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Novel diacylglycerol kinase inhibitor selectively suppressed an U46619-induced enhancement of mouse portal vein contraction under high glucose conditions

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1 Diacylglycerol kinase (DG kinase) is a key enzyme in vascular contraction; however, alterations of the regulatory mechanisms in vascular dysfunction are poorly understood. In this study, the effect of a novel DG kinase inhibitor, stemphone, on vascular contraction was investigated.

2 The conventional DG kinase inhibitor, 6-[2-(4-[(4-fluorophenyl)phenyl-methylene]-1-piperidinyl)ethyl]-7-methyl-5H-thiazolo [3,2-α] pyrimidine-5-one (R59022) (0.1–30 μM), inhibited thromboxane A_2 analogue 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619)-induced sustained contractions in mouse aorta and porcine coronary artery in a dose-dependent manner. Treatment with stemphone did not affect contractions in these tissues. However, stemphone significantly inhibited ($>0.3\,\mu$M) U46619-induced spontaneous phasic contraction in mouse portal vein. This inhibitory effect was not detected following R59022 treatment in portal vein. Therefore, stemphone demonstrated selectivity in terms of portal vein contraction.

3 Under high glucose (22.2 mM) conditions, U46619-induced contraction was enhanced in these three types of vascular tissue. Inhibitory effects of R59022 were attenuated under these conditions; however, effects of stemphone were observed. These results indicated that stemphone could inhibit portal vein contraction under high glucose conditions, for example, diabetes. These data suggested the possibility that DG kinase may be a target of hyperportal pressure.

4 Total mass of DG was enhanced under high glucose conditions. DG was derived from incorporated glucose *via de novo* synthesis in the absence of phospholipase C pathway mediation. This enhanced DG under high glucose conditions activated a calcium-independent protein kinase C (PKC). This PKC was associated with calcium-independent DG kinase activation. Treatment with stemphone also inhibited calcium-independent DG kinase. These signal transduction pathways were distinguishable from a DG–PKC pathway under normal glucose conditions.

5 The present investigation suggested that stemphone selectively inhibited overcontraction of portal vein induced by high glucose levels. This phenomenon was attributable to inhibition of calcium-independent DG kinase activation that occurred under high glucose conditions mediated by both DG synthesized from glucose and calcium-independent PKC activation.
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Abbreviations: DG, diacylglycerol; diC8, dioctanoyl-*sn*-glycerol; Gö6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo-(2,3-α)pyrrolo(3,4-c)-carbazole; IP_3, inositol trisphosphate; [^{32}P]Pi, inorganic phosphate; PA, phosphatidic acid; PIP_2, phosphatidylinositol-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; R59022, 6-[2-(4-[(4-fluorophenyl)phenyl-methylene]-1-piperidinyl)ethyl]-7-methyl-5H-thiazolo [3,2-α] pyrimidine-5-one; U46619, 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$

Introduction

Vascular smooth muscle contractility is dependent not only on intracellular calcium concentration but also on a variety of intracellular signaling factors (Morgan, 1987). Agonist-mediated activation is generally associated with phosphatidyl inositol turnover (PI turnover) (Abdel-Latif, 2001). Diacylglycerol (DG) and inositol trisphosphate (IP_3) play important roles in PI turnover with respect to cellular function in many types of vascular tissue (Berridge & Irvine, 1984; Berridge, 1987). DG is thought to be an essential factor in vascular contraction consequent to its function as a physiological activator of protein kinase C (PKC) (Lee & Severson, 1994). Diacylglycerol kinase (DG kinase) phosphorylates DG, leading to the formation of phosphatidic acid (PA) (Kanoh *et al.*,

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1990). DG kinase alters DG level; thus, this enzyme functions as an indirect PKC regulator (Ohanian & Ohanian, 2001). Moreover, phosphorylation of DG is the first step in phosphatidylinositol resynthesis; therefore, it is widely accepted that this enzyme activity is a crucial component of PI turnover. Alteration of PI turnover activity in vascular diseases has been suggested; however, detailed mechanisms and association with DG kinase activity are poorly understood. We previously documented alteration of DG kinase activity in vascular tissue isolated from diabetic models (Nobe *et al*., 1998). Furthermore, we suggested that this alteration was correlated with dysfunction of vascular contractility (Nobe *et al*., 2003a).

In mammalian tissues, more than seven types of DG kinase isoforms have been documented, which differ in terms of activators, substrate selectivity and tissue distribution (van Blitterswijk & Houssa, 2000; Ohanian & Ohanian, 2001). Cysteine-rich domains are common to all known DG kinase isoforms; however, only Class-I DG kinases (α, β and γ) possess EF-hand motifs that bind to calcium as an activating factor (Yamada *et al*., 1997). Class-II DG kinase isoforms (δ and η), which exhibit N-terminal pleckstrin homology (PH) domains, lack EF hands (Topham *et al*., 1998). Class-I, -III (ε), -IV (ζ) and -V (θ) DG kinase isoforms are activated by phosphatidylserine. Structural domains are currently under investigation; however, intracellular regulatory mechanisms are poorly understood.

Under high glucose conditions such as those associated with diabetes, aortic smooth muscle contraction was enhanced independent of intracellular calcium concentration (Nobe *et al*., 2003c). This situation included the possibility that enhancement of contraction might be derived from hyper-reactivity of DG kinase (Nobe *et al*., 2002). On the basis of these findings, it was suggested that alteration of DG kinase activity induced dysfunction of vascular contraction in diabetes mediated by acceleration of PI turnover. Inhibitors of neurotransmitters and calcium channels were generally employed in the treatment of hyperpressure (Meggs & Kodali, 1999; Mason, 2003). However, tissue specificity of and/or untoward effects caused by these medications were noted. As a result, novel approaches for treatment of hypercontraction were necessary.

Consequently, we hypothesized that reduction (normalization) of DG kinase activity contributes to the relief and/or circumvention of hypercontraction of vascular tissue in diabetes. In terms of the treatment of hypercontraction, the viability of DG kinase has not been determined due to the numerous complications exhibited by previous DG kinase inhibitors (6-[2-(4-[(4-Fluorophenyl)phenyl-methylene]-1-piperidinyl)ethyl]-7-methyl-5H-thiazolo [3,2-α] pyrimidine-5-one (R59022) and R59949) in clinical application. For example, vascular tissue selectivity, nonspecific effects, reversibility and tissue permeability were observed (Lai & El-Fakahany, 1990). These DG kinase inhibitors serve as critical tools in basic medical sciences. Thus, we hypothesized that a DG kinase inhibitor might be a suitable candidate for treatment of hypercontraction, provided a novel DG kinase inhibitor demonstrating vascular tissue selectivity and selective inhibitory effects under limited conditions could be identified.

The effect of the novel DG kinase inhibitor, stemphone, on vascular contraction was examined in the present investigation. Stemphone was extracted from fermented mycelia *Drechslera succhari* and purified as described previously in the laboratory of Mitsubishi Pharma Co. (Ogawara *et al*., 1994). Based on our previous studies, this compound exerts a strong inhibitory effect on isolated DG kinase fraction; in contrast, other kinase types are unaffected (Machida *et al*., 1995). Moreover, these findings confirmed that stemphone influenced cell permeability. However, effectiveness and selectivity of stemphone on vascular tissue have not been evaluated.

The aim of this study was to identify these points and to elucidate the mechanisms. The present investigation underscored the possibility that a DG kinase inhibitor may serve as a novel medication for hypervascular contraction.

Methods

Preparation of porcine coronary artery

Porcine hearts, which were obtained shortly after slaughter, were introduced to a cold (4°C) physiological salt solution (PSS) following the removal of blood (Nobe & Paul, 2001). PSS consisted of (mmoll^{-1}) 137 NaCl, 4.73 KCl, 1.2 $MgSO_4$, 0.025 EDTA, 1.2 KH_2PO_4, 2.5 $CaCl_2$ and 11.1 glucose (buffering was achieved with 25.0 $NaHCO_3$); pH was 7.4 when the solution was bubbled with 95% O_2/5% CO_2 at 37°C. The distal portions of the left anterior descending coronary artery were dissected and placed in ice-cold PSS. Fat and connective tissue were separated from arteries; subsequently, arteries were cut into 5-mm segments. The segments were everted to a de-endothelialized condition *via* rolling gently on filter paper.

