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Lysophosphatidylcholine potentiates vascular contractile responses in rat aorta *via* activation of tyrosine kinase

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1 We previously reported that while lysophosphatidylcholine (LPC) does not itself produce contraction, it significantly potentiates the contractile responses induced by high-K^+, UK14,304 (a selective α_2-adrenoceptor agonist) and phorbol ester in the endothelium-denuded rat aorta. To further investigate this phenomenon, we examined the effects of genistein and tyrphostin B42 (both tyrosine kinase inhibitors) on the LPC-induced potentiation of the contractile responses to high-K^+ and UK14,304 in the endothelium-denuded rat aorta.

2 Although genistein (3×10^{-6} M, 10^{-5} M) did not affect the high-K^+-induced contractile response, it selectively inhibited the potentiating effect of LPC on the contraction and it strongly inhibited the LPC-induced augmentation of the associated increases in $[Ca^{2+}]_i$. Genistein also attenuated the LPC-induced augmentation effects on both the increase in $[Ca^{2+}]_i$ and contractile response induced by the UK14,304. In contrast, daidzein (10^{-5} M) did not inhibit the potentiating effect of LPC. Tyrphostin B42 (3×10^{-5} M) attenuated the potentiating effect of LPC on high K^+-induced contractions.

3 Western blot analysis showed that LPC increased the tyrosine phosphorylation of a number of proteins, including 42 and 44 kDa proteins and 53–64 kDa proteins. These protein phosphorylations were inhibited by genistein.

4 Sodium orthovanadate (10^{-4} M), a tyrosine phosphatase inhibitor, also markedly enhanced the high-K^+-induced contractile responses. This enhancing effect was attenuated by genistein.

5 These results suggest that the LPC-induced augmentation of contractile responses in the rat aorta is due to activation of tyrosine kinase, which in turn regulates Ca^{2+} influx.
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Keywords: Lysophosphatidylcholine; tyrosine kinase; cytosolic Ca^{2+} level; α-adrenoceptor agonist; rat aorta

Abbreviations: BSA, bovine serum albumin; $[Ca^{2+}]_i$, cytosolic free Ca^{2+}; EDTA, ethylenediaminetetraacetic acid; ERK, extracellular signal-regulated protein kinase; fura PE3-AM, fura PE3-acetoxymethyl ester; 5-HT, 5-hydroxytryptamine; JNK, c-*jun* N-terminal kinase; KHS, Krebs-Henseleit solution; LPC, lysophosphatidylcholine; MAP kinase, mitogen-activated protein kinase; NA, noradrenaline; PMSF, phenylmethylsulphonyl fluoride; SOV, sodium orthovanadate; TBS-T, Tris-buffered saline containing 0.1% Tween-20; UK14,304, 5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline

Introduction

Impairment of endothelium-dependent relaxation is thought to play an important role in the pathogenesis of vascular spasm. Further, oxidative modification of low-density lipoprotein (LDL) cholesterol by the endothelium is thought to be an important step in the initiation of atherosclerosis (Steinbrecher *et al*., 1984; Quinn *et al*., 1987; Berliner *et al*., 1990). Over the last decade, oxidized LDL cholesterol has been shown respectively to impair endothelium-dependent relaxation in isolated arteries (Kugiyama *et al*., 1990; Rajavashisth *et al*., 1990; Simon *et al*., 1990; Jacobs *et al*., 1990; Yokoyama *et al*., 1990; Witztum & Steinberg, 1991; Flavahan, 1992; Kobayashi *et al*., 2000). This inhibitory effect, which is not shared with native LDL, is mediated by lysophosphatidylcholine (LPC) (Kugiyama *et al*., 1990; 1992; Yokoyama *et al*., 1990; Flavahan, 1993; Sugiyama *et al*., 1994). LPC is a bioactive phospholipid that is involved in the pathogenesis of such pathological cardiovascular states as arrhythmias and atherosclerosis (Vidaver *et al*., 1985; Steinberg *et al*., 1989; DaTorre *et al*., 1991). It is well documented that LPC inhibits the endothelium-dependent relaxations mediated by both endothelium-derived relaxing factor and endothelium-derived hyperpolarizing factor (Kugiyama *et al*., 1990; Fukao *et al*., 1995: Kamata & Nakajima, 1998) and it is likely that LPC causes vascular spasm. In addition, we found that in the endothelium-denuded rat aorta, LPC did not itself produce contraction but rather potentiated the vascular contractile responses induced by high-K^+, UK14,304 (5-bromo-6-[2-imidazolin-2-ylamino]-quin-oxaline; a selective α_2-adrenoceptor agonist) and phorbol ester (Suenaga & Kamata, 1998; 1999). Although we thought that this effect could be due to an augmentation of the increase in contractile Ca^{2+} ($[Ca^{2+}]_i$) induced by these vasoconstrictors, the underlying mechanisms is still not well understood.

The tyrosine kinase pathway is known to be associated with cell growth, differentiation or proliferation (for review, see Ullrich & Schlessinger, 1990). However, an accumulating body of evidence suggests that these kinases also contribute to the signalling processes that lead to contraction in vascular smooth muscle (for reviews, see Hollenberg, 1994; Berk &

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Corson, 1997). For example, growth factors such as epidermal growth factor and platelet-derived growth factor have been found to produce vascular contractions (Berk *et al*., 1985; 1986). Moreover, the contractile responses induced by some G-protein-coupled agonists, such as angiotensin-II, noradrenaline (NA) and 5-hydroxytryptamine (5-HT) are attenuated by tyrosine kinase inhibitors in several arteries (Laniyonu *et al*., 1994b; Jinsi *et al*., 1996; Watts *et al*., 1996).

It has also been reported that LPC has a mitogenic effect on vascular smooth muscle cells (Chen *et al*., 1995; Yamakawa *et al*., 1998) and that activation of membrane or cytoplasmic tyrosine kinases may play a key role in LPC-induced mitogenic signalling responses (Bassa *et al*., 1999). Moreover, Ozaki *et al*. (1999) recently reported that in bovine aortic endothelial cells, LPC activates extracellular signal-regulated protein kinase (ERK)- and c-*jun* N-terminal kinase (JNK)-mitogen activated protein (MAP) kinases cascade by a tyrosine kinase-dependent pathway. However, there have been no other reports concerning a link between LPC-induced activation of tyrosine kinase and the enhancing effect of LPC on contractile responses. The aim of the present study was to investigate whether the enhancing effect of LPC on contractile responses in the rat aorta might be directly related to an activation of tyrosine kinase.

Methods

General

This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University (which is accredited by the Ministry of Education, Sciences, Sports and Culture, Japan).

