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Lipopolysaccharide Regulates Both Serotonin- and Thrombin-Induced Intracellular Calcium Mobilization in Rat C6 Glioma Cells: Possible Involvement of Nitric Oxide Synthase-Mediated Pathway

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To investigate the mechanisms by which lipopolysaccharide (LPS) affects Ca^{2+} signaling systems, we studied the effects of LPS on the serotonin (5-HT)- or thrombin-induced intracellular Ca^{2+} ($[Ca^{2+}]_i$) increase in rat C6 glioma cells. Pretreatment of the cells with 1 μg/ml LPS for 24 hr significantly inhibited $[Ca^{2+}]_i$ increase induced by 10 μM 5-HT- or 0.5 U/ml thrombin. Its inhibitory effects were both dose- and time-dependent. Treatment with 1 mM dibutyryl cGMP (dbcGMP) for 30 min also significantly inhibited the 5-HT- and thrombin-induced $[Ca^{2+}]_i$ increase to approximately 60–70% of control. However, simultaneous pretreatment with LPS and dbcGMP did not show any synergistic inhibition. The simultaneous pretreatment with LPS and the potent cGMP-dependent protein kinase (PKG) inhibitors H-8 and KT5823 for 24 hr significantly antagonized the inhibitory effect of LPS. Pretreatment of the cells with 1 μg/ml LPS for 24 hr significantly enhanced cGMP accumulation, while dexamethasone and NMMA (NOS inhibitors) significantly attenuated the LPS-induced enhancement in cGMP accumulation. In addition, pretreatment of the cells with 100 nM dexamethasone for 24 hr significantly suppressed LPS-induced inducible nitric oxide synthase (iNOS; type II NOS, NOS-II) protein expression. These results indicate that LPS may inhibit both 5-HT- and thrombin-induced $[Ca^{2+}]_i$ increase via iNOS expression and PKG activation pathway in rat C6 glioma cells. J. Neurosci. Res. 51:517–525, 1998.

Key words: cGMP; cGMP-dependent protein kinase; nitric oxide; affective disorders; neuroimmunology

INTRODUCTION

There are many interactions between the central nervous system (CNS) and immune systems. Cytokines, such as interleukins (ILs), play important roles as mediators between the two systems. We have previously reported that lipopolysaccharide (LPS) and interleukin-1β (IL-1β) inhibited the serotonin- or endothelin-induced $[Ca^{2+}]_i$ increase in rat C6 glioma cells (Kugaya et al., 1995, 1997). The subsequent mechanism of the LPS inhibition is still unclear, although the fact that the inhibitory effects of LPS required an incubation of more than 12 hr suggests that the regulation of some protein expression may be included in this pathway. Therefore, clarification of the mechanism will be useful in understanding the relationship between neurotransmitters and cytokines.

LPS enhances cGMP accumulation after an 18 hr incubation in C6 cells (Simmonds and Murphy, 1993).

Abbreviations: cGMP, cyclic GMP; dbcGMP, N^2,$O^{2\prime}$-dibutyryl guanosine 3′,5′-cyclic monophosphate, monosodium salt; fura-2/AM, 1-(2-(5′-carboxyoxazol-2′-yl)-6-aminobenzofuran-5-oxy)-2-(2′-amino-5′-methylphenoxy)-ethane-N,N,N′,N′-tetraacetic acid, pentaacetoxymethyl ester; H-8, *N*-(2-(methylamino)ethyl)-5-isoquinolinesulfonamide dihydrochloride; H-89, N-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide · 2 HCl; SNP, sodium pentacyanonitrosylferrate (III) dihydrate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; IP_3, inositol 1,4,5-trisphosphate; PKC, protein kinase C; PKG, cyclic GMP-dependent protein kinase; PLC, phospholipase C; 5-HT, serotonin; NMMA, N^G-monomethyl-L-arginine; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; IL-1β, interleukin-1β; IFN-γ, interferon-γ; SDS, sodium dodecyl sulfate.

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Inducible nitric oxide synthase (iNOS; type II NOS) is expressed in cells only after several hours of exposure to certain cytokines and/or bacterial endotoxins (such as LPS) (Kolb and Kolb-Bachofen, 1992; Nathan, 1992). In glial cells, LPS and/or cytokines also induced iNOS after several hours of treatment (Nomura and Kitamura, 1993). Nitric oxide (NO), which is produced by the activation of NOS, activates soluble guanylyl cyclase to elevate cGMP in the same or adjacent cells (Knowles et al., 1989; Murphy et al., 1993; Murad, 1994).

cGMP has been established as an important second messenger in a variety of intracellular signaling pathways (Stryer, 1986). The effects of cGMP are likely to be mediated through the activation of the cGMP-dependent protein kinase (PKG), and in turn activation of this kinase leads to the reduction of $[Ca^{2+}]_i$ in vascular smooth muscle cells (Hirata and Murad, 1994; Lincoln et al., 1996). However, a conflicting result is reported in the CNS cells (Paupardin-Tritsch et al., 1986). Therefore, there is a possibility that PKG may have different effects in different cell types.

From these findings, we hypothesized that LPS inhibits the 5-HT- or thrombin-induced increase in $[Ca^{2+}]_i$ via the NOS-cGMP-PKG-mediated pathway in C6 cells. To clarify the mechanism and to confirm our hypothesis, we evaluated the effects of LPS on intracellular signaling systems such as Ca^{2+} mobilization and cGMP accumulation in rat C6 glioma cells.