Preparation of mouse aorta and portal vein

Mice (ddY, male, 7–9 weeks of age) were killed with ether. The aorta and portal vein were dissected and prepared for analysis as described previously (Nobe *et al*., 2003b, c). Briefly, vessels were rinsed in ice-cold PSS; additionally, loose fat and connective tissue were removed. Endothelium removal did not significantly affect the influence of DG kinase and other inhibitors in response to 9,11-dideoxy-11α, 9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619) (data not shown).

Measurement of isometric force development

Arterial segments were attached to a movable post connected to a force transducer (NEC San-ei Instruments Ltd, Tokyo, Japan). Resting tension was adjusted to 10–15 millinewtons (mN) in porcine coronary artery, 8–9 mN in mouse aorta and 2–3 mN in mouse portal vein. These values were selected on the basis of prior experiments to establish a tissue length in the optimal range for maximum tension development. Isometric force was expressed as mN. The mounted artery was introduced to an organ bath system; subsequently, this assembly was placed in a water-jacketed holder maintained at 37°C. Data were obtained using Power Lab hardware and analyzed with Chart Software (AD Instruments Japan, Tokyo, Japan).

Measurement of total mass of DG

The total mass of DG in each tissue was measured in a manner similar to that described in a previous report (Nobe *et al*., 1993). Isolated tissues were treated under various conditions in 200 μl PSS. The reaction was terminated by the addition of chloroform/methanol (1 : 2 by volume, 75 μl). Tissues were homogenized; subsequently, water and chloroform (200 μl of each) were added. The mixture was shaken followed by centrifugation at $1000 \times g$. The lower phase was removed and dried under N_2 gas. The residue was redissolved in chloroform (concentration; 2 μl mg wet weight tissue^{-1}). This sample was spotted on a TLC plate (Merck, Silica gel 60 with concentrating zone). DG separation was effected with diethylether : heptane : acetic acid (75 : 25 : 1 by volume). Plates were dried and stained with 0.03% Coomassie brilliant blue solution containing 30% methanol and 100 mM NaCl for 30 min; plates were destained for 5 min in dye-free staining solution. Each TLC plate was scanned; moreover, the density of each band was calculated utilizing NIH image software. Total mass of DG was determined from a dioleoyl–glycerol standard curve. Results were expressed as ng mg wet weight tissue^{-1}.

*Assay of DG kinase activity utilizing dioctanoyl-*sn*-lglycerol (diC8) in mouse portal vein*

DG kinase activity in tissue was determined *via* measurement of [^{32}P]dioctanoyl-phosphatidic acid ([^{32}P]diC8-PA) accumulation from diC8 in radioactive inorganic phosphate ([^{32}P]Pi) and diC8-prelabeled tissues (Nobe *et al*., 1994). For this assay, diC8 was dissolved in chloroform and stored at -40°C as a stock solution. Prior to use, the stock solution, which was dried under Ar gas at room temperature, was introduced to a 50% ethanol solution (final concentration of 0.03%). The diC8 ethanol solution was added to PSS supplemented with 2.7 mg ml^{-1} bovine serum albumin (BSA) (diC8 solution). Tissues (15–20 mg wet weight tissue tube^{-1}) were initially incubated with 2.22 MBq ml^{-1} of [^{32}P]Pi in 1 ml diC8 solution for 90 min at 37°C, followed by washing (two repetitions) with 10 ml PSS. The reaction was initiated by the addition of 0.8 ml PSS containing various compounds at 37°C. Incubation with 3 ml ice-cold chloroform : methanol : 10 M HCl (100 : 200 : 1 by volume) terminated the reaction. Subsequently, specimens were homogenized with a glass homogenizer in ice-cold water. Extraction and quantitation of [^{32}P]diC8-PA were performed as described previously (Nobe *et al*., 1994). Results were expressed as c.p.m. mg wet weight tissue^{-1}.

Subcellular fractionation of mouse portal vein

DG kinase activity in extracted subcellular fraction was measured in a manner similar to that described in a previous report (Nobe *et al*., 1994). Mouse portal vein was homogenized with a polytron homogenizer (Brinkman Institute) in an ice-cold solution consisting of 20 mM MOPS (pH 7.2), 250 mM sucrose, 1 mM DTT, 1 mM EGTA, 1 μg ml^{-1} pepstatin, 1 μg ml^{-1} leupeptin and 50 μg ml^{-1} soybean trypsin inhibitor (buffer A), the homogenates were centrifuged ($1000 \times g$ for 5 min) to remove nuclei. The supernatant was decanted and the pellets were washed once with buffer B (sucrose-free buffer A). Then, the combined supernatants were centrifuged again ($20{,}000 \times g$ for 30 min). The pellets were resuspended in buffer B (mitochondrial fraction). Finally, the particulate fraction were collected by centrifugation of the supernatant ($100{,}000 \times g$ for 60 min) and resuspended in buffer B. Octylglycoside and KCl were added to the particulate fractions on ice to final concentrations of 2% and 300 mM, respectively. The mixtures were slowly shaken for 30 min and then centrifuged at $100{,}000 \times g$ for 60 min. DG kinase activity was assayed in the presence of 1-stearoyl-2-arachidonyl-glycerol (18:0/20:4-DG) according to previous reports (Nobe *et al*., 1994). The extracted enzyme was incubated for 2 min at 37°C in the presence of 60 mM MOPS (pH 7.2), 0.86 mM DTT, 18 mM $MgCl_2$, 73 mM octylglycoside, 3.3 mM phosphatidylserine, 0.52 mM [γ-^{32}P]ATP (710 MBq mol^{-1}) and various concentration of 18:0/20:4-DG and/or DG kinase inhibitors (3 μM R59022 and 3 μM stemphone), and the reaction was terminated by the addition of ice-cold chloroform : methanol : 10 M HCl (100 : 200 : 1 by volume). Lipid concentration refers to molar fraction of lipid in the micellar phase of octylglycoside; calculations were based on a critical micellar concentration of 25 mM for octylglycoside. The products, 1-stearoyl-2-arachidonyl-phosphatidic acid (18:0/20:4-PA), were extracted and separated as tissue DG kinase assay.

Materials

Carrier- and HCl-free radioactive [^{32}P]Pi and [γ-^{32}P]ATP were purchased from Du Pont-New England Nuclear (Boston, MA, U.S.A.). BSA, U46619, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo-(2,3-α)pyrrolo(3,4-c)-carbazole (Gö6976) and 1-(6[([17β]-3-methoxyestra-1,3,5[10]-trien-17-ly)amino]hexyl)-1H-pyrrole-2,5-dione (U73122) were obtained from Sigma Chemical Co (St Louis, MO, U.S.A.). Calphostin C and Rottlerin (Mallotoxin) were acquired from Calbiochem-Novabiochem Co (San Diego, CA, U.S.A.). 18:0/20:4-DG, DiC8 and dioleoyl-glycerol were obtained from Avanti Polar Lipids Inc. (Alabaster, AL, U.S.A.). R59022 was purchased from Janssen Life Science Products (Beerse, Belgium). Stemphone was extracted and purified from fermented mycelia *D. succhari* and purified as described previously in the laboratory of Mitsubishi-Pharma Co (Kanagawa, Japan). Thin-layer chromatography (TLC) plates (silica gel 60 with a concentrating zone) were manufactured by Merck Inc. (Darmstadt, Germany). All other chemicals and materials were of reagent grade quality. R59022 was dissolved in a 10% ethanol solution containing 5 mM HCl as a 2.5 mM stock solution (de Chaffoy de Courcelles *et al*., 1985).

Data analysis

Values are presented as means $\pm$ s.e.m. obtained from at least five animals. Statistical analyses for multiple comparisons were performed employing Repeated measures ANOVA followed by the Student–Newman–Keuls test.