Preparation of aortic strips

Male Wistar rats, 8–10 weeks old, were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.), then killed by decapitation. The thoracic aorta was rapidly dissected out and placed into modified Krebs-Henseleit solution (KHS; composition in mM: NaCl 118; KCl 4.7; $CaCl_2$ 1.8; $NaHCO_3$ 25.0; $MgSO_4$ 1.2; NaH_2PO_4 1.2; dextrose 11.0). It was then cleaned of loosely adhering fat and connective tissue and cut into helical strips 2 mm in width and 20 mm in length. The endothelium was removed by rubbing the intimal surface with a cotton swab, successful removal being functionally confirmed by the absence of a relaxation to 10 μM acetylcholine.

Effects of tyrosine kinase inhibitors on vascular contraction

Each aortic strip was suspended in an organ bath containing 10 ml of well-oxygenated (95% O_2 + 5% CO_2) KHS at 37°C. The contractile responses were measured with the aid of a force-displacement transducer (Nihon Kohden, TB-611, Tokyo, Japan) and displayed on a pen recorder (Yokogawa, Model 3021, Tokyo, Japan). The resting tension in the aortic strip was adjusted to 1 g, which was found to be the optimal tension for inducing a maximal contraction in preliminary experiments. The aortic strips were first contracted by 80 mM K^+, these responses being taken as 100%. The mean contractile response induced by 80 mM K^+ was 1017.46 ± 11.78 mg. After washing and equilibrating for 1 h, the aortic strips were treated with tyrosine kinase inhibitors for 20 min and then incubated with LPC for 15 min. After the incubation period, high-K^+ or UK14,304 was cumulatively applied.

Effect of sodium orthovanadate, a tyrosine phosphatase inhibitor, on high-K^+-induced contraction

Sodium orthovanadate was cumulatively applied to the aorta and the threshold concentration for contraction determined. In a separate study, aortic strips were treated with this threshold concentration of sodium orthovanadate for 15 min before high-K^+ was cumulatively applied.

Measurement of intracellular free Ca^{2+} and tension

Tension and $[Ca^{2+}]_i$ were measured by the method of Sato *et al*. (1988) with the minor modifications described previously (Suenaga & Kamata, 1998). Briefly, aortic strips were exposed to 10^{-5} M fura PE3-acetoxymethyl ester (fura PE3-AM) in the presence of 0.04% Cremophor EL in the dark for 5 h at 26°C. The tissue was then rinsed with normal KHS and placed in the organ bath of a fluorometer (Japan Spectroscopic, CAF 110, Tokyo, Japan) containing KHS at 37°C. The intimal surface of the fura-PE3-loaded tissue was alternately subjected to excitation wavelengths of 340 and 380 nm. The fluorescence emissions at a wavelength of 500 nm for each excitation light were separated by means of a dichroic mirror, then measured with the aid of a photomultiplier. The ratio (F340/F380) of the emitted fluorescence signals was used to provide an index of $[Ca^{2+}]_i$. The contractile responses and the emitted fluorescence signals were displayed on a pen recorder (Yokogawa, Model 3021, Tokyo, Japan).

After equilibration, each aortic strip was first exposed to 80 mM K^+. The fluorescence ratio (F340/F380) in the resting muscle and that in muscle depolarized by 80 mM K^+ were taken as 0 and 100%, respectively. After washing, the aortic strips were first treated with LPC for 15 min, then high-K^+ or UK14,304 was applied.

Western blot analysis

Endothelium-denuded aortic tissues were suspended in an organ bath and incubated with 10^{-5} M LPC or 10^{-4} M sodium orthovanadate for 15 min. After incubation, the preparations were exposed to 15 mM K^+ for 10 min. For experiments with a tyrosine kinase inhibitor, tissues were pretreated with 10^{-5} M genistein for 20 min before the addition of LPC. As a positive control for tyrosine kinase activation, some aortic tissues were treated with 3×10^{-3} M sodium orthovanadate for 10 min. These tissues were removed rapidly from the organ bath and rinsed with ice-cold Ca^{2+}-free KHS containing 1 mM sodium orthovanadate and 5 mM ethylenediaminetetraacetic acid (EDTA). They were then frozen in liquid nitrogen and homogenized in ice-cold sucrose buffer (250 mM sucrose, 10 mM Tris HCl, pH 7.5, 5 mM EDTA, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, 1 mM sodium orthovanadate) in a Potter glass–glass

homogenizer. The homogenates were centrifuged at $14,000 \times g$ for 20 min at 4°C and the supernatants collected. Protein concentration in the supernatant was measured by means of the bicinchoninic acid (BCA) protein assay (Pierce), with bovine serum albumin (BSA) as standard. These sample proteins were solubilized in a Laemmli buffer and were boiled for 5 min at 90°C. Equal amounts of proteins (5 μg) and protein-molecular-weight markers were separated by electrophoresis on 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel and electrically transferred to a polyvinylidene difluoride membrane. The membrane was washed with Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and then blocked by an overnight incubation at 4°C in TBS-T containing 1% BSA. The membrane was washed in TBS-T and then incubated with antiphosphotyrosine antibody linked to horseradish peroxidase (PY20) for 1 h. After washing with TBS-T, antibody binding was visualized using an ECL Western blotting detection system (Amersham Pharmacia Biotech). Developed films were scanned and analysed using an NIH Image program.

Drugs

The drugs used (and their suppliers) were as follows: aprotinin, Cremophor EL, daidzein, EDTA, genistein, leupeptin, L-α-lysophosphatidylcholine (palmitoyl), PMSF, sodium orthovanadate, tyrphostin A1, tyrphostin B42 (Sigma Chemical Co., St. Louis, MO, U.S.A.); acetylcholine (Daiichi Pharmaceuticals Co., Tokyo, Japan); fura PE3-AM (Wako Pure Chemical Industries Ltd., Osaka, Japan); UK14,304 (Funakoshi Co., Tokyo, Japan); and BSA, ECL kit, protein-molecular-weight markers, PY20 (Amersham Pharmacia Biotech, Uppsala, Sweden). Fura PE3-AM was dissolved in dimethylsulphoxide (DMSO) and mixed with Cremophor EL. The final concentrations of DMSO and Cremophor EL (1% and 0.04%, respectively) had no detectable effect on $[Ca^{2+}]_i$ or tension in the aortic strips. Daidzein, genistein, tyrphostin A1, tyrphostin B42 and UK14,304 were dissolved in DMSO and diluted in distilled water. PMSF was dissolved in ethanol and diluted in distilled water. All other drugs were dissolved in distilled water.

Statistics

The results shown in the text and Figures are expressed as the mean $\pm$ s.e.mean. Statistical differences were assessed by Dunnett's multiple-comparison test following a one-way analysis of variance (ANOVA). Statistical comparisons between concentration-response curves were made using a two-way ANOVA, with the Bonferroni correction performed *post hoc* to correct for multiple comparisons. Values for 50% effective concentration (EC_{50}) were obtained by linear regression analysis and are expressed as pD_2 value ($-\log EC_{50}$).

