

Inhibitory Effect of Fluvastatin on Lysophosphatidylcholine-Induced Nonselective Cation Current in Guinea Pig Ventricular Myocytes

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ABSTRACT

Using the whole-cell voltage-clamp method, we investigated the effect of fluvastatin, a 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor, on lysophosphatidylcholine (LPC)-induced nonselective cation current (I_{NSC}) in guinea pig cardiac ventricular myocytes. External LPC (3–50 μ M) induced I_{NSC} in a dose-dependent manner with a lag. With fluvastatin (5 μ M) in the external solution, LPC induced I_{NSC} , which was significantly smaller and with a longer lag compared with that in the absence of fluvastatin. With mevalonic acid (MVA) (100 μ M) in the external solution, fluvastatin did not diminish LPC-induced I_{NSC} . Geranylgeranylpyrophosphate, an MVA metabolite, in the pi-

pette solution prevented fluvastatin from diminishing LPC-induced I_{NSC} , suggesting that isoprenylated signaling molecules, such as the small G-protein Rho, might be involved in the LPC effect. Botulinum toxin C3, Rho-kinase inhibitor (*R*)-(+)-*trans*-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide, 2 HCl (Y-27632), or pertussis toxin in the pipette solution suppressed LPC-induced I_{NSC} . We conclude that LPC induces I_{NSC} via a Gi/Go-coupled receptor and Rho-mediated pathway. The inhibitory effect of fluvastatin on LPC-induced I_{NSC} provides a new insight into the signal transduction mechanism and may have important clinical implications.

L- α -Lysophosphatidylcholine (palmitoyl) (LPC) is an amphipathic metabolite of membrane phosphatidylcholine; high concentrations of LPC have been found in ischemic hearts (Sobel et al., 1978; Shaikh and Downer, 1981; Corr et al., 1982; Otani et al., 1989; Sedlis et al., 1990). LPC applied externally increases intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in isolated cardiac myocytes (Sedlis et al., 1983; Woodley et al., 1991; Ver Donck et al., 1992; Hashizume and Abiko, 1996). Therefore, LPC is thought to be one of the causes of Ca^{2+} overload and arrhythmia during cardiac ischemia and reperfusion (Hoque et al., 1995; Hashizume et al., 1998). Magishi et al. (1996) reported that exogenous LPC induces a nonselective cation current (I_{NSC}) in cardiac myocytes, but the mechanism involved has not been elucidated.

Recently, Yokoyama et al. (2002) found that LPC increases

$[Ca^{2+}]_i$ and membrane current in cultured human endothelial cells in a manner similar to that found in cardiac myocytes. Furthermore, they found that 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors such as fluvastatin, cerivastatin, and pravastatin inhibit the LPC-induced increase in $[Ca^{2+}]_i$. HMG-CoA reductase inhibitors prevent the synthesis of mevalonic acid (MVA) from HMG-CoA, thereby reducing cholesterol synthesis. Therefore, it is used clinically to prevent the development of atherosclerosis. Recently, it has been shown that MVA is not only a precursor of cholesterol but also of numerous metabolites that are involved in important cell functions, including endothelial function, inflammatory responses, plaque stability, and thrombus formation (Corsini et al., 1999). We tested fluvastatin in cardiac myocytes and found that it inhibited LPC-induced I_{NSC} in guinea pig ventricular myocytes. We investigated the mechanism of LPC-induced I_{NSC} by using fluvastatin under the whole-cell voltage clamp.

Methods

Isolation of Cells. All experiments were performed according to the regulations of the Animal Research Committee of Fukushima

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ABBREVIATIONS: LPC, L- α -lysophosphatidylcholine (palmitoyl); I_{NSC} , nonselective cation current; HMG, 3-hydroxy-3-methylglutaryl; MVA, mevalonic acid; KB, Kraftsbrühe; BAPTA, (1,2-bis(2-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid; I-V, current-voltage; GGP, geranylgeranylpyrophosphate; C3, *Clostridium botulinum* C3 exoenzyme; Y-27632, (*R*)-(+)-*trans*-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide, 2 HCl; ROCK, Rho-associated coiled-coil-forming protein kinase; PTX, pertussis toxin; SPP, sphingosine-1-phosphate.