Results

Effect of DG kinase inhibitors on U46619-induced porcine coronary artery contraction

Resting isometric force level averaged 13.01 $\pm$ 0.65 mN ($n = 5$). U46619 (30 nmol l^{-1}) increased the force development. The

maximal response (29.07 ± 2.38 mN; $n = 5$) occurred within 5–10 min of stimulation; moreover, >90% of the maximal response was maintained for at least 90 min. After the rinse, identical U46619-induced responses could be elicited (data not shown). In order to detect the effect of DG kinase inhibitor on U46619-induced contraction, 0.1–30 μmol l^{-1} stemphone was cumulatively added during 30 nmol l^{-1} U46619-induced sustained force development (Figure 1a, left panel). Decrease of the sustained force was not observed with 0.1–10 μmol l^{-1} stemphone treatments; additionally, 87.2% of U46619-induced force development remained following treatment with the maximal concentration of stemphone (30 μmol l^{-1}). Similar trials were conducted under high glucose conditions (Figure 1a, right panel).

Preincubation of vascular tissue with PSS containing 22.2 mmol l^{-1} glucose (HG-PSS; glucose concentration twice that of normal PSS) for 30 min did not alter the resting force level (13.37 ± 0.51 mN; $n = 5$). However, 30 nmol l^{-1} U46619-induced force development was significantly enhanced. Maximal level, which was apparent after 8–10 min of stimulation, was 38.93 ± 0.44 mN ($n = 5$). In a manner similar to that in normal PSS, 0.1–3 μmol l^{-1} stemphone did not influence force development and slight decreases were observed at levels in excess of 10 μmol l^{-1}. In total, 81.3% of the U46619-induced force development remained upon treatment with 30 μmol l^{-1} stemphone. In order to compare the effect of stemphone on coronary artery contraction, the general- and conventional-type DG kinase inhibitor, R59022, was employed (Figure 1b) (de Chaffoy de Courcelles *et al*., 1985). R59022 decreased the 30 nmol l^{-1} U46619-induced force development in a dose-dependent manner in the concentration range of 1–30 μmol l^{-1}. Following treatment with 30 μmol l^{-1} stemphone, force level achieved the original resting level (15.62 ± 0.70 mN; $n = 5$). Under high glucose conditions, a dose-dependent decrease in force development was also detected. Maximal level of 30 μmol l^{-1} R59022 treatment was 20.50 ± 0.42 mN ($n = 5$). These results are summarized in Figure 2.

U46619-induced force development was reduced significantly by treatment with R59022 (10–30 μmol l^{-1}); however, stemphone displayed no effect in normal PSS. Significant enhancement of force development was evident under high glucose conditions, although inhibitory effects of R59022 treatment remained. IC_{50} values of R59022 treatment under normal and high glucose conditions were approximately 10.5 and 20.0 μmol l^{-1}, respectively. The maximal concentration of stemphone (30 μmol l^{-1}) slightly reduced the response in HG-PSS; however, it could not alter the major response.

In stemphone-treated tissue, a 60-min rinse of the tissue recovered in excess of 80% of normal 30 nmol l^{-1} U46619-induced response. In contrast, R59022-treated tissue recovered only 30% or less of that response (data not shown).

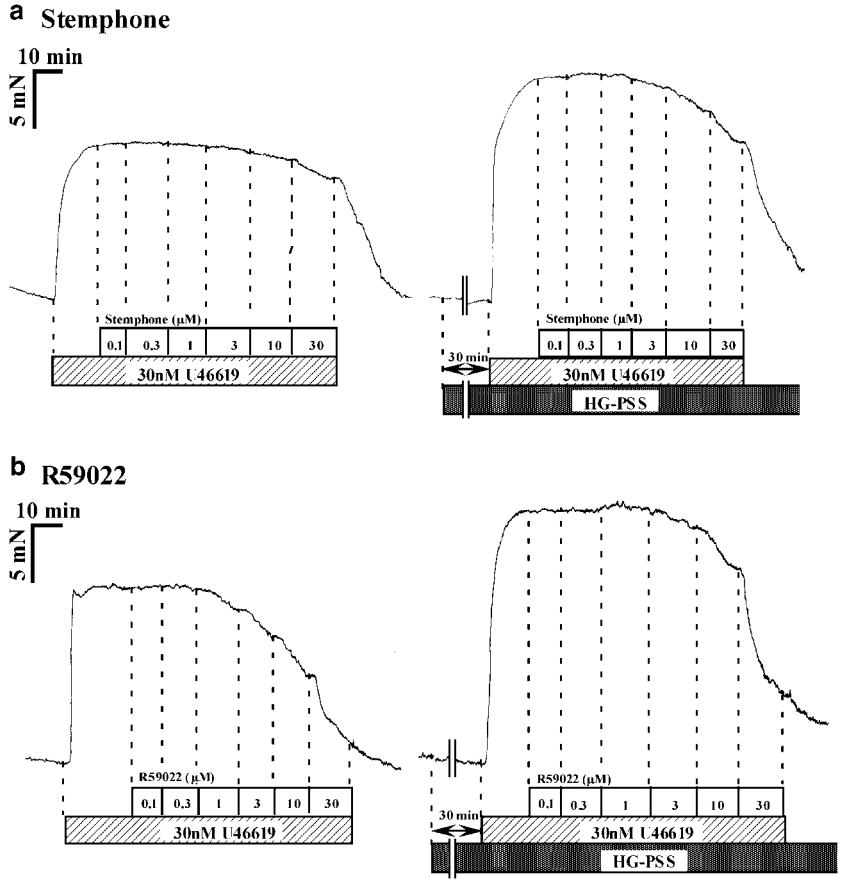


Figure 1 Effects of DG kinase inhibitors on U46619-induced contractile response in porcine coronary artery. Representative experimental traces illustrating the effect of stemphone (a) and R59022 (b) (0.1–30 μM) on 30 nM U46619-induced contraction in mouse aorta. HG-PSS was pretreated 30 min prior to U46619 stimulation.

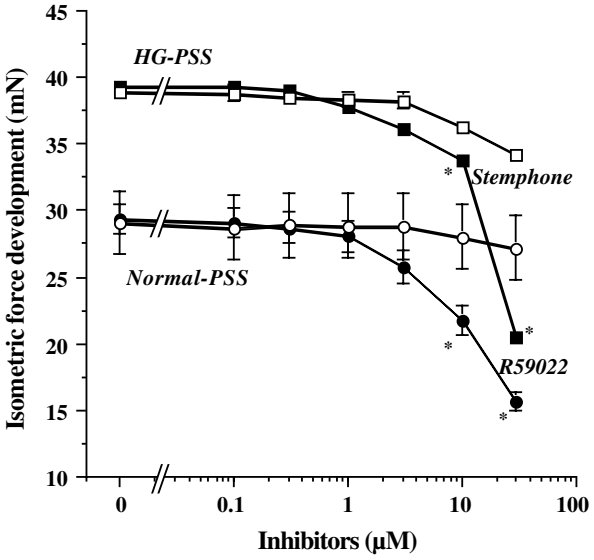


Figure 2 Dose-dependent inhibitory effects of DG kinase inhibitors on U46619-induced contractile response in porcine coronary artery. The 30 nM U46619-induced isometric force development was measured under normal glucose (circles) and high glucose (squares; preincubation with HG-PSS for 30 min) conditions. Following maximal force development, indicated concentrations of stemphone (open) and R59022 (closed) were added for 5 min. Each point is the mean ± s.e.m. from at least five independent determinations. $^*P<0.05$ vs in the absence of inhibitors.

Effect of DG kinase inhibitors on U46619-induced mouse aorta contraction

Resting isometric force level was defined as 4.95 ± 0.26 mN ($n=5$). U46619 (30 nmol l^{-1}) induced a sustained increase in force development. Maximal response (11.92 ± 0.37 mN; $n=5$) was detected at 8–10 min after the stimulation. This response was sustained for at least 90 min following stimulation. The addition of stemphone failed to alter U46619-induced sustained force development. Only a slight decrease (76.6% of maximal response) was observed following administration of 30 μmol l^{-1} stemphone. In a manner similar to porcine coronary artery, preincubation with HG-PSS enhanced U46619-induced force development with no effect on resting levels (from 5.05 ± 0.2 to 15.4 ± 0.39 mN; $n=5$). Under this condition, inhibitory effects of stemphone were not apparent. In excess of 91.9% of force development remained following 30 μmol l^{-1} stemphone treatment.