Results

Effects of genistein on high-K^+- and UK14,304-induced contractions in the presence of LPC

At a concentration of 10^{-5} M, LPC did not cause tension development but it did significantly potentiate the high-K^+-induced contraction, the effect being most marked at 10–20 mM (Figure 1). The pD_2 value for high-K^+ was significantly greater in the presence of LPC (Table 1). However, LPC did not affect the maximal response induced by high-K^+. The maximal contractions induced by high-K^+ (80 mM) in the absence or presence of 10^{-5} M LPC were $103.4 \pm 1.2\%$ ($n=6$) and $105.0 \pm 0.6\%$ ($n=5$), respectively of that induced by the first application of 80 mM K^+. Although genistein, at a concentration of 10^{-5} M, did not significantly inhibit the high-K^+-induced contraction, it markedly attenuated the potentiating effect of LPC on this contraction. The presence of 10^{-5} M genistein did not significantly alter the pD_2 values for high-K^+ obtained in the presence or absence of LPC. A lower concentration of genistein (3×10^{-6} M) also attenuated the LPC effect without affecting the contractile response induced by high-K^+ (Figure 1F). A higher concentration of genistein (3×10^{-5} M) not only prevented the LPC-induced potentiation of the high-K^+-induced contraction but also inhibited the high-K^+-induced contraction itself (Figure 1H). Thus, the potentiating effect of LPC on the high-K^+-induced contraction was dose-dependently inhibited by genistein. In contrast, daidzein (10^{-5} M), the inactive isomer of genistein, did not attenuate the LPC effect. A higher concentration of daidzein (3×10^{-5} M) inhibited the high-K^+-induced contraction (with a potency almost equal to that of 3×10^{-5} M genistein) but caused only a modest inhibition of the potentiating effect of LPC. These results are summarized in Figure 2A and Table 1. Figure 2B shows the effect of tyrphostin B42, another tyrosine kinase inhibitor on the LPC-induced potentiation of the high-K^+-induced contraction. At a concentration of 3×10^{-5} M, it inhibited high-K^+-induced contraction and abolished the potentiating effect of LPC. On the other hand, the inactive analogue tyrphostin A1 did not attenuate the LPC effect. The pD_2 values for high-K^+ obtained in the absence or presence of 10^{-5} M LPC in 3×10^{-5} M tyrphostin A1 treated aorta were 1.73 ± 0.02 ($n=4$) and 1.89 ± 0.04 ($n=4$), respectively ($P<0.05$).

LPC potentiated the UK14,304-induced contraction more effectively that the high K^+-induced contraction (Figure 3A and Table 1). At concentrations of 3×10^{-6} to 3×10^{-5} M, genistein inhibited the UK14,304-induced contractions in a dose-dependent manner and dose-dependently inhibited the potentiating effect of LPC (Figure 3B,C,D and Table 1). In contrast, daidzein (10^{-5} M) did not attenuate the LPC effect. The pD_2 values for UK14,304 obtained in the absence or presence of 10^{-5} M LPC in 10^{-5} M daidzein treated aorta were 6.26 ± 0.13 ($n=4$) and 7.09 ± 0.11 ($n=4$), respectively ($P<0.01$).

Effects of genistein on changes in cytosolic Ca^{2+} associated with high-K^+- and UK14,304-induced contractions in the presence of LPC

Figure 4 shows changes in tension development and cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) in response to LPC in aortic strips loaded with fura PE-3 and also the inhibitory effect of genistein on these responses. Treatment with 10^{-5} M LPC markedly augmented the increases in $[Ca^{2+}]_i$ and contractile tension induced by 20 mM K^+ (Figure 4B). The responses induced by 15 mM K^+ were also augmented by 10^{-5} M LPC (Figure 4E). Genistein (10^{-5} M) did not significantly inhibit the increases in $[Ca^{2+}]_i$ and contractile tension induced by high-K^+ but it strongly attenuated the augmentation of these effects produced by LPC.

