes by immunoblot analysis (Fig. 4C). These results were further supported by immunoblot analysis using brain tissue obtained from the injection sites (Fig. 5). Although both Kv1.5 and Kv1.3 were expressed in rat brains, only Kv1.5 expression increased after LPS injection whereas Kv1.3 expression remained unchanged. These results indicate that LPS induced the expression of outward K⁺ channels in microglia *in vivo* as shown *in vitro* (Pyo et al., 1997) and that a part of the channels, if not all, could be Kv1.5.

As iNOS is a marker of fully activated microglia, we compared the time course of expression of Kv1.5 with that of iNOS. The serial sections were double labeled with combinations of antibodies against either OX-42/Kv1.5 or OX-42/iNOS (Fig. 6). There was no sign of microglial activation until 6 h after the LPS injection; none of the OX-42-, Kv1.5-, and iNOS-positive cells were detected. At 8 h after the injection, OX-42- and Kv1.5-positive cells appeared; however, the expression of iNOS was first detected at 12 h, demonstrating that the expression of OX-42 and Kv1.5 preceded that of iNOS. The expression of OX-42, Kv1.5, and iNOS

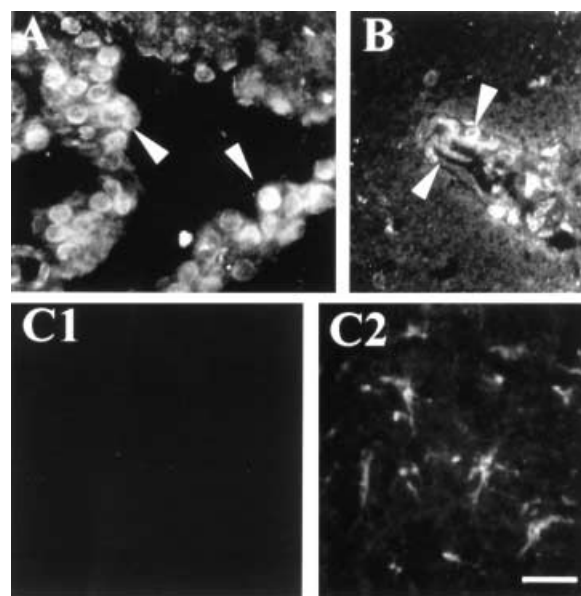


Fig. 3. In uninjected brains, Kv1.5 immunoreactivity was detected in choroid plexus (A) and cells inside of blood vessels (B) but not in the cortex (C1) where GFAP-positive astrocytes were found (C2). Brain sections were labeled with Kv1.5 antibodies (A and B) or double labeled with Kv1.5 antibodies (C1) and GFAP antibodies (C2). Kv1.5 and GFAP were visualized by fluorescein-conjugated (A, B, and C1) and rhodamine-conjugated secondary antibodies (C2), respectively. Scale bar: 25 μ m.

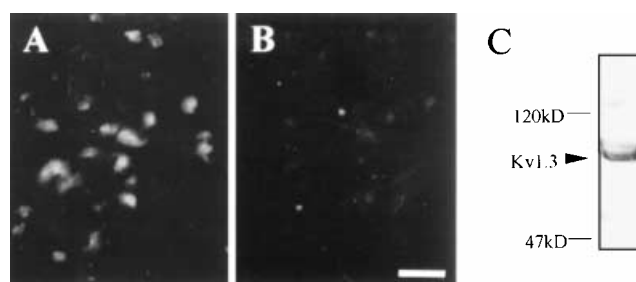


Fig. 4. Kv1.3 immunoreactivity was barely detected in LPS-injected brains (A and B). However, Kv1.3 antibodies clearly detected the channels in lymphocytes in immunoblot analysis (C). Brain sections obtained 12 h after LPS injection were double labeled with antibodies against OX-42 (A) and Kv1.3 (B) as in Fig. 2. Scale bar: 25 μ m.

remained until day 3 but was not detected at day 4. In contrast to the appearance, there was little difference in the time course of their disappearance.

Discussion

It was previously shown that cultured microglia activated by LPS express outward K⁺ channels (Nörenberg et al., 1992, 1993) and could participate in activation process (Pyo et al., 1997). In the present study, by injecting LPS into the brain we tested whether K⁺ channels are expressed in activated microglia *in vivo*. The results obtained are as follows. 1) OX-42-positive cells appeared 8 h after LPS or saline injection and disappeared at approximately day 4. 2) OX-42-positive

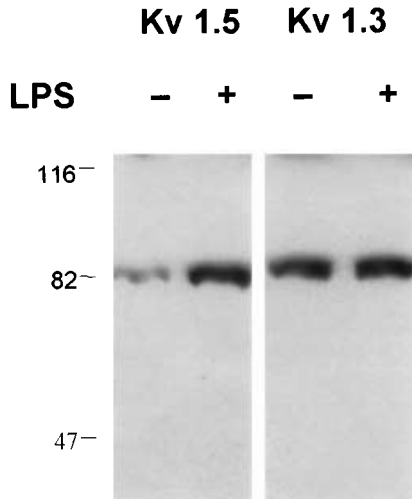


Fig. 5. Immunoblot analysis of expression of Kv1.5 and Kv1.3 in uninjected (-) and LPS-injected brains (+). Kv1.5 expression increased significantly after the LPS injection whereas Kv1.3 expression did not. Similar results were observed from two independent experiments. Immunoblot analysis was performed as described in the Materials and Methods.

cells expressed Kv1.5 immunoreactivity. 3) Kv1.3 was barely detectable. 4) the K^+ channel expression preceded iNOS expression as shown *in vitro* (Pyo et al., 1997).