R59022 reduced 30 nmol l^{-1} U46619-induced force development in a dose-dependent manner. Significant reduction was detected in the presence of 3 μmol l^{-1} R59022; subsequently, 30 μmol l^{-1} R59022 suppressed force development. Under high glucose conditions, a similar inhibitory effect in normal PSS was also observed. These results are summarized in Figures 3 and 4. In normal and HG-PSS, force development was reduced markedly by concentrations of R59022 in excess of 3 μmol l^{-1}. IC_{50} values were 10.0 and 4.5 μmol l^{-1}, respectively. Treatment

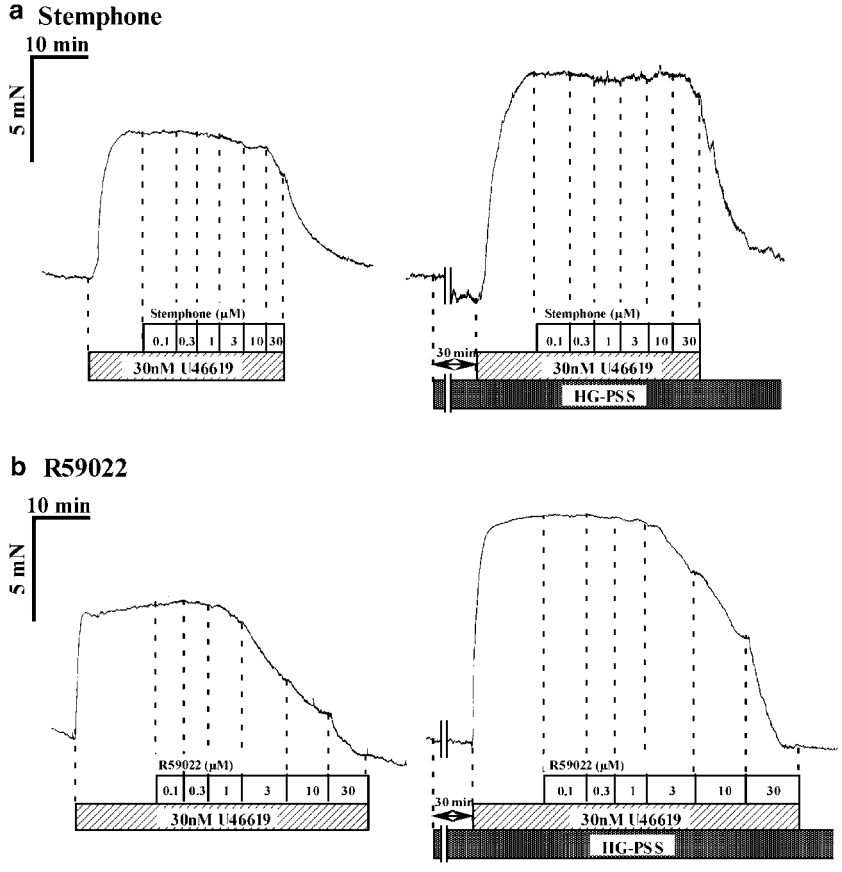


Figure 3 Effects of DG kinase inhibitors on U46619-induced contractile response in mouse aorta. Representative experimental traces illustrating the effect of stemphone (a) and R59022 (b) (0.1–30 μM) on 30 nM U46619-induced contraction in mouse aorta. HG-PSS was pretreated 30 min prior to U46619 stimulation.

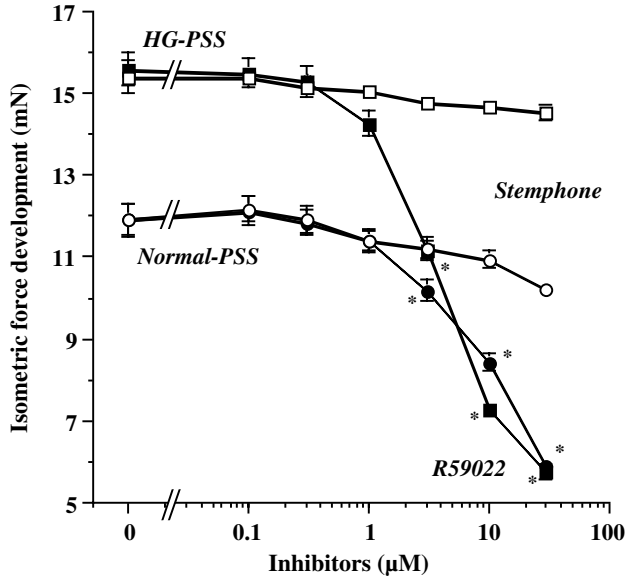


Figure 4 Dose-dependent inhibitory effects of DG kinase inhibitors on U46619-induced contractile response in mouse aorta. The 30 nM U46619-induced isometric force development was measured under normal glucose (circles) and high glucose (squares; preincubation with HG-PSS for 30 min) conditions. Following maximal force development, indicated concentrations of stemphone (open) and R59022 (closed) were added for 5 min. Each point is the mean ± s.e.m. from at least five independent determinations. $^*P<0.05$ vs in the absence of inhibitors.

with 3 μmol l^{-1} R59022 revealed no significant differences between normal and high glucose conditions.

Effect of DG kinase inhibitors on U46619-induced mouse portal vein contraction

Spontaneous phasic contractions were observed in mouse portal vein (Figure 5). In the nonstimulated resting state, 1.0–1.3 mN tension was applied as the minimum basal tone. Absolute peak values of the spontaneous phasic contraction involving basal tone were 1.77 ± 0.16 mN ($n=7$). This response was maintained for at least 12 h in our organ bath system (normal PSS, pH 7.4 at 37°C). Treatment with 30 nM U46619 increased the peak value; moreover, stable force was developed 3–5 min following U46619 addition (2.67 ± 0.08 mN; $n=7$). This contractile response returned to resting levels in approximately 5 min following removal of U46619 *via* exchange of the bath contents. After the rinse, identical U46619-induced responses were elicited (data not shown). This response decreased in a dose-dependent manner as a result of the introduction of stemphone (Figure 5a, left panel). Increase in the 30 nmol l^{-1} U46619-induced force development was abolished by 30 μmol l^{-1} stemphone treatment (1.98 ± 0.14 mN; $n=7$). Changes in phasic force development were measured under high glucose conditions in the presence and absence of U46619 (Figure 5a, right panel). Treatment of portal veins with HG-PSS for 30 min did not affect spontaneous force

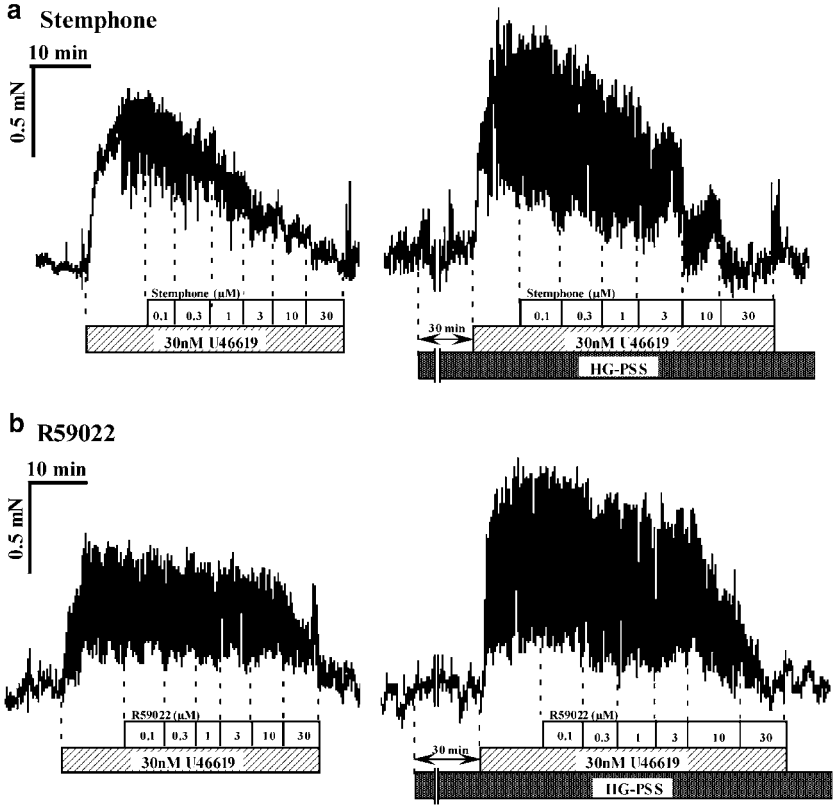


Figure 5 Effects of DG kinase inhibitors on U46619-induced contractile response in porcine mouse portal vein. Representative experimental traces showing the effect of stemphone (a) and R59022 (b) (0.1–30 μM) on 30 nM U46619-induced contraction in mouse aorta. HG-PSS was pretreated 30 min prior to U46619 stimulation.