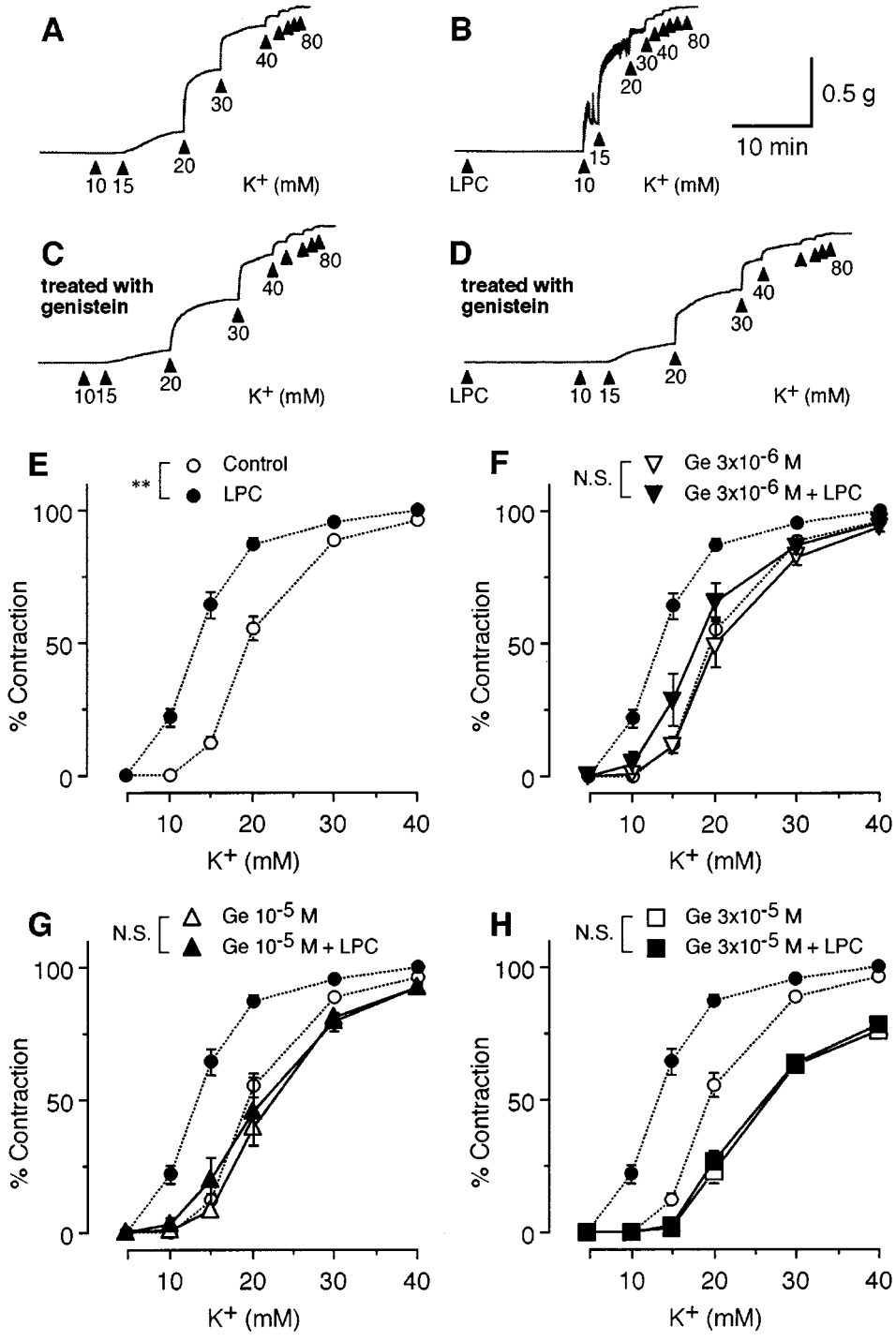

Figure 1 (A–D) Typical traces showing effects of 10^{-5} M lysophosphatidylcholine (LPC) and 10^{-5} M genistein on contractile responses induced by high-K^+ in rat aorta. Aortic tissues were pretreated with vehicle (A), LPC (B), genistein (C) or genistein plus LPC (D). (E–H) Dose-response curves for high-K^+ in the absence (open symbols) or presence (closed symbols) of 10^{-5} M LPC in rat aorta. Aortic tissues were pretreated with vehicle (E) or with 3×10^{-6} M (F), 10^{-5} M (G) or 3×10^{-5} M (H) genistein (Ge). For comparison, the dose-response curves depicted in E are shown in F–H. Values represent means from 5–7 muscle preparations, each isolated from a different animal (vertical line indicating ± s.e.mean is shown only when it exceeds the dimensions of the symbol used). $^{**}P<0.01$; N.S., not significant.

UK14,304 produced increases in $[Ca^{2+}]_i$ and contraction in a dose-dependent manner (Figure 4E). LPC (10^{-5} M) markedly augmented these increases. Genistein (10^{-5} M) both inhibited the UK14,304-induced responses and strongly attenuated the augmentation produced by LPC.

LPC-induced protein phosphorylation

The above results suggested that tyrosine kinase activation might be involved in the augmentation effects produced by LPC on contractile responses in the rat aorta, because

genistein is a tyrosine kinase inhibitor. We therefore set out to investigate whether LPC did indeed induce protein tyrosine phosphorylation. For this, we used Western blot analysis with an antiphosphotyrosine antibody. Figure 5 shows the changes in both muscle tension development and the protein phosphorylation of tyrosine residues induced by LPC in one and the same aortic tissue. LPC (10^{-5} M) did not produce contraction but it did produce an increase in the tyrosine phosphorylation of a number of proteins (including 42 and 44 kDa proteins and 53–64 kDa proteins) over that and above seen in control tissue (Figure 5B). A higher concentration of LPC (3×10^{-5} M) also produced an increase in protein tyrosine phosphorylation with no contraction (data not shown). Treatment with 15 mM K^+ produced a slight contraction but did not markedly increase the tyrosine phosphorylation of proteins. However, in the presence of 10^{-5} M LPC, 15 mM K^+ produced a pronounced contractile response in association with tyrosine phosphorylation of proteins. The combination of LPC and 15 mM K^+ tended to cause a further increase in the phosphorylation of 53–64 kDa proteins. This combination did also cause a detectable increase in phosphorylation of 42 and 44 kDa proteins; but the increase did not appear to be greater than that of LPC alone. Genistein (10^{-5} M) inhibited both the increase in tyrosine phosphorylation and the augmentation effect on contractile responses induced by LPC.

Table 1 Effects of 10^{-5} M lysophosphatidylcholine (LPC), genistein and daidzein on pD_2 values for high-K^+ and UK14,304-induced contractions in rat aorta

	pD_2	
	Control	*LPC*
High K^+-induced contraction		
Control (vehicle)	1.70±0.02	1.87±0.02**
Genistein		
3×10^{-6} M	1.68±0.03	1.75±0.03
1×10^{-5} M	1.66±0.02	1.68±0.05
3×10^{-5} M	1.60±0.02	1.60±0.02
Daidzein		
1×10^{-5} M	1.64±0.03	1.85±0.02**
3×10^{-5} M	1.60±0.01	1.70±0.02
UK14,304-induced contraction		
Control (vehicle)	6.33±0.08	7.30±0.10**
Genistein		
3×10^{-6} M	6.19±0.03	6.44±0.08
1×10^{-5} M	6.12±0.07	6.13±0.08
3×10^{-5} M	6.05±0.04	6.04±0.04

Values represent mean±s.e.mean of 4–7 muscle preparations, each isolated from a different animal.**$P<0.01$ *vs* control value.

Effect of tyrosine phosphatase inhibition on high-K^+-induced contraction

To determine whether tyrosine phosphorylation really is involved in LPC-induced potentiation of contractile responses in the rat aorta, we investigated the effect of sodium orthovanadate (a tyrosine phosphatase inhibitor) on high-K^+-induced contractions. Cumulative addition of sodium orthovanadate produced concentration-dependent contractile responses in the rat aorta (Figure 6A), the threshold concentration being 10^{-4} M. In a separate experiment, non-cumulative application of 10^{-4} M sodium orthovanadate produced only a small contraction in the rat aorta (3.3±1.4% of the contraction induced by 80 mM K^+, $n=7$), but markedly enhanced the contractile responses to high-K^+ (Figure 6B). This enhancing effect of sodium orthovanadate was attenuated by 10^{-5} M genistein. Treatment with 10^{-4} M sodium orthovanadate also markedly increased the tyrosine phosphorylation of a number of proteins including 42 and 44 kDa proteins and 53–64 kDa proteins (by comparison with that seen in control tissue) (Figure 6C–E). In the presence of 10^{-4} M sodium orthova-