MATERIALS AND METHODS

Cell Culturing Conditions

Rat C6BU-1 glioma cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 50 IU/ml of penicillin, and 50 µg/ml of streptomycin. Cultures were maintained at 37°C in a humidified incubator with an atmosphere of 90% air and 10% CO_2. After 3–4 days of culture, the cells were subcultured on dishes. Once confluent, the cells were then incubated with Dulbecco's modified Eagle's medium in the absence of fetal calf serum for 1 day as previously described (Kagaya et al., 1993). In the experiment of subacute effect, LPS and/or other agents were added to the medium for the appropriate hours at the end of the fetal calf serum-free phase.

Measurement of $[Ca^{2+}]_i$ in C6 Cells

Samples were prepared as previously described (Kagaya et al., 1995) with minor modifications. In brief, cultured cell monolayers were washed with phosphate buffered saline (PBS). Fresh PBS was then added, and the cells were incubated at 37°C until they had become detached from the dish surface. Following centrifugation at 400*g* for 5 min, the resultant cell pellet was suspended in Krebs-Ringer HEPES buffer (145 mM NaCl, 5 mM KCl, 0.5 mM Na_2HPO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose; pH 7.4) and incubated with 3 µM fura-2/AM for 30 min at 37°C. In the experiments to determine the effect of cGMP, dbcGMP was also applied at this time. The cells were once again centrifugated at 400*g* for 5 min and resuspended in the Krebs-Ringer HEPES buffer. They were then put into a cubette, and the fluorescence of fura-2 was measured in a Hitachi F-2000 fluorescence spectrophotometer with excitations at 340 and 380 nm and emission at 510 nm. The cells were prewarmed in a cubette at 37°C with stirring and then the ligands were added to the incubation buffer. Calibration of the fura-2 signals was calculated as described previously (Kagaya et al., 1990).

Determination of Cellular cGMP Contents

Samples were prepared as previously described (Kagaya et al., 1996). In brief, the cells suspended in PBS were centrifuged at 400*g* for 5 min, and the resultant pellet was resuspended in the Krebs-Ringer HEPES buffer. The cells were preincubated for 30 min at 37°C with gentle shaking. They were then resuspended with fresh Krebs-Ringer HEPES buffer containing 1 mM 3-isobutyl-1-methylxanthine, which prevents the degradation of cGMP by phosphodiesterases, and incubated without or with NMMA for an additional 15 min at 37°C. The incubation was then terminated by adding HCl at a final concentration of 0.1 M and immediately incubating the tubes at 100°C for 10 min to prevent further cGMP accumulation. The supernatant obtained by centrifugation at 1,000*g* for 10 min was subjected to an enzyme immunoassay for cGMP. Protein concentrations were determined as described previously (Lowry et al., 1951).

Western Blot Analysis

iNOS was purified from C6 glioma cells as previously described (Fujisawa et al., 1995) with minor modifications. In brief, NOS partially purified by 2′,5′-ADP-agarose chromatography from cultured cells was separated by 6% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidenedifluoride (PVDF) membrane, and NOS protein was detected with anti-rat iNOS antibody, using peroxidase-labeled anti-rabbit IgG as the second antibody and 4-chloro-1-naphthol as the chromophore.

Materials

5-HT creatinine sulfate, thrombin, 2′,5′-ADP-agarose, and LPS of *Salmonella typhimurium* were obtained from Sigma Chemical Corp. (St. Louis, MO). H-8, H-89, and dbcGMP were from Seikagaku Kogyo Co. (Tokyo, Japan). KT5823 was from Calbiochem-Novabio-

chem Co. (La Jolla, CA). SNP was from Katayama Chemical Co. (Osaka, Japan). NMMA was from Research Biochemicals, Inc. (Natick, MA). Fura-2/AM and HEPES were from Dojindo Laboratory (Kumamoto, Japan). 4-Chloro-1-naphthol was from NEN Life Science Products (Boston, MA). Dulbecco's modified Eagle's medium was from GIBCO (Grand Island, NY). Anti-rat iNOS antibody was a kind gift from Dr. H. Esumi (Fujisawa et al., 1995). Penicillin and streptomycin were the generous gifts of Meiji Seika Kaisha, Ltd. (Tokyo, Japan).

Data Analysis

Results are presented as means ± SEM and were analyzed with the Student's t-test or analysis of variance (ANOVA) followed by the Bonferroni/Dunn test. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of LPS on the 5-HT- or Thrombin-Induced $[Ca^{2+}]_i$ Increase in C6 Cells

The cells were preincubated without or with 1 μg/ml LPS for 24 hr and then the $[Ca^{2+}]_i$ was determined. The peak amplitude of 5-HT- or thrombin-induced $[Ca^{2+}]_i$ increase was measured. LPS significantly inhibited the $[Ca^{2+}]_i$ increase induced by 5-HT or thrombin in C6 cells (Fig. 1, Table I). The increase of $[Ca^{2+}]_i$ induced by 10 μM 5-HT in control cells was 99.8 ± 5.4 nM (n = 10). Pretreatment of the cells with 1 μg/ml LPS for 24 hr significantly inhibited this increase to approximately 55% of the control. The increase of $[Ca^{2+}]_i$ induced by 0.5 U/ml thrombin in control cells was 254.7 ± 18.3 nM (n = 10) and pretreatment with 1 μg/ml LPS for 24 hr also significantly inhibited this increase to approximately 60% of the control (Table I). The dose-dependent inhibition by LPS is shown in Figure 2A,B. Pretreatment of the cells for 24 hr with LPS at concentrations of 1–10 μg/ml resulted in a significant dose-dependent inhibition of the $[Ca^{2+}]_i$ increase induced by 10 μM 5-HT (Fig. 2A) or by 0.5 U/ml thrombin (Fig. 2B). The time courses of the LPS inhibition of 5-HT- or thrombin-induced $[Ca^{2+}]_i$ increase are shown in Figure 3A,B. The cells were preincubated with 1 μg/ml LPS for various time before stimulation with 10 μM 5-HT or 0.5 U/ml thrombin. The inhibition was time-dependent, and treatment of ≥12 hr was necessary for significant effects.