Medical University. Single cardiac ventricular cells were isolated essentially by the method of Yazawa et al. (1990). Guinea pigs weighing 250 to 400 g were anesthetized by intraperitoneal injection of pentobarbital (30 mg/kg). The chest was opened under artificial ventilation, the aorta was cannulated in situ, and the heart was removed. Blood was washed out with Tyrode-HEPES solution and the heart was mounted in a Langendorff perfusion system. Tyrode-HEPES solution contained 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 , 0.33 mM NaH_2PO_4 , 5.5 mM glucose, and 5 mM HEPES, pH 7.4. The perfusate was changed to Ca^{2+} -free Tyrode solution to stop the heartbeat and then to one containing 0.01% (w/v) collagenase (Wako, Osaka, Japan) and 0.002% (w/v) alkaline protease (Nagase, Tokyo, Japan). After about 20 min, the collagenase solution was washed out by perfusing with a high- K^+ /low- Cl^- solution [modified KB solution (Isenberg and Klöckner, 1982)]. The modified KB solution contained 70 mM KOH, 50 mM *l*-glutamic acid, 40 mM KCl, 20 mM taurine, 20 mM KH_2PO_4 , 3 mM MgCl_2 , 10 mM glucose, 0.2 mM EGTA, and 10 mM HEPES, pH 7.2. Incisions were made in the cardiac ventricular tissue in the modified KB solution and the tissue was shaken gently to isolate the cells. The cell suspension was stored at 4°C for later use.

Patch-Clamp Recording. Membrane currents were recorded by the whole-cell patch-clamp method using pCLAMP (ver. 8.0, Axon Instruments, Union City, CA) and custom RAM5 software and patch-clamp amplifiers [TM-1000 (ActME, Tokyo, Japan) and EPC-7 (List, Darmstadt, Germany)]. Single cardiac ventricular cells were placed in a recording chamber (0.5-ml volume) attached to an inverted microscope (Nikon ECLIPSE TE200, Tokyo, Japan) and superfused with the Tyrode solution at a rate of 5 ml/min. The temperature of the bath solution was maintained at $35 \pm 0.5^\circ\text{C}$ with a water jacket. Patch pipettes were forged from 1.5-mm diameter glass capillaries (Nihon Rikagaku Kikai, Tokyo, Japan) with a microelectrode puller (pp-83; Narishige, Tokyo, Japan). The pipette resistance was 2–4 M Ω when filled with the pipette solution. The pipette solution contained 120 mM CsOH, 20 mM CsCl, 60 mM aspartic acid, 3 mM MgCl_2 , 5 mM MgATP, 10 mM BAPTA, and 20 mM HEPES, pH 7.2, with aspartic acid. BaCl_2 (0.5 mM) in Tyrode solution was added to block K^+ currents. The series resistance was compensated. Current signals were filtered at a 2.5-kHz bandwidth and were stored on a PC (XPS T450; Dell Computer Corp., Round Rock, TX).

Ramp pulses of 500-ms duration were given at 10-s intervals (Watano et al., 1996). The ramp pulse was initially depolarized from -60 mV to 60 mV, then hyperpolarized to -110 mV and depolarized back to the holding potential at a speed of 680 mV/s. The descending limb of the ramp was used to plot the I-V curve without capacitive current compensation. Analog recordings of current and voltage were made with a chart recorder (W1-641G; Nihon Kohden, Japan) in one of the two apparatus used (for Figs. 3A, 5A, and 7A). Current recordings were made digitally in another apparatus so that long-time current records were reproduced digitally using pCLAMP software (Figs. 1A, 2A, and 6A).

Drugs. LPC (Sigma-Aldrich, St. Louis, MO) was dissolved in chloroform to make a 1 mM stock solution, which was evaporated to dryness under a stream of N_2 gas and stored at -20°C . After thawing the LPC was diluted to appropriate concentrations with the external solution. DL-Mevalonic acid lactone, geranylgeranylpyrophosphate (GGP) and *Clostridium botulinum* C3 exoenzyme (C3) were purchased from Sigma-Aldrich. Fluvastatin was a kind gift from Novartis (Basel, Switzerland) and Y-27632, a Rho kinase inhibitor, was from Welfide (Osaka, Japan).

Pertussis toxin (50 μg ; Sigma-Aldrich) was mixed with 5 mM dithiothreitol and incubated in 5 ml of the pipette solution at 37°C for 15 to 20 min for preactivation. The solution was diluted to 50 ml with the pipette solution to make a final concentration of preactivated pertussis toxin, A-protomer, of 1 $\mu\text{g}/\text{ml}$ (Kurachi et al., 1986). All the chemicals used were the highest grade available.

Data Analysis. All the values are presented as mean \pm S.E. (number of experiments). Student's *t* test and analysis of variance

were used for statistical analyses. *P* values less than 0.05 were considered significant. The concentration-response data were fitted and IC_{50} and Hill coefficient values were obtained using Delta Graph Professional (Polaroid Computing, Tokyo, Japan).