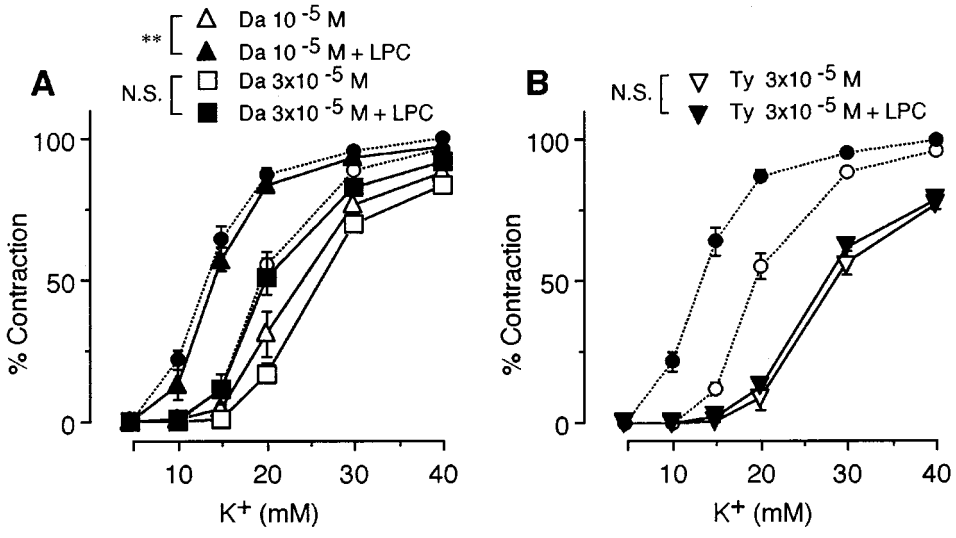

Figure 2 Dose-response curves for high-K^+ in absence (open symbols) or presence (closed symbols) of 10^{-5} M lysophosphatidylcholine (LPC) in rat aorta. Aortic tissues were pretreated with daidzein (Da) (A) or tyrphostin B42 (Ty) (B). For comparison, open circles show the dose-response curves obtained in the absence of daidzein, tyrphostin B42 and LPC (control), while closed circles show the dose-response curves obtained in the absence of the two inhibitors but in the presence of 10^{-5} M LPC. Values represent means from 4–6 muscle preparations, each isolated from a different animal (vertical line indicating±s.e.mean is shown only when it exceeds the dimensions of the symbol used). **$P<0.01$; N.S., not significant.

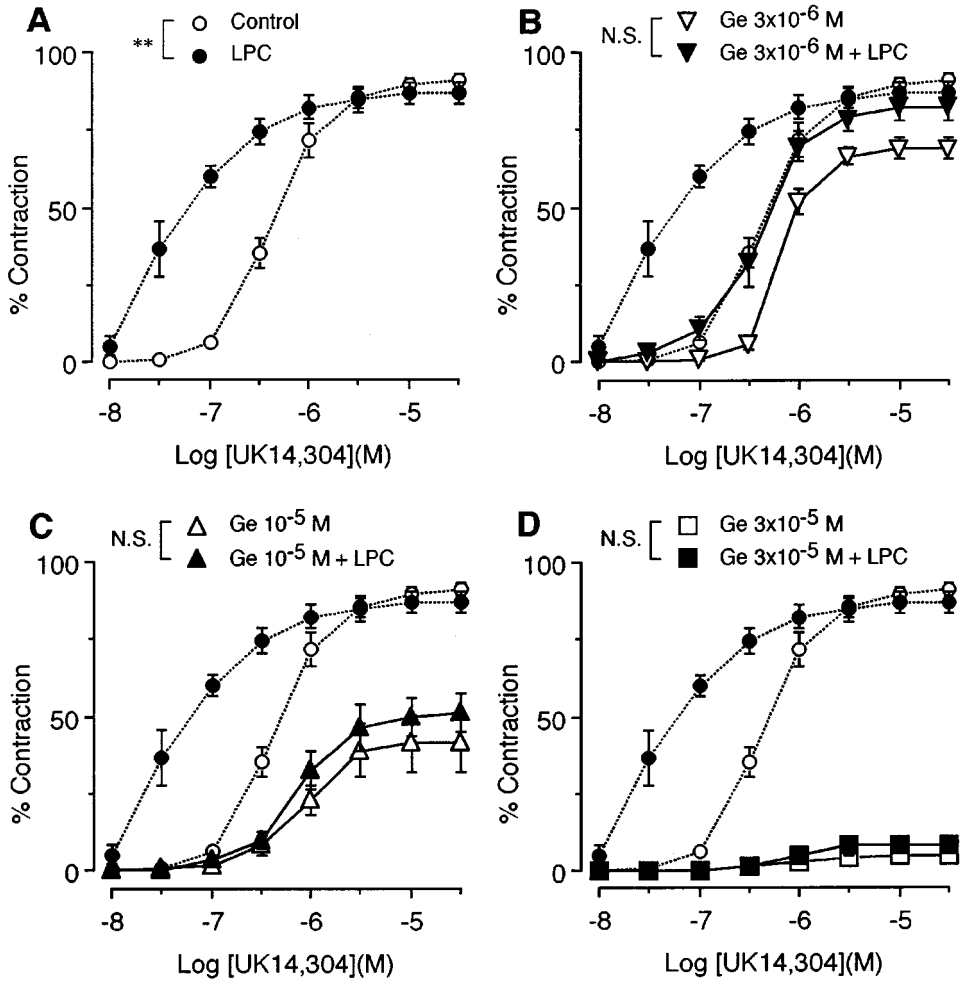

Figure 3 Dose-response curves for UK14,304 in absence (open symbols) or presence (closed symbols) of 10^{-5} M lysophosphatidylcholine (LPC) in rat aorta. Aortic tissues were pretreated with vehicle (A) or with 3×10^{-6} M (B), 10^{-5} M (C) or 3×10^{-5} M (D) genistein (Ge). For comparison, the dose-response curves depicted in A are shown again in B–D. Values represent means from 4–6 muscle preparations, each isolated from a different animal (vertical line indicating ± s.e.mean is shown only when it exceeds the dimensions of the symbol used). $^{**}P<0.01$; N.S., not significant.

nadate, 15 mM K^+ produced a pronounced contractile response in association with a marked tyrosine phosphorylation of proteins (Figure 6).

Discussion

Our previous study showed that LPC markedly potentiated the contractions induced by high-K^+ and UK14,304 in the endothelium-denuded rat aorta (Suenaga & Kamata, 1998). In the present study, we obtained evidence suggesting that the potentiating effect of LPC on such contractions may involve activation of tyrosine kinase.

Protein phosphorylation of tyrosine residues plays a pivotal role in an animal's development by regulating cell proliferation, differentiation and migration (Ullrich & Schlessinger, 1990). However, recent studies have suggested that activation of some G protein-coupled receptors stimulate phosphorylation of a tyrosine residue of many vascular smooth muscle cell proteins and that tyrosine phosphorylation by receptor and non-receptor tyrosine kinases may also participate in contractile responses in vascular smooth muscle (for reviews, see Hollenberg, 1994; Schieffer *et al*., 1996; Berk & Corson, 1997; Hughes & Wijetunge, 1998; Touyz & Schiffrin, 2000). In the present study, we found that genistein, at low concentrations (3×10^{-6} M and 10^{-5} M), selectively attenuated the contractile response to UK14,304 in the rat aorta without appreciably affecting the contraction induced by high-K^+. Moreover, genistein (10^{-5} M) markedly attenuated the increases in $[Ca^{2+}]_i$ and contractile tension induced by UK14,304 in aortae loaded with fura PE-3. These results suggest that UK14,304-induced contractile responses may be regulated by tyrosine kinase. Interestingly, an involvement of tyrosine kinase in the vascular contractile response to an α_2-adrenoceptor agonist has already been reported by others. Jinsi *et al*. (1996) found that genistein inhibited both noradrenaline (NA)- and UK14,304-induced contractions in the rat aorta, with the latter inhibitory effect being the greater.