Effects of dbcGMP and SNP on LPS Inhibition of the 5-HT- or Thrombin-Induced $[Ca^{2+}]_i$ Increase in C6 Cells

The cells were loaded with the cell-permeant cGMP analogue dbcGMP at the concentration of 1 mM for 30 min, and subsequently stimulated with 10 μM 5-HT or 0.5 U/ml thrombin. dbcGMP significantly inhibited the 5-HT- and thrombin-induced $[Ca^{2+}]_i$ increase (Fig. 4A,B). However, dbcGMP had little effect on the Ca^{2+} response in the cells pretreated with LPS (Fig. 4A,B), suggesting that the inhibitory effect of LPS is mediated by a cGMP-dependent pathway.

The cells were also loaded with an NO donor, sodium nitroprusside (SNP), at the concentration of 100 μM for 30 min, and subsequently stimulated with 10 μM 5-HT or 0.5 U/ml thrombin. SNP significantly inhibited the 5-HT- and thrombin-induced $[Ca^{2+}]_i$ increase (Fig. 4A,B). However, SNP had little effect on the Ca^{2+} response in the cells pretreated with LPS (Fig. 4A,B), suggesting that the inhibitory effect of LPS is mediated by an NO-cGMP-mediated pathway.

Effects of Protein Kinase Inhibitors on LPS Inhibition of the 5-HT- or Thrombin-Induced $[Ca^{2+}]_i$ Increase in C6 Cells

The cells were preincubated with protein kinase inhibitors and/or 1 μg/ml LPS for 24 hr, and then stimulated with 10 μM 5-HT or 0.5 U/ml thrombin. H-8, KT5823, or H-89 alone had little effect on 5-HT- or thrombin-induced $[Ca^{2+}]_i$ increase. However, both H-8 (30 μM, 24 hr) and KT5823 (1 μM, 24 hr), potent PKG inhibitors, significantly antagonized the inhibitory effect of LPS on the 5-HT and thrombin responses (Table II). On the other hand, H-89 (1 μM, 24 hr), a selective PKA inhibitor, did not antagonize the inhibitory effect of LPS (Table II). These results suggest that the inhibitory effect of LPS is mediated by a PKG-dependent pathway.

Inhibition by Dexamethasone of LPS-Induced Changes in Ca^{2+} Mobilization, iNOS Expression, and cGMP Accumulation in C6 Cells

To confirm that NO/NOS is involved in the pathway of LPS inhibition, we studied the effects of dexamethasone (100 nM, 24 hr), an inhibitor of iNOS expression, on LPS inhibition of the 5-HT- or thrombin-induced $[Ca^{2+}]_i$ increase in C6 cells. Dexamethasone alone had little effect on 5-HT- or thrombin-induced $[Ca^{2+}]_i$ increase. However, dexamethasone significantly antagonized the inhibitory effect of LPS on the 5-HT and thrombin responses (Fig. 5A,B), suggesting that the inhibitory effect of LPS is mediated by an iNOS-mediated pathway.

iNOS protein was not detected in non-pretreated (control) and dexamethasone-pretreated C6 cells, while pretreatment of the cells with 1 μg/ml LPS for 24 hr significantly induced iNOS protein expression (Fig. 6). Simultaneous pretreatment of the cells with 100 nM dexamethasone and 1 μg/ml LPS for 24 hr almost completely blocked LPS-induced iNOS protein expression (Fig. 6).