Results

Effects of LPC on Membrane Current. Figure 1 shows the effect of 10 μM LPC on the membrane current in a ventricular myocyte. LPC in the external solution induced I_{NSC} with a lag and it did not increase steadily but oscillated. The current response was reversible and the second application of 10 μM LPC induced I_{NSC} with an amplitude similar to the first application. The lag before inducing I_{NSC} was longer after the first than the second application. The control I-V curves before the application of LPC and the largest current induced by 10 μM LPC are plotted in Fig. 1, B and C. The LPC-induced current crossed with the control at 0 mV, confirming that the LPC-induced current is I_{NSC} (Magishi et al., 1996).

The concentration-response relation of LPC was examined. The average values of the magnitude of the LPC-induced I_{NSC} and the lag are summarized in Fig. 1, D and E, respectively. The I_{NSC} density was calculated by averaging the current magnitude measured at 50 mV of the three consecutive responses to the ramp pulse including the maximum current response to LPC in the middle of three traces in each cell. The EC_{50} value of LPC was about 20 μM (Fig. 1D). Figure 1E indicates a significant shortening of the lag with increasing concentrations of LPC. We used 10 μM LPC in the following experiments, because the lag before I_{NSC} develop-

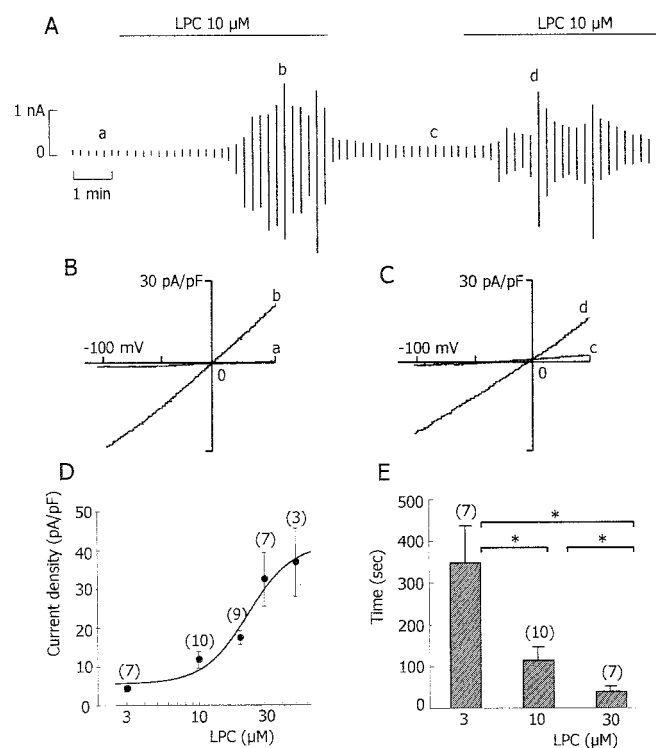


Fig. 1. Effect of LPC on membrane current. A, current recording versus time. LPC at 10 μM was added twice as indicated. B and C, I-V curves of control (a, c) and maximum current (b, d) induced by the initial and the second applications of LPC. D, concentration dependence of LPC-induced I_{NSC} density. E, concentration dependence of the lag. The method of current density measurement is described in the text.

ment was longer at lower concentrations of LPC; if higher concentrations of LPC were used, the cell developed contracture during the first LPC application.

Effect of Fluvastatin on LPC-Induced I_{NSC} . Figure 2 shows the effect of fluvastatin on LPC-induced current. As shown in Fig. 1, repetitive application of LPC induces I_{NSC} with each application. However, as shown in Fig. 2, the initial application of 10 μ M LPC induced I_{NSC} in the absence of fluvastatin but did not in response to the second application of LPC, when 5 μ M fluvastatin was present in the external solution. After washing out fluvastatin, LPC again induced I_{NSC} (not shown). These results indicate that fluvastatin reversibly inhibits LPC-induced I_{NSC} . When fluvastatin was applied after I_{NSC} had developed in response to LPC, it did not inhibit the current (not shown), indicating that fluvastatin does not directly inhibit I_{NSC} channels.

Effect of MVA on LPC-Induced Current. If fluvastatin inhibited LPC-induced I_{NSC} by inhibiting HMG-CoA reductase, MVA should prevent this effect of the drug and this was examined in the experiment shown in Fig. 3 in which 100 μ M MVA was perfused in the external solution for 3 min before adding 5 μ M fluvastatin. In the presence of MVA and fluvastatin, 10 μ M LPC induced I_{NSC} . The I-V curve of the current induced by LPC (Fig. 3B, d) was identical to that induced in the absence of the two agents. This result indicates that fluvastatin inhibits LPC as a result of its inhibition of an HMG-CoA reductase.