development (2.00 ± 0.02 mN; $n = 7$) in the resting state. However, the increase in contractility elicited by U46619 was significantly augmented. Under high glucose conditions, 30 nmol l^{-1} U46619 induced a maximal peak value of 3.13 ± 0.04 mN ($n = 7$). In a manner similar to that of the normal condition, stemphone reduced U46619-induced force development. The effects were dose dependent; furthermore, 30 μmol l^{-1} was submaximal (1.82 ± 0.16 mN; $n = 7$).

In order to compare the effect of stemphone on portal vein contraction, R59022 was introduced for U46619-induced force development (Figure 5b). R59022 (0.1–10 μmol l^{-1}) did not induce inhibition of the contractile response. Maximal concentration of R59022 (30 μmol l^{-1}) slightly reduced the response; however, this level was 2.18 ± 0.06 mN ($n = 7$). During enhancement of force development under high glucose conditions, inhibitory effects of R59022 were not detected at 0.1–3.0 μmol l^{-1}. Treatment with R59022 in excess of 10 μmol l^{-1} led to inhibition of increased force development under high glucose conditions (2.09 ± 0.11 mN; $n = 7$).

In order to assess U46619-induced contractile response in normal- and HG-PSS, three parameters were extracted from the data corresponding to DG kinase inhibitor pretreatment in 1–100 nmol l^{-1} U46619-induced force development. The following parameters were defined in a previous paper (Nobe *et al*., 2003b):

Amplitude: The 'maximum–minimum' value in each phasic contraction was calculated. Results were expressed as an average in a 3–5 min window of stabilized response. Results were expressed in mN.

Frequency: The number of contractile events in a 3–5 min window of stabilized response was counted. The threshold consisted of 30% of each spontaneous response. Results were expressed in cycles per minute (cycles min^{-1}).

ON-time: Total seconds in excess of 20% of maximal response induced by 100 nmol l^{-1} U46619 in a 3–5 min window of stabilized response. Results were expressed as seconds per minute (s min^{-1}).

In the resting state, the amplitude (in the absence of applied basal tension) was 0.56 ± 0.11 mN ($n = 5$). This amplitude transiently increased in a manner dependent on U46619 concentration in the absence of stemphone under normal conditions (Figure 6a). Maximal values were detected in 10 nmol l^{-1} U46619 (3.03 ± 0.19 mN; $n = 5$). Following attainment of maximal amplitude, readings fell to 20% of maximal values upon stimulation with 100 nmol l^{-1} U46619. Pretreatment with 3 μM stemphone significantly reduced increases in amplitude induced by 3–30 nmol l^{-1} U46619. In the presence of stemphone, 10 nmol l^{-1} U46619-induced amplitude was 2.09 ± 0.06 mN ($n = 5$). Under high glucose conditions, a similar pattern of change emerged. Although maximal amplitude occurred at 10 nmol l^{-1} U46619 stimulation (8.13 ± 0.39 mN; $n = 5$), most values in HG-PSS were significantly larger than those in normal PSS. Treatment with 3 μmol l^{-1} stemphone also significantly decreased amplitude in 1–100 nmol l^{-1} U46619 stimulation. A measure of 10 nmol l^{-1} U46619-induced amplitude declined to 2.75 ± 0.07 mN ($n = 5$). Following stemphone treatment, significant differences between normal and high glucose conditions were suppressed. Similar trials were performed using R59022 (Figure 6b). Following pretreatment with 3 μmol l^{-1} R59022, enhancement of the amplitude relative to corresponding values in normal PSS was detected during U46619 stimulation. A measure of 10 nmol l^{-1} U46619-induced amplitude in the presence of R59022 was 2.29 ± 0.30 mN ($n = 5$). Under high glucose conditions, pretreatment with R59022 did not lead to a significant decrease in the amplitude induced by U46619.

A graph depicting the correlation between frequency and ON-time is presented in Figure 7. In the resting state, frequency and ON-time displayed readings of 2.05 ± 0.04 cycles min^{-1} and 19.1 ± 0.48 s min^{-1}, respectively ($n = 5$). In a manner dependent on U46619 concentration, the frequency effectively increased relative to the ON-time values. Maximal frequency was detected during stimulation with 10 nmol l^{-1} U46619 (2.96 ± 0.06 cycles min^{-1}; $n = 5$). Subsequently, ON-time increased to maximal levels (60 s min^{-1}). These results revealed that the relationship between frequency and ON-time changed in counterclockwise manner. Pretreatment with 3 μmol l^{-1} stemphone altered the counterclockwise nature

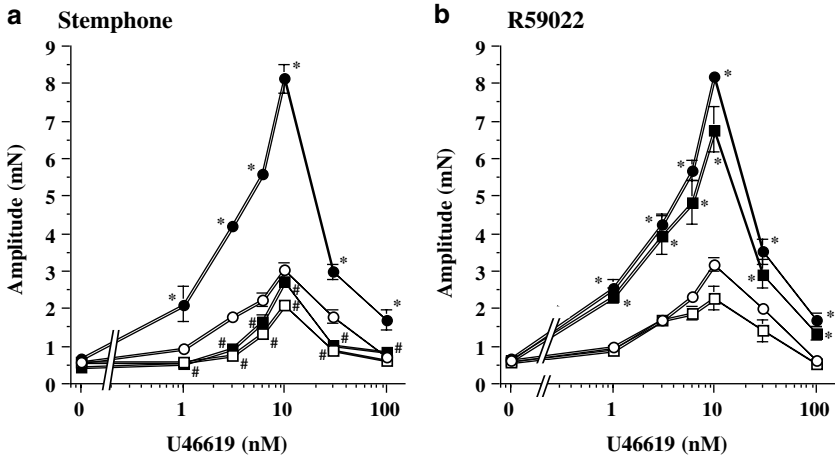


Figure 6 Effects of DG kinase inhibitor on U46619-induced changes in amplitude in mouse portal vein. Portal veins were preincubated in normal PSS (open) and HG-PSS (closed) for 30 min; subsequently, the tissues were treated with U46619 (1–100 nM) in the presence (squares) or absence (circles) of DG kinase inhibitors; stemphone (a) and R59022 (b) (3 μM each) were introduced 10 min prior to stimulation. Amplitudes of the contractile responses were extracted. Each value represents the mean $\pm$ s.e.m. from at least five independent determinations. $^{*}P < 0.05$ vs normal PSS. $^{\#}P < 0.05$ vs in the absence of DG kinase inhibitor.