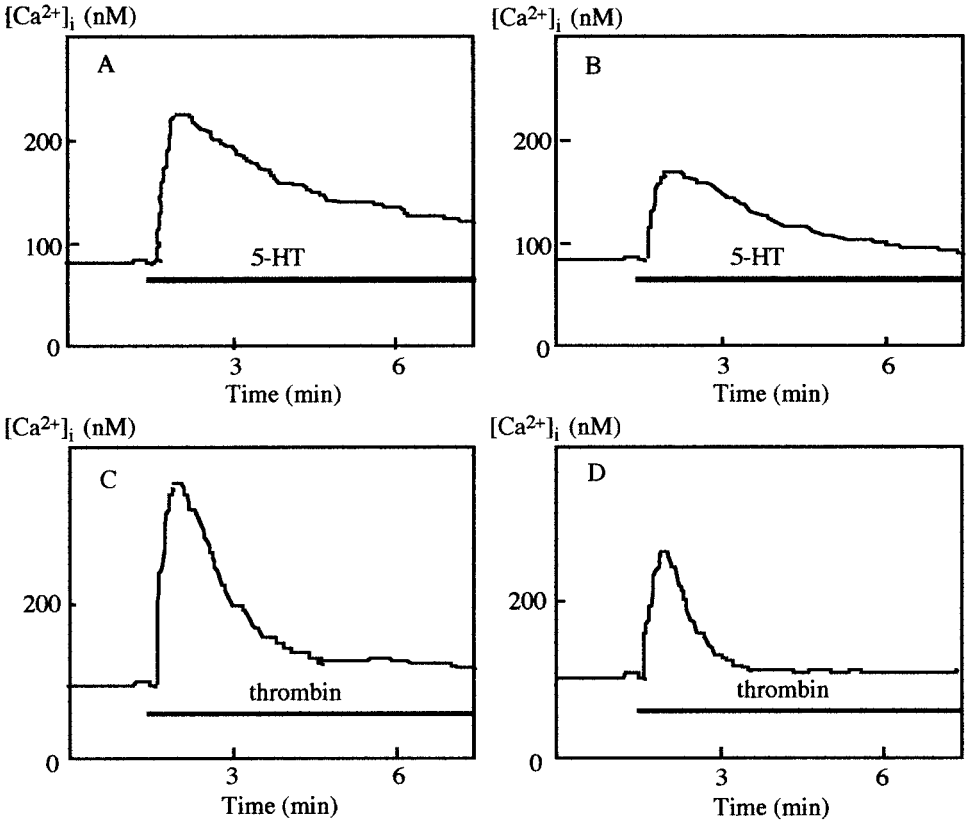

Fig. 1. Effects of LPS on the 5-HT- or thrombin-induced $[Ca^{2+}]_i$ increase in C6 cells. Applications of 10 μM 5-HT or 0.5 U/ml thrombin are indicated by the underlines. **A,C:** Without pretreatment. A typical example from 10 experiments is shown. **B,D:** Pretreatment with 1 μg/ml LPS for 24 hr. A typical example from 10 experiments is shown.

TABLE I. Effect of LPS on the 5-HT- or Thrombin-Induced $[Ca^{2+}]_i$ Increase in C6 Cells†

	Ca^{2+} increase (nM) after pretreatment with	
Stimulated with	Control	LPS
5-HT	99.8 ± 5.4	54.6 ± 4.8*
Thrombin	254.7 ± 18.3	154.0 ± 17.8*

†The cells were pretreated with 1 μg/ml LPS for 24 hr and stimulated with 10 μM 5-HT or 0.5 U/ml thrombin. Data are means ± SEM from 10 experiments.
*$P < 0.001$ vs. control by Student's t-test.

The mean basal cGMP accumulation was 0.74 ± 0.11 pmol/mg of protein. After pretreatment with 1 μg/ml LPS for 1 day, the cGMP accumulation was 8.7 ± 1.3 pmol/mg of protein. This was significantly higher (1,168.3 ± 161.9% of the basal level; $P < 0.0001$ by ANOVA followed by the Bonferroni/Dunn test) than the basal level. To investigate the pathway that induces cGMP, its accumulation was studied with dexamethasone, an inhibitor of iNOS expression. Simultaneous pretreatment of the cells with 100 nM dexamethasone and 1 μg/ml LPS for 24 hr also significantly attenuated the LPS-induced enhancement in cGMP accumulation (534.4 ± 86.8% of the basal level; $P < 0.001$ by ANOVA followed by the Bonferroni/Dunn test) (Fig. 7). Furthermore, in the presence of 30 μM NMMA, a competitive inhibitor of NOS, the LPS-induced enhancement in cGMP accumulation was also significantly attenuated (521.2 ± 114.6% of the basal level; $P < 0.001$ by ANOVA followed by the Bonferroni/Dunn test) (Fig. 7).

Acute Effects of Drugs on the 5-HT- or Thrombin-Induced $[Ca^{2+}]_i$ Increase in C6 Cells

Acute treatment (3 min) of the cells with LPS, dbcGMP, SNP, H-8, KT5823, H-89, dexamethasone, or NMMA had little effect on the 5-HT- or thrombin-induced $[Ca^{2+}]_i$ increase in C6 cells (data not shown). These drugs themselves also had little effect on the $[Ca^{2+}]_i$ (data not shown).

DISCUSSION

In the present study, we demonstrated that LPS may attenuate both 5-HT- and thrombin-induced intracellular