Our main finding in the present study was that the augmentation effect produced by LPC on the high-K^+-induced contraction of the aorta was concentration-depen-

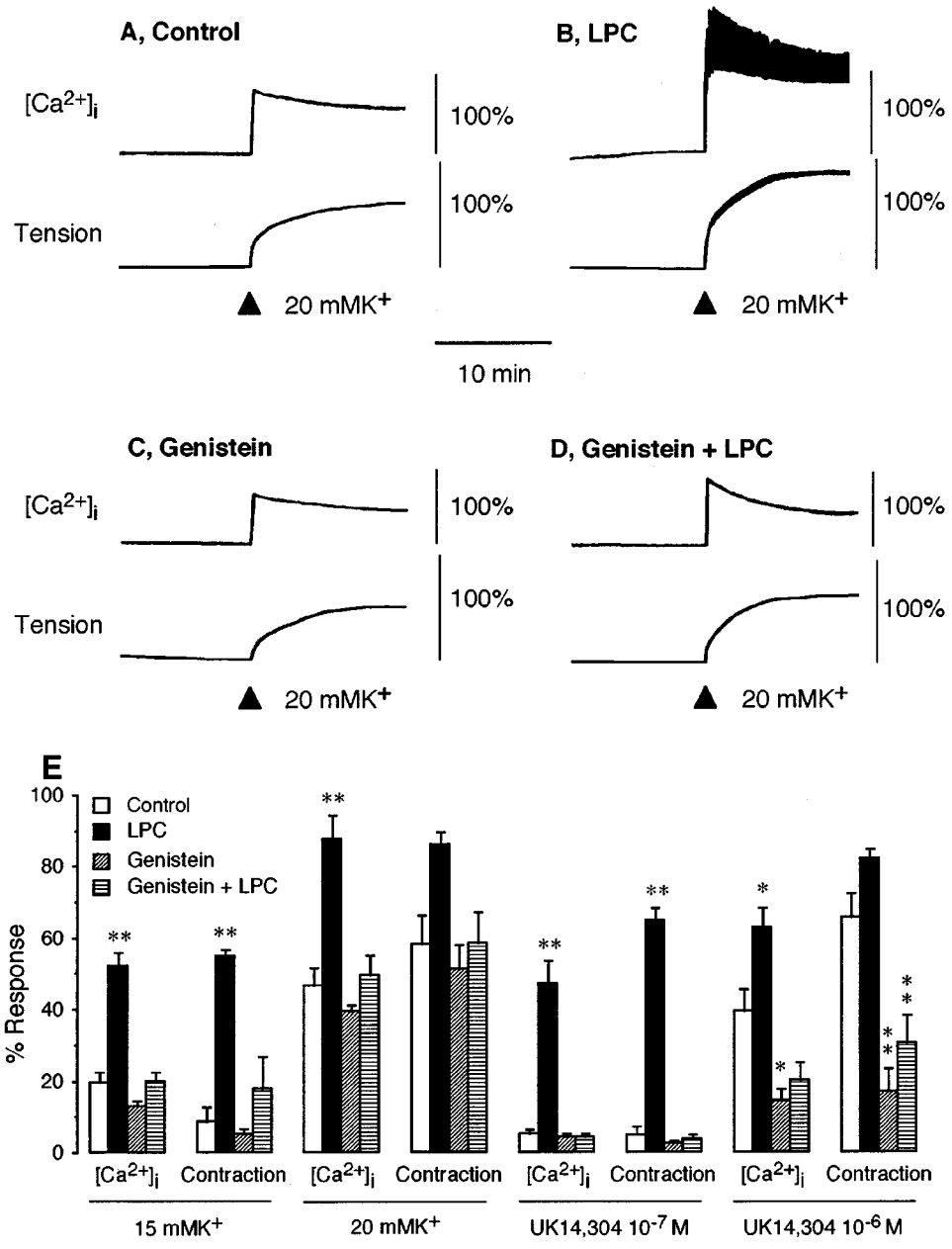

Figure 4 (A–D) Typical traces showing changes in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) and force development induced by 20 mM K^+ in absence (A, C) or presence (B, D) of 10^{-5} M lysophosphatidylcholine (LPC) in the fura PE-3-loaded rat aorta. Aortic tissues were pretreated with vehicle (A, B) or 10^{-5} M genistein (C, D). The response to 80 mM K^+ obtained before the start of the experiment was taken as 100% and the resting level as 0%. (E) Effects of 10^{-5} M LPC and 10^{-5} M genistein on changes in $[Ca^{2+}]_i$ level and contraction induced by high K^+ and UK14,304 in rat aorta. The response to 80 mM K^+ obtained before the start of the experiment was taken as 100% and the resting level as 0%. Values represent means ± s.e.mean from 4–6 muscle preparations, each isolated from a different animal. $^*P<0.05$, $^{**}P<0.01$ *vs* value in the absence of both LPC and genistein (control).

dently inhibited by genistein. Genistein also inhibited the LPC-induced potentiation of the contractile response to UK14,304. Western blot analysis showed that LPC produced an increase in the tyrosine phosphorylation of several proteins and that these phosphorylations were inhibited by genistein at a concentration that also attenuated the LPC-induced potentiation of the contractile responses to high-K^+ and UK14,304. These results suggest that an increase in tyrosine kinase activity may be involved in this augmentation of contractile responses in the rat aorta. Genistein, a flavonoid, strongly inhibits tyrosine kinase *via* an interaction with the ATP-binding site but also has a vasodilator effect that is not due to inhibition of tyrosine kinase. Flavonoids have been reported to cause inhibition of protein kinase C and cyclic AMP-dependent phosphodiesterase and to decrease transmembrane Ca^{2+} uptake. (Herrera *et al.*, 1996). In the present study, a higher concentration of genistein (3×10^{-5} M) inhibited the high- K^+-induced contraction and the potency of this inhibition was almost equal to that of 3×10^{-5} M daidzein, a genistein analogue that is inactive as a