Microinjection of LPS into the brain induced the appearance of OX-42-positive cells around the injection site. Because of the absence of a specific marker to distinguish microglia from macrophages, it is arguable that the OX-42-positive cells could be macrophages infiltrated from the blood due to tissue damage rather than microglia. However, LPS injection as well as saline injection did not induce noticeable tissue damage except needle injection sites, and the abundant OX-42-positive cells were found in only LPS-injected brain. Furthermore, it is not possible that infiltrated macrophages proliferated in LPS-injected brains as LPS inhibits proliferation of macrophages (Harada et al., 1995). Thus, the OX-42-positive cells could be considered as activated microglia that existed in the brain.

The major type of outward K^+ channel expressed in LPS-treated microglia *in vitro* has been suggested to be Kv1.3 because the biophysical properties of current traces were similar to that of Kv1.3 (Nörenberg et al., 1992) and because Kv1.3 mRNA was detected by PCR (Nörenberg et al., 1993). However, there is a possibility that other types of K^+ channels than Kv1.3 are expressed in activated microglia, based on the following pharmacological study. Kv1.3 has been known to be very sensitive to charybdotoxin ($K_d = 2-3$ nM) (Grissmer et al., 1994), but only 42% of microglial K^+ currents were blocked by 10 nM charybdotoxin and 80% by even 100 nM of the toxin (Nörenberg et al., 1994). Therefore, Kv1.5 can be a possible candidate because Kv1.5 is not sensitive to charybdotoxin even at 100 nM, and also our previous study showed that Kv1.5 antibodies strongly stained cultured microglia after LPS treatment (Pyo et

al., 1997). It is possible that Kv1.3 and Kv1.5 form heteromultimers *in vivo*, rendering pharmacological characteristics different from either Kv1.3 or Kv1.5 alone (Li et al., 1992; Hopkins et al., 1994).

Employing Kv1.5 antibodies purchased from two different companies (UBI and Alomone Labs), the expression of Kv1.5 was detected in OX-42-positive cells (Fig. 2A,B). These findings were also confirmed by immunoblot analysis using brain tissues excised from the injection sites (Fig. 5). In contrast to Kv1.5, Kv1.3 expression showed little change after LPS injection (Fig. 4A,B and Fig. 5). However, the basal expression level of Kv1.3 in uninjected brain showed discrepancy in immunohistochemistry and immunoblot; Kv1.3 expression was detected in immunoblot but not in immunoreactivity. It is unlikely that the antibody could not detect the native form of Kv1.3 because cultured microglia were stained with this antibody after LPS treatment (data not shown). It has been reported that Kv1.3 mRNA level is low in neurons (Kues and Wunder, 1992) and astrocytes (Smart et al., 1997) although Kv1.3 is quite highly expressed in the brain (Smart et al., 1997). Thus, it seems that *in vivo* neurons and astrocytes express Kv1.3 but with low density; as a result, Kv1.3 immunoreactivity was not detected in immunohistochemistry but detected in immunoblot where Kv1.3 was concentrated. These results suggest that Kv1.5 is expressed in activated microglia *in vivo* even though Kv1.5 may not be the only one type of K^+ channel expressed in these cells.

In many nonexcitable cells, both outward and inward K^+ currents are related to proliferation or differentiation of cells. The blockade of outward K^+ currents inhibits or retards proliferation of T and B lymphocytes (DeCoursey et al., 1984; Amigorena et al., 1990), brown fat cells (Pappone and Ortrizmiranda, 1993), and melanoma cells (Nillius and Wohlrab, 1992) or differentiation of mast cells (McCloskey and Qian, 1994). The outward K^+ currents in Schwann cells and oligodendrocytes are also related to their proliferation (Chiu and Wilson, 1989; Gallo et al., 1996; Knutson et al., 1997). In microglia, the blockade of inward K^+ currents in microglia inhibited their proliferation (Schlichter et al., 1996). Furthermore, microglia treated with macrophage colony-stimulating factor expressed inward K^+ currents and stayed in a proliferating state. On the other hand, those treated with granulocyte/macrophage colony-stimulating factor additionally expressed outward K^+ currents and turned into an active, antigen-presenting state (Fisher et al., 1995).