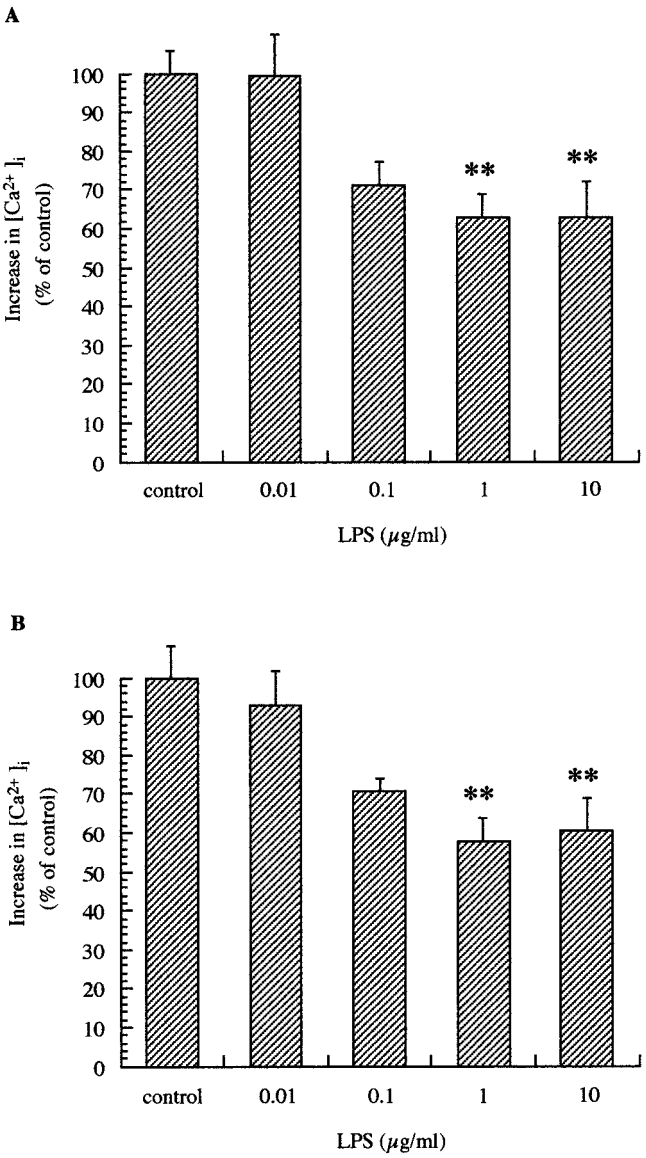

Fig. 2. Effects of LPS concentration on the 5-HT- or thrombin-induced $[Ca^{2+}]_i$ increase in C6 cells. The cells were incubated without or with various concentrations of LPS for 24 hr before stimulation with 10 μM 5-HT or 0.5 U/ml thrombin. **A:** Stimulation with 10 μM 5-HT. The increase of $[Ca^{2+}]_i$ in control cells was 112.0 ± 7.0 nM (n = 6). **B:** Stimulation with 0.5 U/ml thrombin. The increase of $[Ca^{2+}]_i$ in control cells was 293.3 ± 24.3 nM (n = 6). Data are means ± SEM and are expressed as percentages of the control response. $^{**}P < 0.01$ vs. control by ANOVA followed by the Bonferroni/Dunn test.

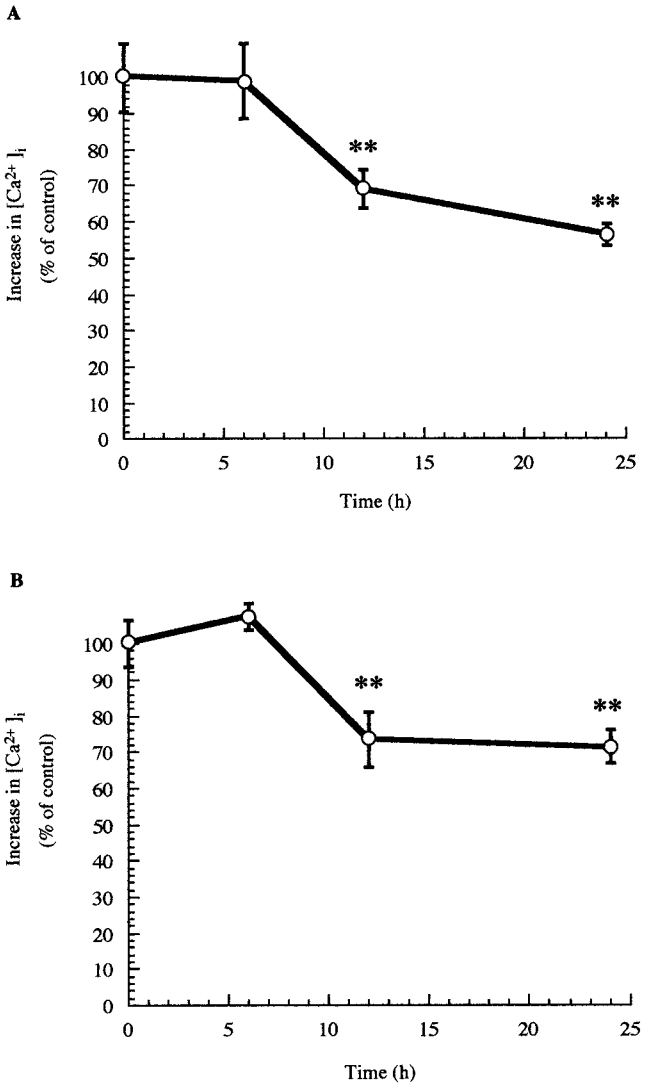

Fig. 3. Time courses of the inhibitory effects of LPS on the 5-HT- or thrombin-induced $[Ca^{2+}]_i$ increase in C6 cells. The cells were incubated with 1 μg/ml LPS for various times before stimulation with 10 μM 5-HT or 0.5 U/ml thrombin. **A:** Stimulation with 10 μM 5-HT. The increase of $[Ca^{2+}]_i$ in control cells (0 hr time point) was 97.5 ± 9.1 nM (n = 7). **B:** Stimulation with 0.5 U/ml thrombin. The increase of $[Ca^{2+}]_i$ in control cells (0 hr time point) was 184.8 ± 11.9 nM (n = 6). Data are means ± SEM and are expressed as percentages of the control response. $^{**}P < 0.01$ vs. control by ANOVA followed by the Bonferroni/Dunn test.

calcium mobilization via iNOS expression and the PKG activating pathway in rat C6 glioma cells.