Figure 4 summarizes the results described so far. The current densities of I_{NSC} induced by 10 μ M LPC in the presence and absence of fluvastatin and MVA (each alone and together) are compared in Fig. 4A. The current densities were obtained by the method described above for Fig. 1. Fluvastatin significantly diminished the LPC-induced current, which was restored by MVA. However, MVA alone neither induced I_{NSC} nor modified the magnitude of LPC-induced I_{NSC} . Fig. 4B shows the lags

before the currents were induced after application of 10 μ M LPC. MVA reduced the lag significantly. These results suggest that fluvastatin inhibits LPC-induced I_{NSC} by inhibiting HMG-CoA reductase and preventing MVA synthesis. In other words, LPC induces I_{NSC} via a mechanism that requires the MVA-cholesterol synthesis pathway.

Involvement of Geranylgeranylpyrophosphate. Geranylgeranylpyrophosphate (GGP) is a downstream isoprenoid product of MVA in the cholesterol synthesis pathway (Glomset et al., 1990). To pursue the mechanism of LPC-induced I_{NSC} further, GGP was included in the pipette solution and the effect of fluvastatin was examined. Inclusion of GGP alone did not induce I_{NSC} . Fluvastatin did not inhibit I_{NSC} induced by 10 μ M LPC in the presence of GGP (Fig. 5). This indicates that GGP is necessary for the current induction by LPC. Because GGP is necessary for membrane association of the small GTP binding protein, Rho (Goldstein and Brown, 1990; Maltese, 1990; Ridley and Hall, 1992), this result suggests the possible involvement of Rho in the effect of LPC.

Effect of C3 Exoenzyme on LPC-Induced I_{NSC} . Rho is selectively inactivated by C3 through ADP-ribosylation (Sah et al., 2000). To assess whether Rho is involved in the effect of LPC, we included 4 μ g/ml C3 in the pipette solution and applied LPC (Fig. 6). LPC did not induce any significant change in current in the presence of C3 whether or not MVA was present in the bath solution (Fig. 6, B and C). Heat-inactivated C3 did not inhibit LPC-induced current. These results indicate that the small GTP binding protein Rho is involved in the process of LPC-induced I_{NSC} .

Effect of a Rho-Kinase Inhibitor on LPC-Induced Current. Y-27632 is a selective inhibitor of Rho-kinase (Uehata et al., 1997). Because Rho may activate Rho-kinase in the process of LPC-induced I_{NSC} , we tested the effect of LPC with 10 μ M Y-27632 in the pipette solution. Figure 7 shows that LPC induced I_{NSC} , but it was significantly smaller and the lag was longer in the presence of Y-27632 than in its absence.

Figure 8 summarizes the current densities under various

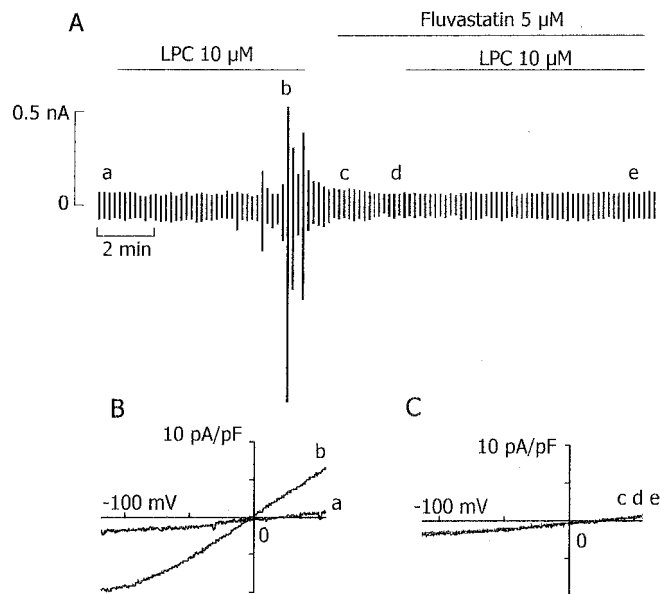


Fig. 2. Effect of fluvastatin on LPC-induced I_{NSC} . A, Current recording versus time. Fluvastatin was applied 3 min before the second application of LPC. B, I-V curves of the corresponding labels in A. Control I-V curve before (a) and during (b) MVA application, in the presence of MVA and fluvastatin (c), and maximum response to LPC in the presence of MVA and fluvastatin (d).