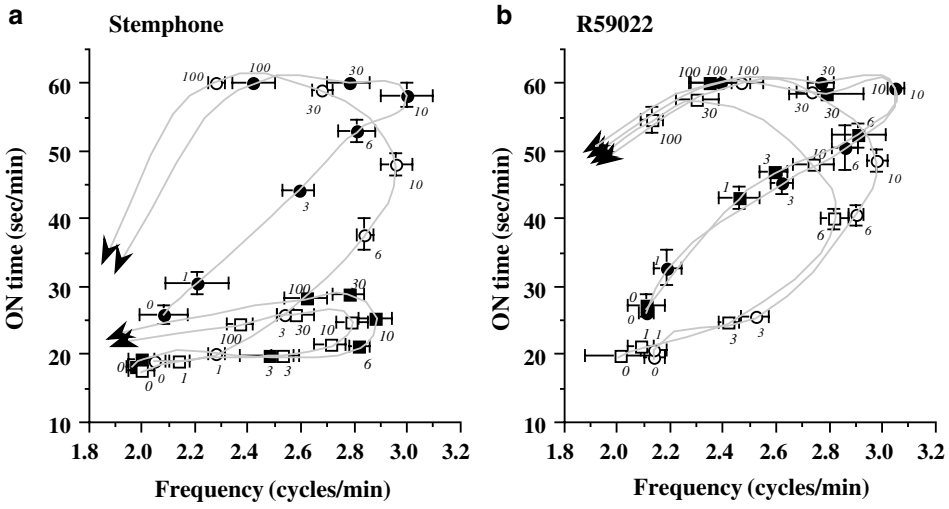


Figure 7 U46619 induced changes in the relation between frequency and ON-time in mouse portal vein. ON-time and frequency were extracted from the identical original data of Figure 6. Values of these parameters obtained during stemphone (a) and R59022 (b) (3 μM each) treatments were plotted. Each concentration of U46619 (nM) is indicated as open (normal PSS) and closed (HG-PSS) numbers. Each value represents the mean $\pm$ s.e.m. from at least five independent determinations.

Table 1 Effects of DG kinase inhibitors on particulate fractions of vascular tissue

Groups	*Animals*	*DG kinase activity (p mol min^{-1} mg protein^{-1})*		
		Porcine coronary	*Mouse aorta*	*Mouse PV*
Control	5	6.75$\pm$0.33	4.65$\pm$0.72	3.72$\pm$0.51
R59022	5	1.50$\pm$0.33*	1.15$\pm$0.24*	3.05$\pm$0.61
Stemphone	5	5.65$\pm$0.41	3.47$\pm$0.69	0.66$\pm$0.20*

Fresh vascular tissues were homogenized and particulate fractions were collected. Rates of phosphorylation of 18:0/20:4-DG in the presence or absence (control) of 3 μM R59022 and 3 μM stemphone as described in Methods. Results were expressed as rates of 18:0/20:4-DG phosphorylation.
*$P<0.05$ vs control values.

(Figure 7a). Stemphone significantly reduced the ON-time (24.8$\pm$0.45 s min^{-1} at 10 nmol L^{-1} U46619 stimulation; $n=5$) without affecting the frequency (2.79$\pm$0.06 cycles min^{-1}; $n=5$).

Under high glucose conditions, changes in the relationship also exhibited counterclockwise behavior; however, ON-time readings were significantly enhanced during stimulation with U46619. During this period, frequency did not differ from those values observed in normal PSS. In a manner similar to that of normal PSS, stemphone administration reduced the ON-time values. In the case of 10 nmol l^{-1} U46619 stimulation, stemphone reduced the ON-time to 25.4$\pm$0.41 s min^{-1} ($n=5$).

Influence of 3 μmol l^{-1} R59022 on the relationship between ON-time and frequency was investigated (Figure 7b). R59022 did not affect the relation in normal PSS. Enhancement of ON-time in HG-PSS was detected; however, treatment with R59022 did not alter the response.

In order to confirm the selectivity of stemphone in PV contraction, inhibitory effects of the stemphone and R59022 on crude DG kinase activity in particulate fractions were investigated (Table 1). The crude DG kinase activity was measured in particulate fraction extracted from three types of vascular tissue using octylglycoside mixed miceller assay method. Each control response indicates maximal DG kinase activity in each particulate fraction. The addition of 3 μM R59022 reduced under 25% of control response in porcine coronary artery and mouse aorta. However, activity in mouse portal vein remained over 80% of control. On the other hand, 3 μM stemphone reduced the DG kinase activity only in mouse portal vein (17.9% of control) without affecting the activities in another tissues.

Effects of phospholipase C (PLC) and DG kinase inhibitors on U46619-induced total mass of DG in mouse portal vein

Total mass of DG in mouse portal vein was measured (Figure 8). The resting level of DG mass was 149.18$\pm$7.26 ng mg wet weight tissue^{-1} ($n=5$) in normal PSS. Treatment with U46619 (30 nmol l^{-1}, 10 min) induced significant elevation in the DG level (231.3$\pm$10.6 ng mg wet weight tissue^{-1}; $n=5$). Elevated DG levels returned to the resting level upon PSS rinse of the tissue (data not shown). The U46619-induced increase in total mass of DG was significantly reduced by pretreatment of tissue with 1 μmol l^{-1} U73122 (157.5$\pm$12.7 mg wet weight tissue^{-1}; $n=5$). This inhibitory effect could not be detected by a pretreatment of 1 μM U73343 (inactive analogue of U73122). Stemphone (3 μmol l^{-1}) had no effect on the increase in DG mass (216.6$\pm$4.9 ng mg wet weight tissue^{-1}; $n=5$). Under high glucose conditions, non-stimulated resting level of the total mass of DG was significantly enhanced (248.5$\pm$22.1 ng mg wet weight tissue^{-1};

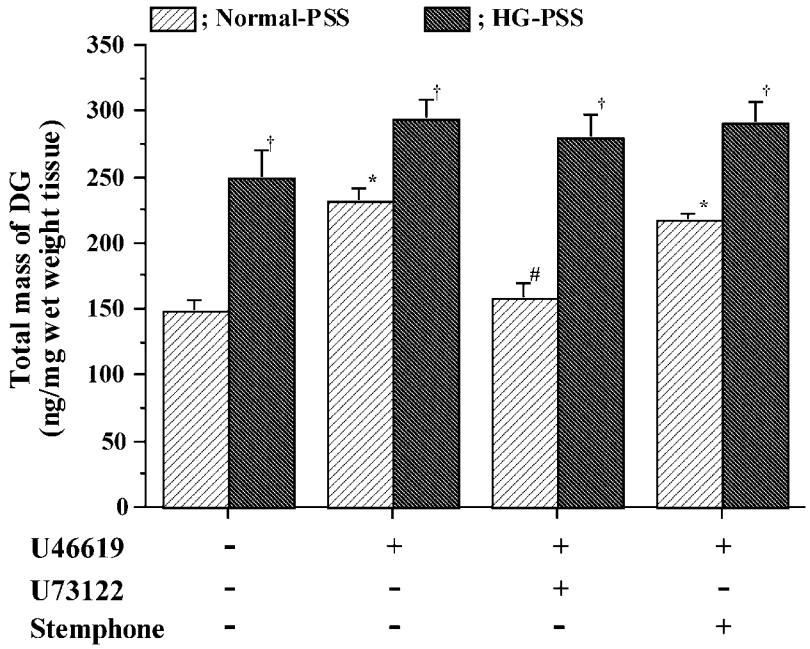


Figure 8 U46619 induced changes in total mass of DG in mice PV. Tissues were preincubated in normal (bright column) and HG-PSS (dark column) at 37°C for 30 min. A PLC inhibitor (1 μM U73122) or stemphone (3 μM) was introduced to several of these samples for the final 10 min of the preincubation; subsequently, 30 nM U46619 was added for 5 min. Following termination of the response, total mass of DG was quantified as described in Methods. Results are expressed as ng mg wet weight tissue^{-1}. Each value represents the mean $\pm$ s.e.m. from at least five independent determinations. $^{*}P<0.01$ vs resting level. $^{\#}P<0.01$ vs 30 nM U46619 alone. $^{\dagger}P<0.05$ vs normal PSS.

$n=5$) compared with that of normal PSS. This level exceeded the U46619-induced maximal response in normal PSS. Significant increases in the total mass of DG were not detected upon U46619 treatment in HG-PSS (294.2 ± 14.2 ng mg wet weight tissue^{-1}; $n=5$). Moreover, pretreatment with U73122 or stemphone did not alter total mass of DG (278.9 ± 18.4, 290.7 ± 15.5 ng mg wet weight tissue^{-1}, respectively; $n=5$). Significant differences between normal and HG-PSS remained under all treatments.