We have reported previously that LPS and IL-1β inhibited the 5-HT-induced $[Ca^{2+}]_i$ increase (Kugaya et al., 1995). Genistein, a selective tyrosine kinase antagonist, inhibited the effect of LPS and IL-1β. These results indicate that LPS and IL-1β inhibited the 5-HT-induced $[Ca^{2+}]_i$ increase via pathways that include the activation of a tyrosine kinase. The activation of tyrosine kinase is an important early response to LPS: tyrosine kinase inhibitors suppress LPS-induced iNOS protein expression, and a tyrosine kinase may be associated with the induction of iNOS mRNA by LPS (Nishiya et al., 1995). Therefore, it is possible that the NOS pathway is involved

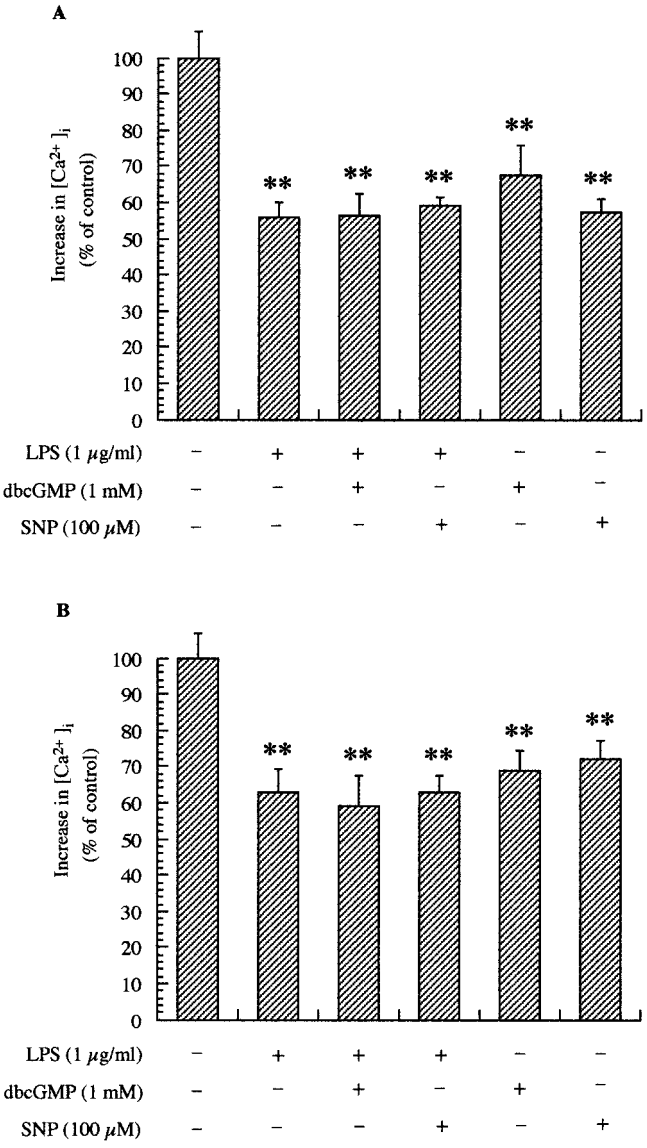

Fig. 4. Effects of dbcGMP and SNP on LPS inhibition of the 5-HT- or thrombin-induced $[Ca^{2+}]_i$ increase in C6 cells. The cells were pretreated with 1 μg/ml LPS for 24 hr and/or loaded with 1 mM dbcGMP or 100 μM SNP for 30 min. **A:** Stimulation with 10 μM 5-HT. The increase of $[Ca^{2+}]_i$ in control cells was 103.0 ± 7.3 nM (n = 8). **B:** Stimulation with 0.5 U/ml thrombin. The increase of $[Ca^{2+}]_i$ in control cells was 252.8 ± 17.2 nM (n = 8). Data are means ± SEM and are expressed as percentages of the control responses. $^{**}P < 0.01$ vs. control by ANOVA followed by the Bonferroni/Dunn test.

in the inhibitory effect of LPS. However, subsequent mechanisms of LPS modulation on $[Ca^{2+}]_i$ mobilization in C6 cells are not clear yet.

In this study it took more than 12 hr until the apparent effect of LPS was observed, suggesting that regulation of some protein expression may be included in the pathway. Several reports indicate that LPS induces

TABLE II. Effects of Protein Kinase Inhibitors on LPS Inhibition of the 5-HT- or Thrombin-Induced $[Ca^{2+}]_i$ Increase in C6 Cells†

	Ca^{2+} increase (% of control) after stimulation with	
Pretreatment	5-HT	Thrombin
Control	100 ± 6.5	100 ± 7.4
LPS (1 μg/ml)	51.8 ± 3.9*	59.3 ± 4.8*
H-8/LPS	90.4 ± 4.4**	93.8 ± 6.5**
KT5823/LPS	86.6 ± 6.2**	87.8 ± 7.7**
H-89/LPS	55.6 ± 7.2$^{n.s.}$	55.6 ± 4.4$^{n.s.}$
H-8 (30 μM)	103.4 ± 7.1	110.7 ± 11.8
KT5823 (1 μM)	103.1 ± 5.5	100.3 ± 5.1
H-89 (1 μM)	95.9 ± 6.0	103.6 ± 7.1

†The cells were pretreated without or with 1 μg/ml LPS, 30 μM H-8, 1 μM KT5823, and 1 μM H-89 for 24 hr and stimulated with 10 μM 5-HT or 0.5 U/ml thrombin. The 5-HT-induced increase in $[Ca^{2+}]_i$ in control cells was 90.5 ± 6.0 nM. The thrombin-induced increase in $[Ca^{2+}]_i$ in control cells was 165.4 ± 12.0 nM. Data are means ± SEM from 6 experiments and are expressed as percentages of control.
$^{*}P < 0.001$ vs. control; $^{**}P < 0.001$ vs. pretreatment with LPS; n.s., not significant compared with pretreatment with LPS (ANOVA followed by the Bonferroni/Dunn test).