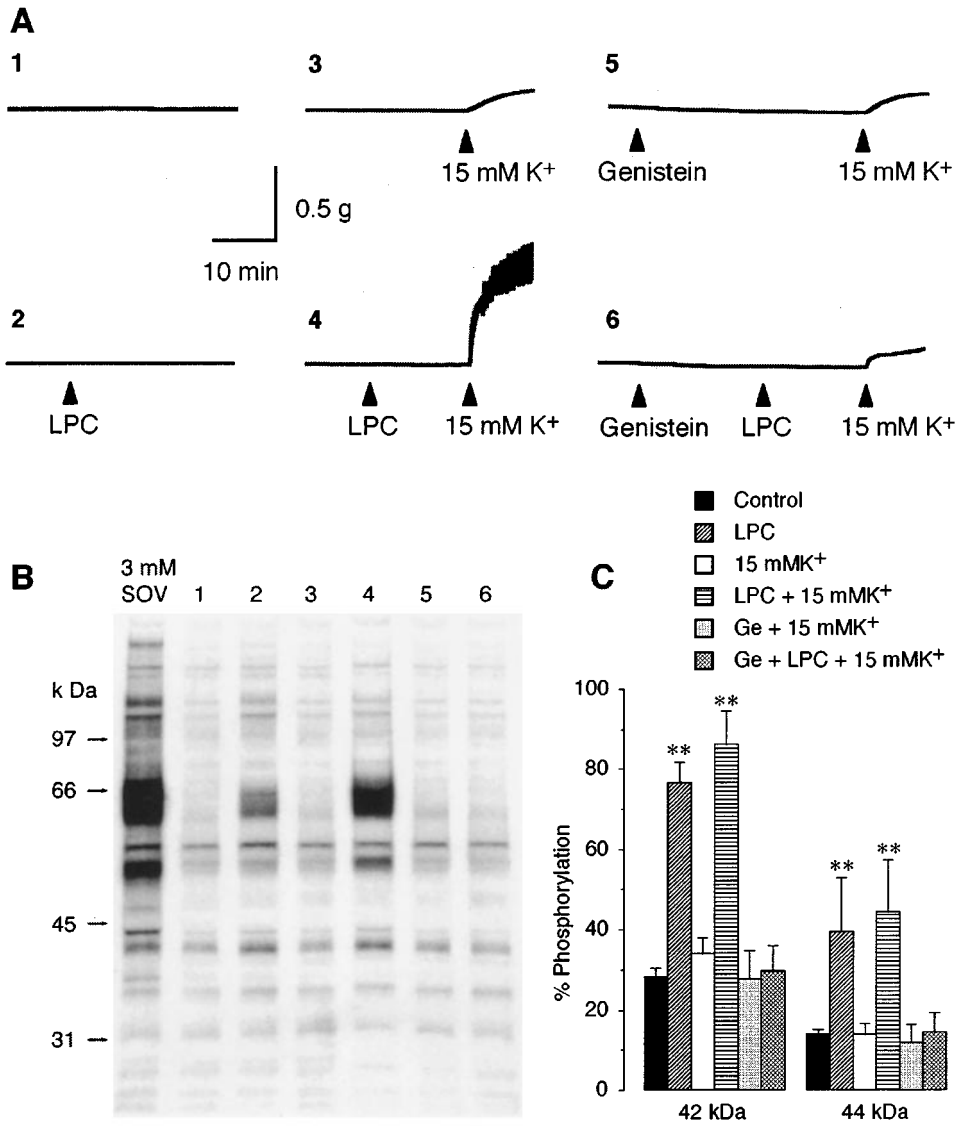

Figure 5 Effects of lysophosphatidylcholine (LPC) on protein tyrosine phosphorylations and contractions in rat aorta. (A) Traces showing effects of 10^{-5} M LPC and 10^{-5} M genistein (Ge) on contractile responses induced by 15 mM K^+. These tissues and aortae treated with 3 mM sodium orthovanadate (3 mM SOV) were homogenized for Western blot analysis. (B) Protein tyrosine phosphorylation of these homogenates (1–6; numbered as in A) was detected by immunoblot analysis using a phosphotyrosine antibody. Arrows indicate the protein-molecular-weight markers. (C) Bar graph shows tyrosine phosphorylation of 42 kDa and 44 kDa proteins in rat aorta under various conditions. Values are expressed as a percentage of the phosphorylation induced by 3 mM sodium orthovanadate. Values represent means ± s.e.mean from five muscle preparations, each isolated from a different animal. $^{**}P<0.01$ *vs* control (in the absence of genistein, LPC and 15 mM K^+).

tyrosine kinase inhibitor (Nevala *et al*., 1998). However, low concentrations of genistein (3×10^{-6} M, 10^{-5} M) selectively inhibited the potentiating effect of LPC without appreciably affecting the high-K^+-induced contraction itself. In contrast, at a concentration of 3×10^{-5} M daidzein caused only a modest inhibition of the potentiating effect of LPC, while a lower concentration of daidzein did not inhibit the LPC effect at all. Furthermore, we previously found that LPC was still able to potentiate the high-K^+-induced contraction in tissues treated with calphostin C or Ro-31-8220 (protein kinase C inhibitors) (Suenaga & Kamata, 1998). Taken together, these previous and present results suggest that the inhibition of the LPC-induced augmentation effect produced by genistein is probably due to an attenuation of tyrosine kinase activity. Our finding that LPC does not potentiate the high-K^+-induced contraction in the presence of tyrphostin B42 (a non-flavone tyrosine kinase inhibitor) supports this conclusion. The involvement of tyrosine kinase is further supported by the effects of sodium orthovanadate. In the present study, sodium orthovanadate produced contraction in the rat aorta. This was previously shown by Filipeanu *et al*. (1995), who reported that it produces a contraction that is sensitive to genistein. In addition, we found that although treatment of the aorta with 10^{-4} M sodium orthovanadate produced only a small contraction, it markedly enhanced the contractile response to high-K^+. This potentiating effect was attenuated by genistein, again suggesting that tyrosine kinase may help regulate vascular smooth muscle contraction in the rat aorta.