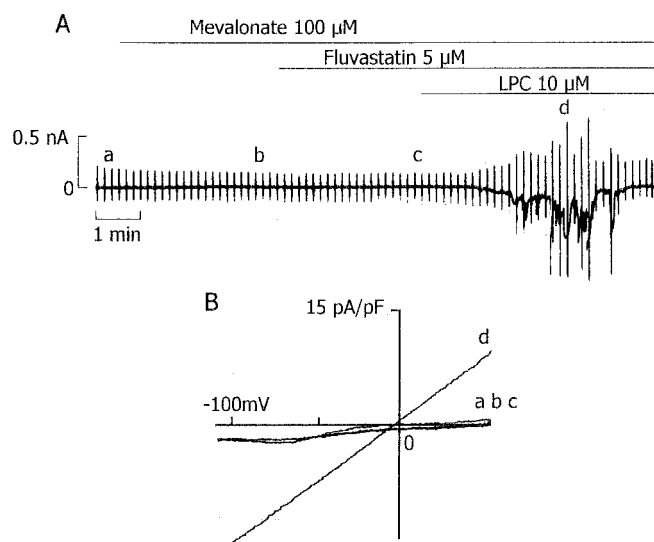


Fig. 3. Effect of fluvastatin on LPC-induced current in the presence of MVA. A, chart recording of current. MVA was applied 3 min before adding fluvastatin. B, I-V curves of the corresponding labels in A. Control I-V curve before (a) and during (b) MVA application, in the presence of MVA and fluvastatin (c), and maximum response to LPC in the presence of MVA and fluvastatin (d).

conditions. Average current densities were obtained from three pulses, including the maximum response in the middle, as described above. LPC-induced I_{NSC} density was not significantly different when LPC alone was present and when GGP was present even with fluvastatin, whereas I_{NSC} was significantly attenuated by C3 and Y-27632 in the pipette solution. This strongly suggests that Rho and Rho-kinase are involved in LPC-induced I_{NSC} .

Effect of Pertussis Toxin on LPC-Induced Current.

To examine the possible involvement of heterotrimeric G-proteins in the LPC-mediated pathway, pertussis toxin, an inhibitor of G_i/G_o , was included in the pipette solution. In the presence of pertussis toxin (1 $\mu\text{g/ml}$), the LPC (10 μM)-induced current was 1.62 pA/pF ($n = 4$) at -100 mV (figure not shown). This was significantly smaller than that of the control, indicating that the Gi/Go-coupled membrane receptor is involved in the LPC-mediated pathway.

Discussion

In this study, we demonstrated that LPC induces I_{NSC} in guinea pig ventricular myocytes through a mechanism that involves an activation of the small G-protein Rho. The LPC-induced effect must be mediated by a receptor that interacts with the PTX sensitive G-proteins. Figure 9 shows a proposed signal transduction pathway for LPC-induced I_{NSC} based on our results.

LPC activated I_{NSC} in a concentration-dependent manner with an EC_{50} of approximately 20 μM . There was a lag before the development of I_{NSC} with an average of 115 ± 32 s ($n = 10$) with 10 μM LPC, and it was shortened to 40 ± 11 s ($n = 7$) with 30 μM LPC, suggesting that the induction of I_{NSC} by LPC involves a distinct signal transduction process that takes a few minutes. We demonstrated that fluvastatin, a HMG-CoA reductase inhibitor, inhibited LPC-induced I_{NSC} . Fluvastatin inhibits MVA synthesis from HMG-CoA. With MVA in the external solution, fluvastatin did not inhibit LPC-induced I_{NSC} . Therefore, it seems that MVA is essential for the induction of I_{NSC} by LPC. Recently, it has been shown that the MVA-derived isoprenoids, GGP and farnesylpyrophosphate, modify several proteins by covalent attachment or prenylation, which is necessary for their association with membranes and thus for their function (Glomset et al., 1990; Goldstein and Brown, 1990; Maltese, 1990). When GGP was

included in the pipette solution, fluvastatin did not inhibit LPC-induced I_{NSC} . Therefore, it seems that some protein that is isoprenylated by GGP is involved in the LPC effect. Candidate proteins for isoprenylation by GGP are Rho, Rac, Cdc42, Rab, Rap, and the γ -subunit of heterotrimeric G-proteins (Corsini et al., 1999). As shown in Fig. 6, botulinum toxin C3 exoenzyme, but not heat-inactivated C3, in the pipette solution almost completely inhibited LPC-induced I_{NSC} . C3 is an ADP-ribosyltransferase that inactivates Rho subfamily proteins (RhoA, RhoB, and RhoC) more specifically than other small GTP binding proteins, such as Rac or Cdc42 (Ridley and Hall, 1992). The result with C3 indicates that Rho is involved in the induction of I_{NSC} by LPC (Fig. 9).