iNOS in glial cells (Kitamura et al., 1996). In rat C6 glioma cells, there are several reports that LPS induces NOS-like activity (Simmonds and Murphy, 1992; Demerle-Pallardy et al., 1993), and other studies suggest that LPS plus IFN-γ induce iNOS expression (Feinstein et al., 1994; Nishiya et al., 1995). The results of our study that accumulation of cGMP was enhanced by pretreatment with LPS in both an NMMA (a competitive inhibitor of NOS) and a dexamethasone (an inhibitor of iNOS expression) susceptible manner, and that dexamethasone suppressed LPS-induced iNOS protein expression and Ca^{2+} mobilization suggest that LPS enhances cGMP accumulation via the iNOS expression pathway in C6 cells. However, there are some reports that neuronal NOS (nNOS; type I NOS) and endothelial NOS (eNOS; type III NOS) exist in glial cells (Barna et al., 1996; Togashi et al., 1997). Therefore, there are possibilities that nNOS and/or eNOS as well as iNOS have some roles in the inhibition mechanism of LPS. Further investigation is necessary for the characterization of the pathway. Nitric oxide, which is produced by an activation of NOS, activates soluble guanylyl cyclase to elevate cGMP (Knowles et al., 1989; Murphy et al., 1993; Murad, 1994). cGMP and PKG have multiple effects on the intracellular signal transduction system including Ca^{2+} signaling (Scott, 1991; Hirata and Murad, 1994). In order to assess whether cGMP plays a role in the inhibitory effect of LPS, we loaded the cells with dbcGMP (a cell-permeant cGMP analogue) and SNP (NO donor), and to assess whether PKG plays a role in the inhibitory effect of LPS, we pretreated the cells with H-8 and KT5823 (potent PKG inhibitors). The results of this study show that both dbcGMP and SNP as well as LPS significantly inhibited

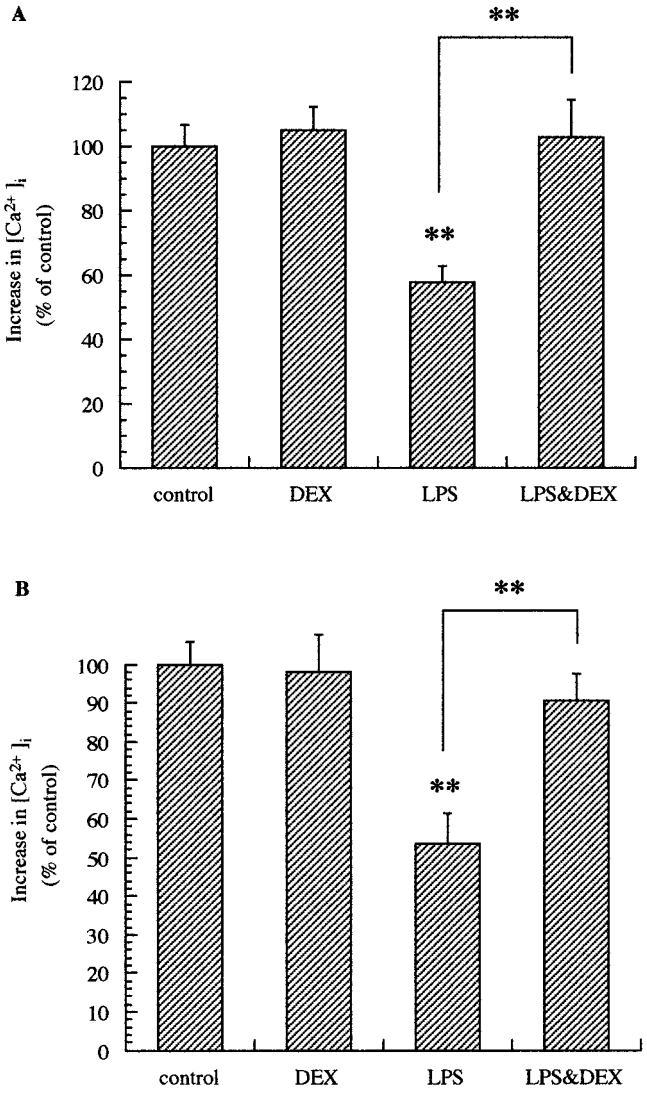

Fig. 5. Effects of dexamethasone on LPS inhibition of the 5-HT- or thrombin-induced $[Ca^{2+}]_i$ increase in C6 cells. The cells were pretreated with 1 μg/ml LPS and/or 100 nM dexamethasone. **A:** Stimulation with 10 μM 5-HT. The increase of $[Ca^{2+}]_i$ in control cells was 59.7 ± 4.1 nM (n = 4). **B:** Stimulation with 0.5 U/ml thrombin. The increase of $[Ca^{2+}]_i$ in control cells was 163.5 ± 9.6 nM (n = 5). Data are means ± SEM and are expressed as percentages of the control responses. $^{**}P < 0.01$ vs. control by ANOVA followed by the Bonferroni/Dunn test. DEX, dexamethasone.

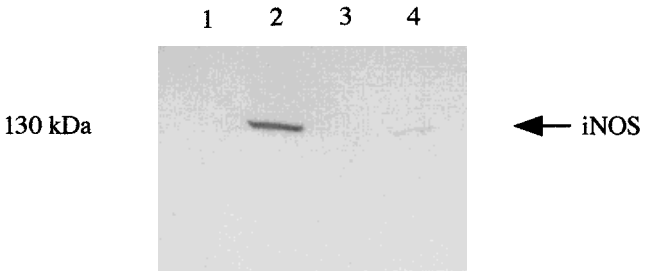

Fig. 6. Dexamethasone suppressed LPS-induced iNOS protein expression in C6 cells. The cells were pretreated without or with 1 μg/ml LPS and/or 100 nM dexamethasone for 24 hr. **Lane 1:** Control. **Lane 2:** LPS pretreatment. **Lane 3:** Dexamethasone pretreatment. **Lane 4:** LPS/dexamethasone pretreatment. NOS partly purified by 2′,5′-ADP-agarose chromatography was separated by 6% SDS-polyacrylamide gel electrophoresis and blotted onto a membrane. iNOS protein was detected by anti-rat iNOS antibody. A typical example is shown from 4 separate experiments.