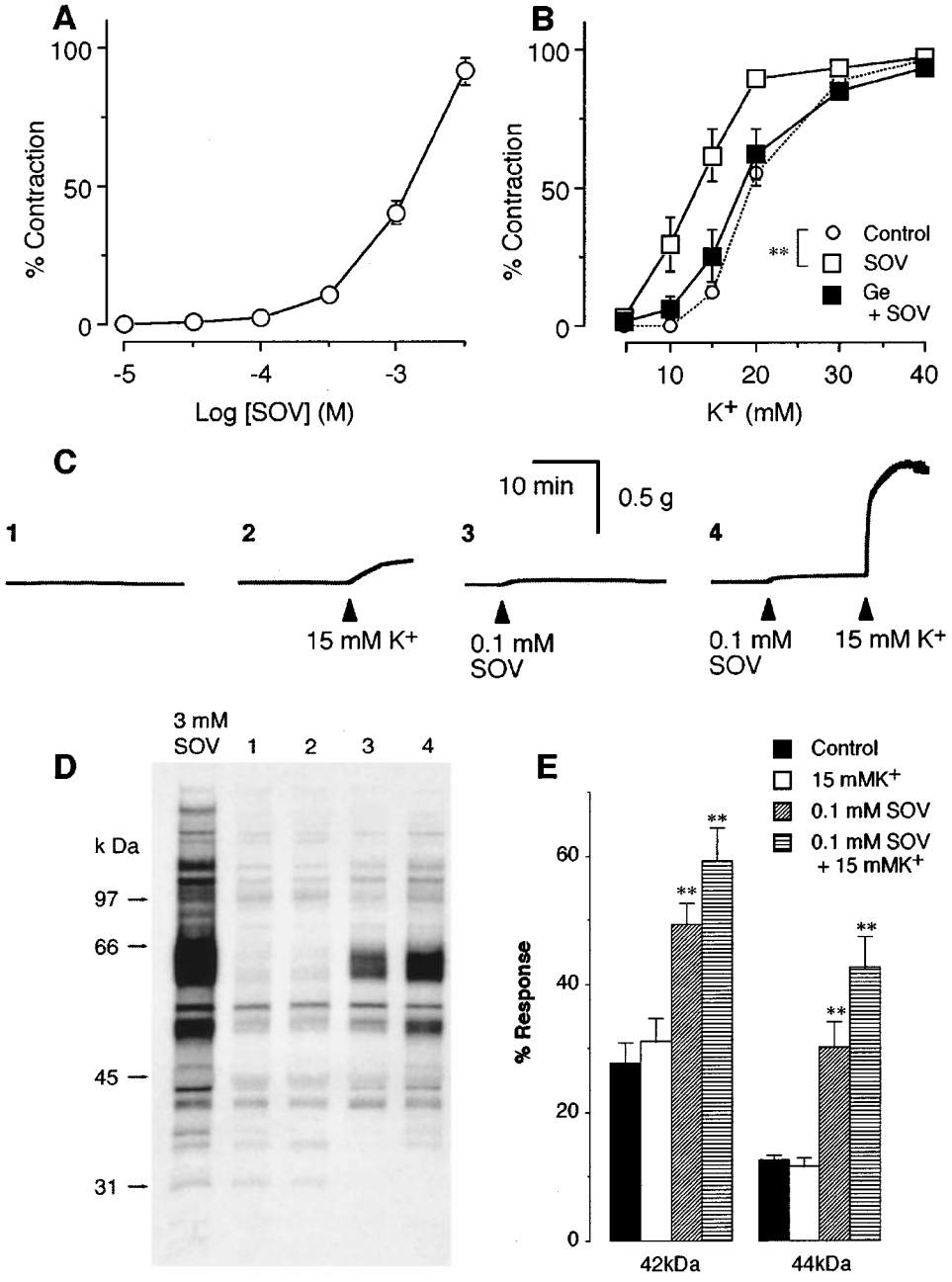

Figure 6 Effects of sodium orthovanadate (SOV) on protein tyrosine phosphorylations and contractions in rat aorta. (A) Dose-response curve for SOV in rat aorta ($n=4$). (B) Dose-response curves for high K^+ in rat aorta in absence (open circles) or presence (open squares) of 10^{-4} M SOV. Closed squares show the dose-response curves obtained in the presence of both 10^{-4} M SOV and 10^{-5} M genistein (Ge) ($n=6-7$). $^{**}P<0.01$. (C) Traces showing effect of 10^{-4} M SOV on contractile responses induced by 15 mM K^+. These tissues and 3 mM SOV-treated aortae were homogenized for Western blot analysis. (D) Protein tyrosine phosphorylation in these homogenates (1–4; numbered as C) was detected by immunoblot analysis using a phosphotyrosine antibody. Arrows indicate protein-molecular-weight markers. (E) Bar graph shows tyrosine phosphorylation of 42 kDa and 44 kDa proteins in rat aorta under various conditions. Values are expressed as a percentage of the phosphorylation induced by 3 mM SOV ($n=5$). $^{**}P<0.01$ *vs* control in absence of SOV and 15 mM K^+. Data points and values (in A, B, E) represent means ± s.e.mean.

In the present study, genistein (10^{-5} M) did not affect the increase in $[Ca^{2+}]_i$ induced by high K^+ but it strongly inhibited the LPC-induced augmentation of the increases in $[Ca^{2+}]_i$ and contractile tension induced by high-K^+ in the fura PE3-loaded rat aorta. Moreover, the augmentation effects of LPC on the increases in $[Ca^{2+}]_i$ and contractile tension induced by UK14,304 were also attenuated by genistein. These results suggest that an involvement of tyrosine kinase in the LPC-induced augmentation of the increase in $[Ca^{2+}]_i$ induced by both high-K^+ and UK14,304 in the rat aorta. A few years ago, it was reported that activation of tyrosine kinase by platelet-derived growth factor

activates L-type voltage-dependent Ca^{2+} channels in cells isolated from the rabbit ear artery (Wijetunge & Hughes, 1995). We did not directly investigate the possible link between tyrosine kinase activity and opening of voltage-dependent Ca^{2+} channels. However, we previously found that UK14,304- and high-K^+-induced contractile responses were hardly potentiated at all by LPC in the nicardipine-treated rat aorta (Suenaga & Kamata, 1998). Taken together, the above results suggest that the LPC-induced augmentation effect in the rat aorta involves tyrosine kinase activation, and that this regulates Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels.

In the present study, LPC produced an increase in the phosphorylation of several proteins as well as augmenting high-K^+- and UK14,304-induced contractions. However, LPC alone did not produce a contraction. We did not investigate this dissociation between the tyrosine phosphorylation of proteins and contraction. However, we did find that a low concentration of sodium orthovanadate (10^{-4} M) also produced an increased phosphorylation of proteins with only a small contraction (i.e. it seems to mimic the dissociation seen with LPC). We therefore consider that tyrosine kinase may act as a vasomodulator rather than as a vasoconstrictor in the rat aorta. In line with our data, it has been reported that low concentrations of tyrosine phosphatase inhibitors (pervanadate and sodium orthovanadate) did not cause an appreciable contractile response but did augment the angiotensin II and high-K^+-induced contractions in arteries (Laniyonu *et al*., 1994a; Masui & Wakabayashi, 2000).

Recent studies have suggested that ERK-MAP kinases are involved in the contractile response induced by 5-HT in the rat aorta (Watts *et al*., 1996) and that Src tyrosine kinases are upstream from ERK in the signalling pathway in the arteries (Ishida *et al*., 1995; 1998; Banes *et al*., 1999; Wesselman *et al*., 2001). In the present study, LPC increased the tyrosine phosphorylation of a number of proteins, including 42 and 44 kDa proteins and 53–64 kDa proteins. Since these regions of the gel are consistent with the presence of increased ERK-MAP kinase and Src tyrosine kinase phosphorylation, as suggested by others studies (Florian & Watts, 1998; Banes *et al*., 1999), it is possible that both kinases involved in the augmentation effect of LPC on contractions in the rat aorta.

In conclusion, we have confirmed the previously reported LPC-induced potentiation of vascular contractile responses, an effect apparently produced *via* an enhancement of the vasoconstrictor-induced increase in $[Ca^{2+}]_i$ in the rat aorta. Tyrosine kinase inhibitors attenuated this effect of LPC. In addition, LPC produced an increase in protein phosphorylation in tyrosine residues, an effect inhibited by genistein. Hence, the augmentation effect of LPC in the rat aorta may involve tyrosine kinase activation, which in turn regulates Ca^{2+} influx.

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