Many effectors of Rho-binding proteins have been identified but the best characterized are Rho kinases, which are serine/threonine directed and include Rho kinase/ROK α /Rock-II and p160ROCK/ROCK β (Sah et al., 2000). Y-27635 specifically inhibits Rho-kinase Rock, by competing for ATP binding (Uehata et al., 1997). Y-27635 at 10 μM inhibited LPC-induced I_{NSC} significantly but not completely (Fig. 8), indicating that Rho-kinase might be involved in the effect of LPC (Fig. 9).

LPC may stimulate some membrane receptor through which a signal transduction pathway is activated and this pathway requires Rho and Rock to open I_{NSC} channels. In this study, PTX inhibited the effect of LPC, indicating that guinea pig ventricular myocytes possess an LPC-receptor that is coupled to the pertussis toxin-sensitive heterotrimeric G-protein, G_i/G_o (Fig. 9). Recently, two G-protein-coupled orphan receptors, G2A (Kabarowski et al., 2001) and GPR4 (Zhu et al. 2001), were identified as the LPC receptors. Both of these receptors are coupled to the PTX-sensitive G protein, and mediate the LPC-induced increase in intracellular Ca^{2+} concentration. In addition, GPR4 was shown to be expressed in the heart, and its effects were inhibited by both PTX and botulinus toxin C3 (Zhu et al. 2001). Involvement of GPR4 and/or G2A in LPC-induced I_{NSC} should be investigated in cardiac ventricular cells.

G-protein-coupled lysophospholipid receptors have also been found for sphingosine-1-phosphate (SPP), sphingosylphosphocholine, and lysophosphatidic acid (Fukushima et al., 2001). Bünemann et al. (1995) found that SPP activates $I_{\text{K(Ach)}}$ in guinea pig atrial myocytes via Gi-coupled receptors. Recently, they detected Edg1, Edg3, Edg5, and Edg8 mRNAs and OGR1 lysophospholipid receptor protein in rat atrium and ventricle

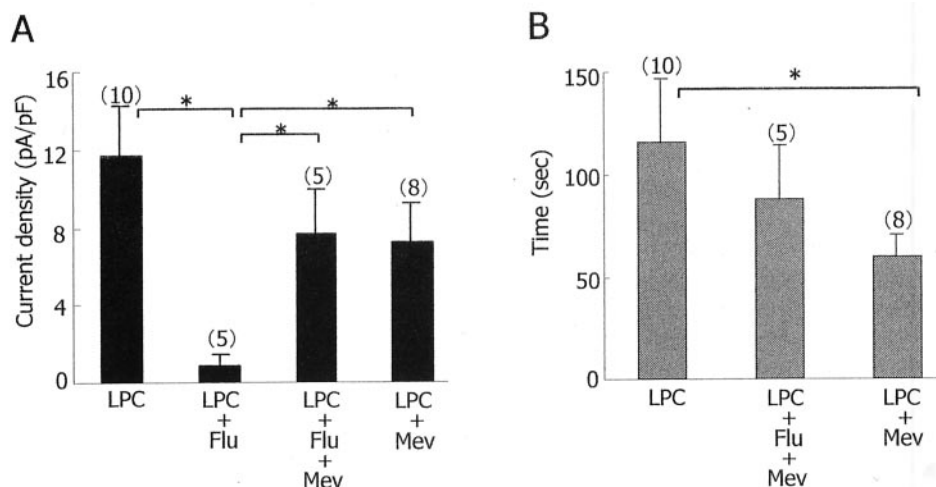


Fig. 4. Averaged data of LPC-induced i_{NSC} density (A) and lag (B) under the conditions indicated. *, significant difference. Mev, MVA.

(Liliom et al., 2001). More recently, Muraki and Imaizumi (2001) succeeded in recording PTX-sensitive nonselective cation channels induced by SPP in human endothelial cells.

Some important questions about the LPC-induced signal transduction pathway need to be addressed. One is how LPC

activates the Rho signaling pathway. Although $G_{12\alpha}$ and $G_{13\alpha}$ have recently been identified as the G-proteins that transduce signals from various receptors to the Rho GTPase by stimulating the GDP-GTP exchange, our results suggest that certain PTX-sensitive G-proteins can initiate the Rho signaling pathway in a GGP-dependent manner. Because GGP alone did not induce I_{NSC} , it seems that LPC triggers geranylgeranylation of Rho. It is important to identify what types of PTX-sensitive G-proteins and which of the α - or $\beta\gamma$ -subunits are associated with the LPC receptors.