Activation of DG kinase under normal and high glucose conditions in mouse portal vein

Cellular DG kinase activity was measured as an accumulation of [^{32}P]diC8-PA in [^{32}P]Pi and diC8-prelabeled tissues (Figure 9) (Nobe *et al*., 1994). In the portal vein, the resting level of DG kinase activity was 27.6 ± 0.87 c.p.m. mg wet weight tissue^{-1} ($n=5$) in normal PSS. This activity increased markedly consequent to treatment with 30 nmol l^{-1} U46619 for 5 min. Maximal value was 104.2 ± 8.73 c.p.m. mg wet weight tissue^{-1} ($n=5$). Under high glucose conditions, activities of resting and 30 nmol l^{-1} U46619-treated DG kinase were elevated significantly (73.4 ± 3.7, 181.6 ± 13.7 c.p.m. mg wet weight tissue^{-1}, respectively; $n=5$). U46619-induced activation of DG kinase was partially inhibited by calcium-free normal PSS (57.3 ± 1.2 c.p.m. mg wet weight tissue^{-1}; $n=5$). However, alteration of DG kinase activity induced by U46619 under calcium-free conditions was not observed in HG-PSS. Significant differences between normal- and HG-PSS were apparent.

Two types of DG kinase inhibitor were employed in these experiments. Pretreatment with 3 μmol l^{-1} R59022 for 10 min failed to influence the resting level of DG kinase activity (data not shown); U46619-induced activation was inhibited (73.9 ± 6.03 c.p.m. mg wet weight tissue^{-1}; $n=5$) in normal PSS. However, inhibitory effect of R59022 in HG-PSS was not detected. On the other hand, treatment with 3 μmol l^{-1} stemphone did not influence resting DG kinase activity; however, U46619-induced activation was inhibited in both normal and HG-PSS (63.7 ± 3.00 and 45.2 ± 4.79 c.p.m. mg wet weight tissue^{-1}, respectively) ($n=5$). The inhibitory effects of stemphone were greater than those of R59022.

The association of PKC, with respect to regulatory influence, with DG kinase activity was examined. A calcium-dependent PKC inhibitor, Gö6976 (Martiny-Baron *et al*., 1993), and a calcium-independent PKC inhibitor, Rottlerin (Gschwendt *et al*., 1994), were utilized. Each concentration was decided from preliminary trials as a sufficient concentration without induced nonspecific effects. Pretreatment with 1 μmol l^{-1} Gö6976 significantly reduced U46619-induced DG kinase activation exclusively in normal PSS (63.0 ± 2.75 c.p.m. mg wet weight tissue^{-1}; $n=5$). Values under high glucose conditions were unaffected. Rather, Rottlerin significantly reduced DG kinase activation only in HG-PSS (114.6 ± 6.13 c.p.m. mg wet weight tissue^{-1}; $n=5$).

Discussion

Our results indicated that the novel DG kinase inhibitor, stemphone, selectively inhibited mouse portal vein contraction. Furthermore, these data suggested the possibility that this compound alleviated hyperportal vein contraction under high glucose conditions, as in diabetes.

In this study, typical three types of vascular tissues were utilized. The agonist TXA_2 analogue, U46619, was employed

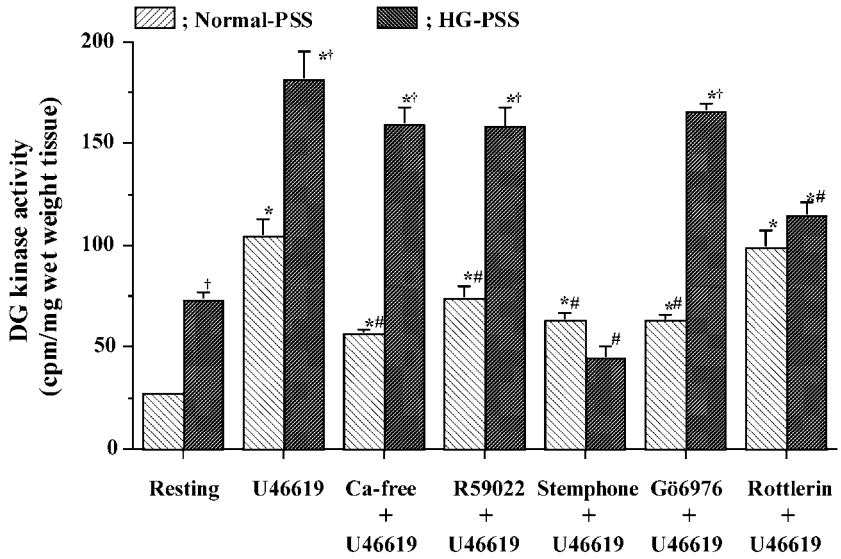


Figure 9 Response of DG kinase activity in U46619-treated mice PV. [^{32}P]Pi and dioctanoyl-glycerol prelabeled tissues were preincubated in normal (bright column) and HG-PSS (dark column) at 37°C for 30 min. These samples were exposed to $CaCl_2$-replaced PSS (Ca free), 3 μM R59022 (R59022), 3 μM stemphone (Stemphone), 1 μM Gö6976 (Gö6976) or 1 μM Rottlerin (Rottlerin) for 10 min; subsequently, 30 nM U46619 was added for 5 min. These treatments were terminated and DG kinase activity was measured as described in 'Methods'. Results are expressed as c.p.m. mg wet weight tissue^{-1}. Each value represents the mean ± s.e.m. of at least five independent determinations. $^{*}P<0.01$ vs resting and $^{\#}P<0.01$ vs U46619 alone. $^{\dagger}P<0.05$ vs normal PSS.

consequent to the submaximal contraction in each tissue induced by this compound (Imura *et al*., 1988; Stein & Trachte, 1989; Paul *et al*., 2000). Moreover, it was previously confirmed that norepinephrine (NE) and prostaglandin $F_{2\alpha}$ also induced contractile responses (Nobe *et al*., 2003b). Inhibitory effects of the stemphone were also detected in these agonists-induced responses (data not shown) as similar as in U46619-induced response.

Inhibitory effects of stemphone and R59022 were compared on U46619-induced vascular contractions. On the basis of these trials, R59022 inhibited contractile response in both aorta and coronary artery; however, only a slight reduction was detected in portal vein (Figures 1–4). Rather, stemphone strongly inhibited U46619-induced contraction exclusively in mouse portal vein (Figures 1–4). Results that DG kinase activity in extracted fraction was selectively inhibited by stemphone without affecting by R59022 (Table 1) also supported the possibility of the stemphone selectivity in portal vein contraction. These results demonstrated the selectivity of stemphone in relation to contraction in mouse portal vein. A portal vein-specific reagent has not been documented. Consequently, we hypothesized that this compound might be the initial candidate in terms of a medication for treatment of hyperportal contraction. However, it is believed that portal blood pressure is enhanced under abnormal conditions, such as in the high glucose condition in diabetes (Petrides *et al*., 1992; Bomzon *et al*., 2001). High glucose-induced enhancement of contraction in aorta and portal vein was also described previously (Nobe *et al*., 2003b, c).

On the basis of this point, it appeared that the inhibitory effect on portal vein contraction should be evident under high glucose conditions in a manner similar to that under normal glucose conditions. The high glucose condition was employed in the form of a similar range of blood glucose levels in normal and diabetic mouse portal veins. Portal blood glucose level in the resting state was 9.58 ± 0.74 mM ($n=5$) in normal mouse; this value was significantly higher than that in aorta as the blood had recently passed through digestive organs. In addition, blood glucose level was elevated significantly in diabetic (*ob*/*ob*) mice (22.07 ± 0.47 mM; $n=5$) (Nobe *et al*., 2003b). In this study, U46619-induced contractile response was significantly enhanced in three types of vascular tissue (Figures 1–7). These results indicated the possibility of vascular responsibility in terms of enhancement in diabetes.