A question arises of how K^+ channels regulate proliferation or differentiation of cells. Many studies showed that K^+ channels can regulate membrane potential. For example, DeFarias et al. (1995) showed that the membrane potential of Chinese hamster ovary cells transfected with Kv1.3 was hyperpolarized and that inhibition of the channels returned the membrane potential to that of untransfected cells. Chung et al. (1998) also found that the resting membrane potential of activated microglia was depolarized by the treatment of 4-amino-

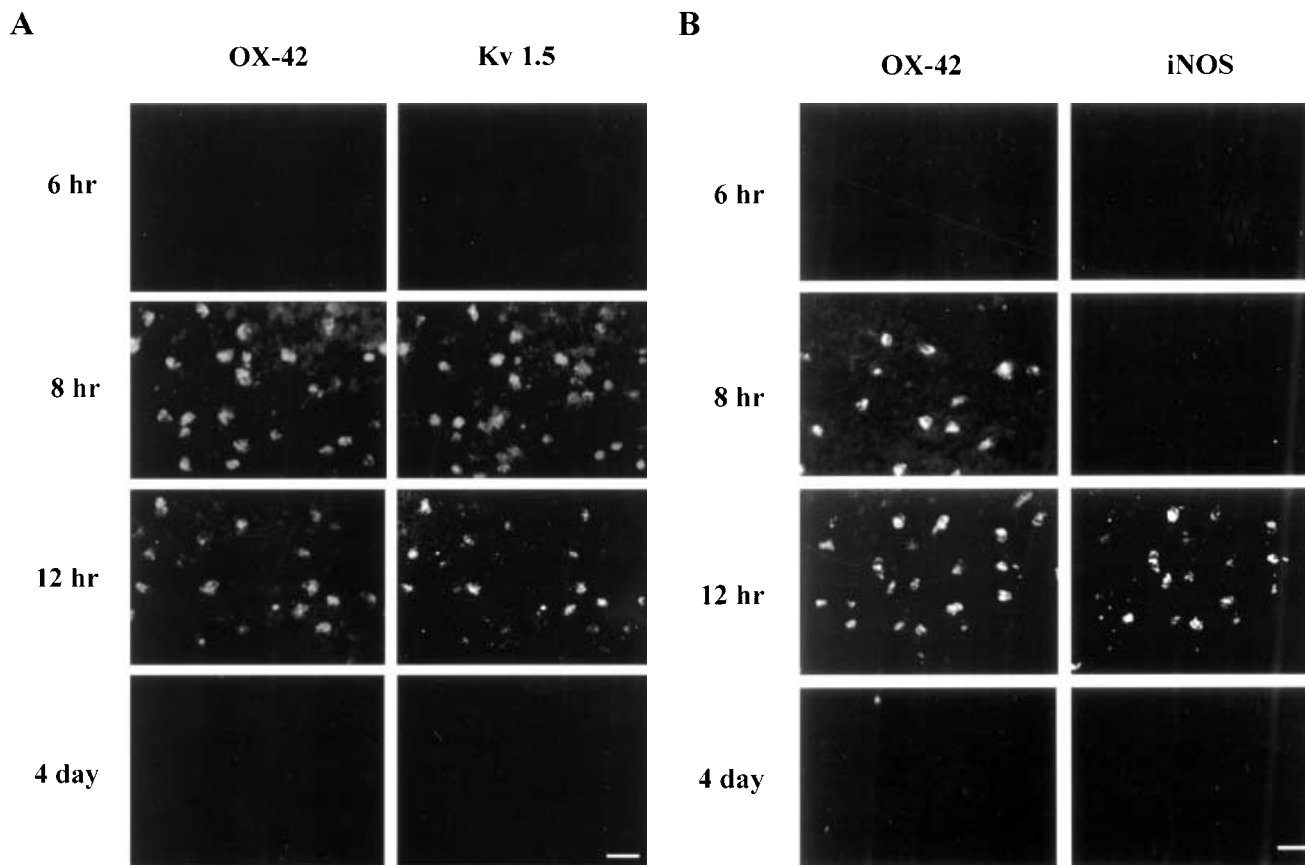


Fig. 6. Kv1.5 expression (A) preceded that of iNOS (B). The immunoreactivity of OX-42 and Kv1.5 appeared from 8 h whereas that of iNOS appeared from 12 h. Serial brain sections obtained at the

indicated time points after LPS injection were double labeled with antibodies against either a combination of OX-42 and Kv1.5 (A) or OX-42 and iNOS (B) as in Fig. 2. Scale bar: 25 μ m.

pyridine, a blocker of outward rectifier K⁺ channels. Thus, regulation of membrane potential could be related to Ca²⁺ signaling, which is critical for Ca²⁺-dependent mitogenesis of many cells (Whitaker and Patel, 1990) and for NO release from LPS-treated macrophages (Park et al., 1996). Depolarization can inhibit increase of intracellular Ca²⁺ by reducing the driving force of Ca²⁺ influx (Mohr and Fewtrell, 1987) and Ca²⁺-induced Ca²⁺ release (Ishida and Chused, 1988).

ACKNOWLEDGMENTS

We thank Drs. Induk Chung (Hanyang University School of Medicine, Korea), Woon Ki Paik (Ajou University School of Medicine, Korea) and Chul Seung Park (Kwangju Institute of Science and Technology, Korea) for helpful comments. This work was supported by KOSEF 971-0704-073-2 to E.H. Joe and BM 97-108 to I. Jou.

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