Another important question is how fluvastatin affects the intracellular levels of GGP so rapidly (i.e., within a few minutes). It has already been demonstrated that inhibition of HMG-CoA reductase by statins blocks the Rho-dependent cellular responses by preventing isoprenyl modification of Rho. However, this effect of statin was generally observed between 6 and 24 h (Laufs and Liao, 1998). The present results demonstrated that the inhibition of LPC-induced I_{NSC} by statin was very rapid, occurring within a few minutes. Furthermore, mevalonate or GGP prevented the inhibitory effect of statin, suggesting that statin exerted its effect by lowering intracellular GGP. We recently demonstrated similar results with LPC-induced I_{NSC} in human aortic endothelial cells (Yokoyama et al., 2002). In the endothelial cells, LPC-induced I_{NSC} was accompanied by a rapid translocation of GTP-bound RhoA into membrane within one min. Furthermore, a brief treatment with fluvastatin (3 min) prevented the LPC-induced I_{NSC} and translocation of RhoA to the membrane. This effect of statin also disappeared in the presence of MVA or GGP. These results suggest that LPC stimulates I_{NSC} through a similar mechanism in both ventricular myocytes and endothelial cells. Given these results, we suggest that, at least in these cells, intracellular

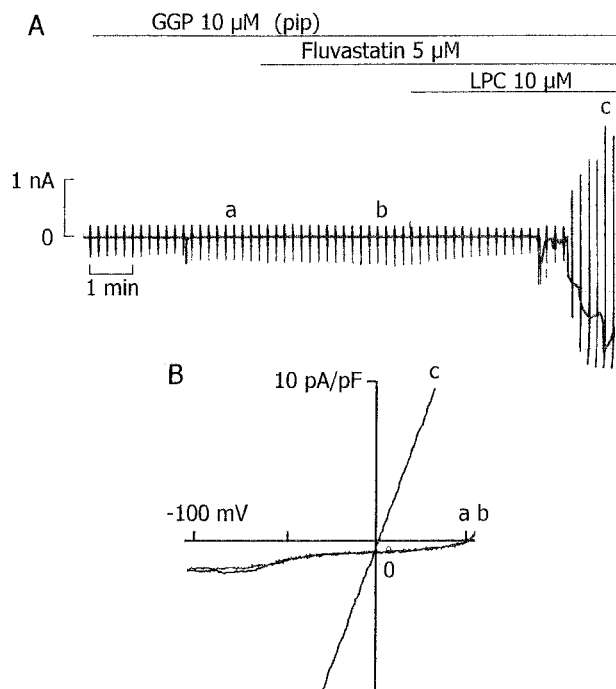


Fig. 5. Effect of fluvastatin on LPC-induced I_{NSC} in the presence of GGP in the pipette solution. A, chart recording. B, I-V curves of the corresponding labels in A.

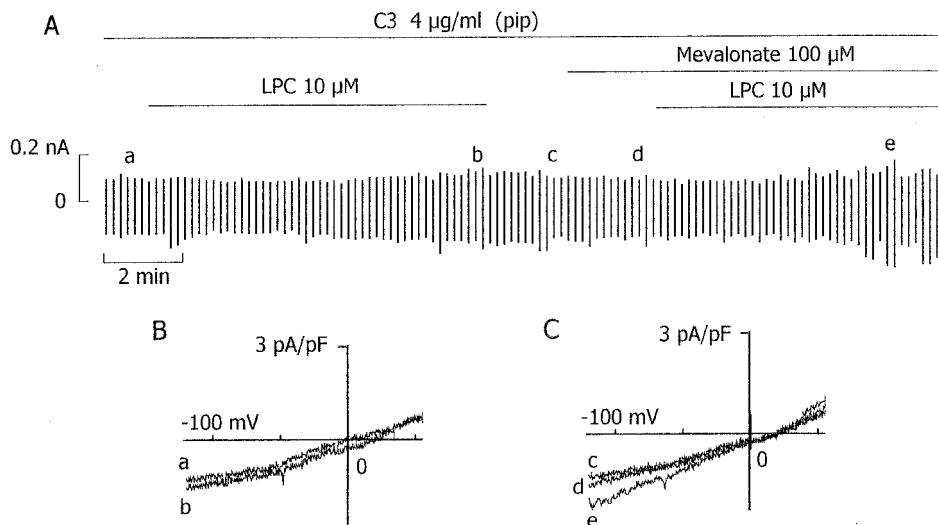


Fig. 6. Effect of C3 on LPC-induced I_{NSC} . A, current recording versus time. C3 is included in the pipette solution. B, I-V curves before (a) and during (b) LPC application. C, I-V curves before (c) and during (d) MVA application and further addition of LPC (e).