Under the high glucose condition, addition of R59022 reduced contraction in aorta and coronary artery in a manner similar to those responses observed under normal conditions (Figures 1–4). Treatment with stemphone significantly reduced high glucose-induced enhancement of contraction only in portal vein (Figures 5–7). This inhibitory effect was larger than that in normal PSS. Among the three parameters, stemphone reduced the increases in amplitude and ON-time (Figures 6 and 7), whereas frequency was unaffected (Figure 7). Amplitude is representative of the volume of blood supply to the liver and ON-time is indicative of portal pressure; consequently, these results suggested that this compound reduced hyperportal pressure in diabetes and other vascular diseases. In general, overcontraction of portal vein was evident in hepatic cirrhosis and/or diabetes (Bomzon *et al*., 2001). These diseases induced hyperpressure of the portal vein, subsequently leading to varix in the worst-case scenario (McCormick *et al*., 1994). The critical care of varix has not been established. Therefore, selective inhibition of hyperportal contraction may alleviate a serious situation. The present study suggests that the novel DG kinase inhibitor, stemphone, may serve as a medication or fundamental compound of this disease. Findings that stemphone did not alter the value of frequency is preferable for a medicinal of this type due to the essential nature of the spontaneous rhythmic contraction relative to the vascular system. Similar results were detected under NE-induced

contraction in mouse portal vein (data not shown). It indicated that the stemphone may be an effective tool for general physiological contraction in portal vein.

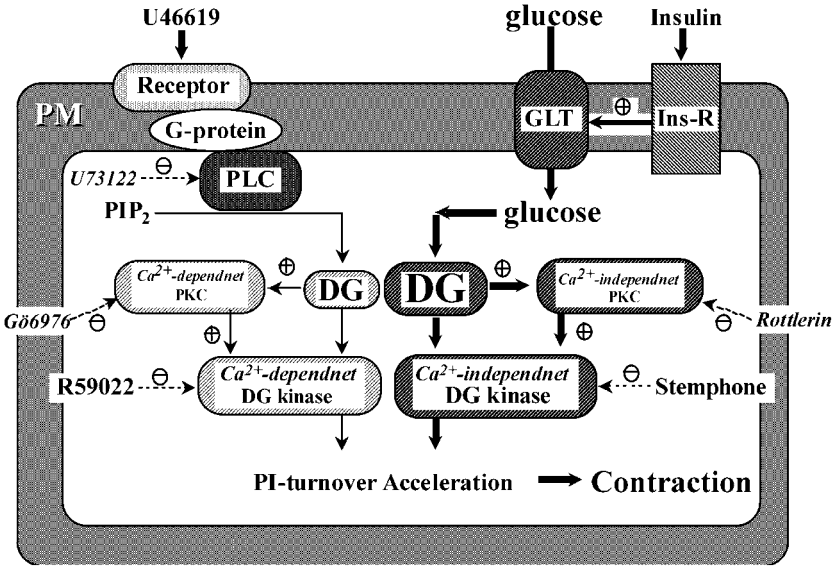
Why did stemphone reduce contractile response solely in portal vein? Subsequently, this point was examined. It was initially thought that the selectivity of stemphone with respect to portal vein contraction was derived from selectivity to DG kinase isoforms. In mouse portal vein, expression of DG kinase isoforms has not been evaluated, because DG kinase antibodies, which are important biochemical tools, could not be established in mouse. The present study assessed mechanisms of DG kinase inhibition by stemphone under high glucose conditions *via* pharmacological means.

DG level, as a specific DG kinase substrate, and its origin were measured (Figure 8). Under high glucose conditions, total mass of DG was enhanced. As this enhancement was not affected by PLC inhibitor, it was indicated that enhancement of DG level was not a result of hydrolysis of phosphatidylinositols. We previously reported that enhanced DG level under high glucose conditions was derived from incorporated glucose *via de novo* synthesis in vascular tissue (Nobe *et al*., 2003c). Enhancement of [^{14}C]DG synthesis was detected in the presence of [^{14}C]glucose in HG-PSS. Similar mechanisms, occurring in various cell types, have been postulated by other investigators (Lee *et al*., 1989; Rossi *et al*., 1991), which supported our hypothesis.

To detect a direct effect of stemphone on cellular DG kinase activation, total activity was measured employing a tissue DG kinase assay system. DiC8 served as an extracellular substrate (Nobe *et al*., 1994). DG kinase activity was increased by U46619 treatment; furthermore, both resting and U46619-treated tissue activity was significantly enhanced under high glucose conditions (Figure 9). These changes were well correlated with changes in total mass of DG (Figure 8). DG kinase activation under high glucose conditions accelerated the total PI turnover cycle. This phenomenon might lead to the enhancement of vascular contraction (Abebe & MacLeod, 1991; 1992).

In calcium-free PSS, DG kinase activation was inhibited only under normal glucose conditions (Figure 9). Activated DG kinase under high glucose conditions was unaltered, which suggested that activated isoforms of DG kinase under normal and high glucose conditions were distinguishable. We hypothesized that a calcium-independent DG kinase isoform was activated under high glucose conditions. Enhanced DG kinase activity under high glucose conditions was inhibited by only stemphone (Figure 9). Moreover, this inhibitory effect was detected under calcium-replaced high glucose conditions (data not shown). These findings also supported our hypothesis. As a calcium-independent DG kinase, Class-II DG kinase isoforms (DG kinase-δ, -η) were suggested (Kanoh *et al*., 1996). Although stemphone might associate with the DG kinase isoforms, critical evidence has not been detected. In order to elucidate the regulatory mechanisms governing DG kinase isoforms, association of PKC with DG kinase activity under high glucose conditions was investigated on the basis of our previous description with regard to the activation of DG kinase by activated PKC (Nobe *et al*., 1995). This feedback mechanism induces a reduction in DG level, which leads to the reduction of activation signal in PKC. Therefore, PKC may regulate the level of its own activator.

Subsequently, the effects of PKC inhibitors on U46619-induced DG kinase activation under both glucose conditions were measured (Figure 8) (Wakino *et al*., 2001). Calcium-dependent PKC inhibitor (Gö6976) reduced DG kinase activation solely in normal PSS; furthermore, calcium-independent PKC inhibitor (Rottlerin) reduced DG kinase activation in high glucose PSS. Effects of these inhibitors were critically distinguished; this finding indicated that activation of PKC type differed under each glucose condition. These differences might be attributable to the origins of the DG molecule, that is, DG was formed by distinct pathways in normal and high glucose PSS. Distinct synthetic pathways produce different molecular DG species (different acyl-chain components) (Marignani *et al*., 1996).



Scheme 1 Target of stemphone on U46619-induced activation of intracellular signaling system under high glucose conditions. Abbreviations used: DG, diacylglycerol; GLT, glucose transporter; Ins-R, insulin receptor; PIP_2, phosphatidylinositol-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; Plus marks, activation and formation; Minus marks, inhibition.

Under normal glucose conditions, hydrolysis of phosphatidylinositol generates physiologically active DG, that is, stearoyl-arachidonyl-glycerol (Walsh *et al*., 1995; Deacon *et al*., 2002). It may activate calcium-dependent PKC followed by calcium-dependent DG kinase (Class-I). We believed that the general DG kinase inhibitor, R59022, inhibited activation of DG kinase. Rather, DG derived from incorporated glucose contains a variety of acyl-chains in high glucose PSS (Thomas *et al*., 1994). DG may activate calcium-independent PKC and DG kinase (Class-II). Stemphone might inhibit this form of DG kinase. Therefore, stemphone selectively inhibited portal vein contraction under high glucose conditions (Scheme 1). The activation of PKC in vascular smooth muscle under high glucose conditions has been described in several reports (Ishii *et al*., 1998; Srivastava, 2002). PKC mediated DG and/or fatty acids formation. These reports supported our hypothesis. However, in other vascular cell types, PKC activity under high glucose conditions was independent of the *de novo* synthesis of DG (Du *et al*., 2001). On the basis of these opposing findings, a consistent argument could not be established. This phenomenon may depend on the stage of diabetes and/or the species of vascular tissue including PKC isoform distribution.

In conclusion, the novel DG kinase inhibitor, stemphone, displays selectivity with respect to enhancement of portal vein contraction under high glucose conditions. This substance may mediate inhibition of calcium-independent DG kinase activation, which was activated by both DG derived from incorporated glucose and calcium-independent PKC activation. The present study noted that intracellular DG kinase activation under high glucose conditions would be a novel target in the management of portal vein hyperpressure. A selective inhibitor of hyperportal contraction was established; consequently, we are optimistic that this compound, stemphone, would prove to be an effective medication for treatment of hyperportal pressure in diabetes. On the other hand, stemphone may provide the basic structure for novel medicines applicable to these diseases.

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