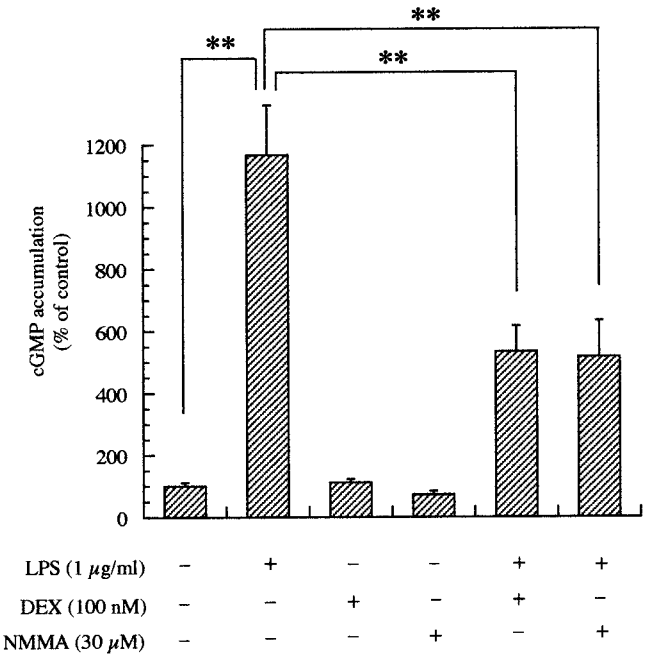

Fig. 7. Effects of NOS inhibitors on LPS-induced cGMP accumulation in C6 cells. The cells were pretreated without or with 1 μg/ml LPS for 24 hr and/or 100 nM dexamethasone before preincubation. cGMP accumulation was determined without or with 30 μM NMMA. $^{**}P < 0.001$ by ANOVA followed by the Bonferroni/Dunn test. DEX, dexamethasone.

the 5-HT- and thrombin-induced $[Ca^{2+}]_i$ increases and that both H-8 and KT5823 significantly antagonized the inhibitory effect of LPS. This indicates that the pathway which activates PKG is involved in the LPS-induced modulation of intracellular Ca^{2+} movement in C6 glioma cells.

$5\text{-}HT_{2A}$ and thrombin receptors activate phosphoinositide hydrolysis to produce IP_3, leading to an increase in $[Ca^{2+}]_i$ from the intracellular calcium store (Baldassare et al., 1989; Kagaya et al., 1990; Panettieri et al., 1995). We have reported previously that the peak amplitude of 5-HT-induced $[Ca^{2+}]_i$ increase was not significantly different between extracellular Ca^{2+}-containing buffer and extracellular Ca^{2+}-free buffer, suggesting that the peak

amplitude of $[Ca^{2+}]_i$ increase was mainly from intracellular calcium store (Kagaya et al., 1995). However, these two kinds of receptors may couple to different type G-proteins: 5-HT_{2A} receptors couple the Gq type, while thrombin receptors couple the Gi/o type (Brass et al., 1991; Watanabe et al., 1991; Sanders-Bush and Canton, 1995) and are regulated via distinct mechanisms at the receptor levels (Vouret-Craviari et al., 1995). In this study, very similar results were obtained on stimulation with 5-HT or thrombin, indicating that LPS inhibited a common pathway, i.e., PLC activation or Ca^{2+} release from IP_3-sensitive intracellular calcium store. Past studies showing that phosphorylation of the IP_3 receptor by PKG inhibits Ca^{2+} release from IP_3-sensitive intracellular calcium store (Komalavilas and Lincoln, 1994, 1996; Murthy and Makhlouf, 1995) support our suggestion.

The function of 5-HT_{2A} receptors appears to be regulated through various pathways. It has been reported that Ca^{2+} movement including 5-HT_{2A} receptors can be desensitized via a calmodulin-dependent pathway (Kagaya et al., 1993) and that NaF attenuates the 5-HT-induced increase in $[Ca^{2+}]_i$ in a tyrosine kinase-dependent manner (Kagaya et al., 1996). We have now clarified the pathway involving NOS, cGMP, and PKG following LPS treatment which regulates the function of 5-HT_{2A}- and thrombin receptors.

Like 5-HT, several reports support the fact that thrombin plays a role in the developing and adult CNS (Cavanaugh et al., 1990; Mazzoni and Kenigsberg, 1991; Weinstein et al., 1995). We and other laboratories (Dubovsky et al., 1989; Kusumi et al., 1992; Mikuni et al., 1992; Okamoto et al., 1995; Yamawaki et al., 1996) have reported that patients with affective disorders show hyperfunction of 5-HT_{2A}- or thrombin receptor-mediated intracellular Ca^{2+} mobilization and proposed that this is one possible pathophysiology of affective disorders. On the other hand, changes in immunological function appear to have a role in the disorders (Leonard, 1995). It is noted that cytokines such as LPS and IL-1β modulate neurotransmitter-mediated Ca^{2+} signaling via the NOS-dependent pathway.

In conclusion, we demonstrated that LPS may attenuate both 5-HT- and thrombin-induced $[Ca^{2+}]_i$ increase via the NOS-cGMP-PKG-mediated pathway in rat C6 glioma cells.

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