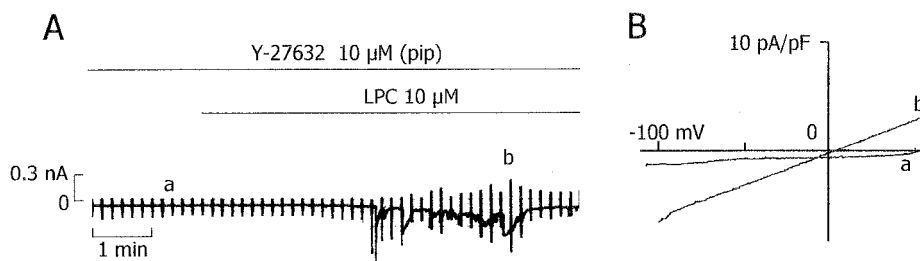


Fig. 7. Effect of Y-27632 on LPC-induced I_{NSC} . A, chart recording of current. B, I-V curves of control (a) and the maximum response to LPC (b) with Y-27632 in the pipette solution.

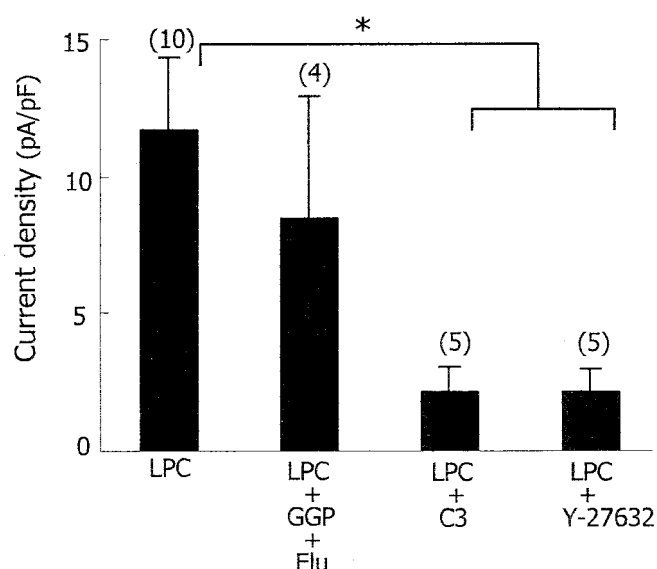


Fig. 8. Summary of results in Figs. 6 and 7. *, significant difference. Current density was measured as described in the legend for Fig. 1.

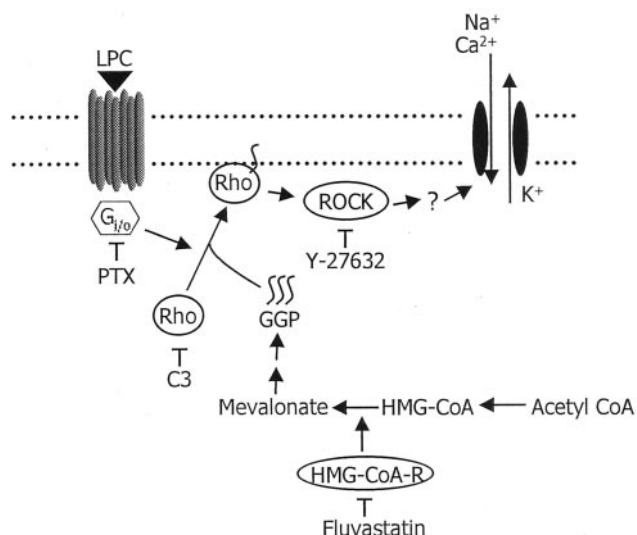


Fig. 9. Proposed mechanisms of the LPC-induced signal transduction pathway. The LPC receptor is shown as $G_{i/o}$ -coupled receptor. Rho is a small G-protein.

GGP levels may be controlled dynamically through metabolic processes. Further studies with direct measurements of intracellular GGP levels are required to evaluate our hypothesis.

Fluvastatin and various other statins are used clinically for hyperlipidemia to lower serum cholesterol levels. There are increasing reports on various effects of statins. The inhibitory effect of fluvastatin on LPC-induced I_{NSC} provides a new insight into the signal transduction mechanism and may have important clinical implications.

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