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Relaxation Effect of Synthetic Ceramide Analogues in Cat Esophageal Smooth Muscle Cells

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Ceramide has emerged as a novel second messenger for intracellular signalling. It is produced from sphingomyelin and is involved in the control of cell differntiation, proliferation, and apoptosis. C_2-ceramide, short chain ceramide, plays a role in mediating contraction of cat esophageal smooth muscle cells. We examined the effect of synthesized ceramide analogues on the C_2-ceramide and ACh-induced contraction in esophageal smooth muscle cells isolated with collagenase. CY3523, CY3525, or CY3723 inhibited C_2-ceramide induced contraction, in a time dependent manne. Each analogue also inhibited the contraction in concentration dependent manners. CY 3523, CY 3525, and CY 3723 had no effect to the contraction induced by PMA. The inhibition with CY3523, CY3525 and CY3723 on the C_2-ceramide induced contraction was recovered by PMA. These analogues decreased the density of MAPK bands (p44/42 or p38) in the western blot. These results suggest that ceramide analogues can inhibit C_2-ceramide induced contraction via PKC and MAPK dependent pathway.

Key Words: Mitogen-activated protein kinase, Smooth muscle, Protein kinase C, C_2-ceramide

INTRODUCTION

Sphingolipids such as ceramide and sphingosine have shown as novel intracellular signal mediators. The ceramide is important for the intracellular signaling pathways responding to various cytokines and stress (Hannun, 1996). It is known that ceramide involved in cellular signaling is produced from sphingomyelin (SM) by the action of endogenous neutral and acid (Okazaki, 1989) sphingomyelinase (SMase) (Schwandner et al, 1998), which catalizes the breakdown of sphingomyelin to form ceramide and phosphrylcholine (Hannun, 1996) or by *de novo* synthesis (Bose et al, 1995).

Ceramide, released as a consequence of sphingomyelin pathway, is thought to play a role in fundamental process such as cell proliferation, membrane-receptor function, oncogenesis, and immune inflammatory responses. It has been shown that ceramide induced a sustained contraction of smooth muscle cells through a pathway that involves the activation of mitogen-activated protein kinase (MAPK) (Sbrissa et al, 1997). Thus ceramide could be an important mediator of smooth muscle cell contraction.

C_2 ceramide, the permeable short-chain ceramide, induced the contraction of feline esophageal smooth muscle cells, and that the contraction can be mediated via a PKC- and MAPK- pathway (Shin et al, 2002). Acethylcholine (ACh) released from the postganglionic parasympathetic nerve, activates the muscarinic receptors. Muscarinic receptors transduce ACh signals by activating guanine nucleotide-binding proteins (G proteins) to regulate generation of second messengers and Ca^{2+} signaling (Hosey et al., 1996). It has been shown that esophageal smooth muscle cell contraction in response to maximum effective dose of ACh is mediaed by PKC-dependent pathway.

ACh-induced contraction of circular muscle of the esophagus is independent on calmodulin and mediated through a protein kinase C (PKC)-dependent pathway (Sohn et al, 1995), involving a calcium-insensitive PKC-ε (Sohn et al, 1997) In this pathway, calcium is required for activation of phospholipases and production of the second messengers diacylglycerol (DAG) and arachidonic acid (AA) (Sohn et al, 1994). When the second messengers are present, contraction can proceed even in the absence intracellular calcium (Sohn et al, 1995).

PKC is present in the cell cytoplasm and upon agonist stimulation, rapidly translocates to the particulate or membrane fraction observed by western immunoblot analysis and immunofluorescence studies (Crabos et al, 1992; Sohn et al, 1997).

Mitogen activated protein kinases (MAPKs) including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 are important to the transduction of extracellular signals from the membrane to the nucleus. The ERKs are stimulated by mitogenic factors such as growth factors and

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ABBREVIATIONS: C_2, C_2-ceramide; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; myr-PKC, N-myristoylated peptides; SMC, smooth muscle cells; PMA, Phorbol 12 myristate 13-acetate.

phorbol esters (Maris et al, 1996) by MEK1, a dual-specificity threonine/tyrosine kinase, that phosphorylates ERK at a signature sequence T-E-Y (Powis, 1995; Powis et al, 1995). The p44/42 MAPK pathway consists of a protein kinase cascade linking growth and differentiation signals with transcription in the nucleus. Activated p44/42 MAP Kinase translocates to the nucleus and activates transcription by phosphorylation of transcription factors such as Elk-1 and Stat3.

In this study, we investigated the inhibitory effect of new synthetic ceramide analogues on the contractions induced by C_2-ceramide and ACh in feline esophagus smooth muscle cells.

METHODS

Materials

C_2-ceramide was purchased from Tocris Cookson Ltd. (Avonmouth Bristol, UK); SDS sample buffer from Owl scientific, Inc (Woburn, MA); PBS from Boehringer Mannheim (Indianapolis, IN); acethylcholine (ACh), Phorbol 12 myristate 13-acetate (PMA), anisomycin, HEPES, collagenase type F, ammonium persulfate, ponseu S, bovine serum albumin, leupeptin, aprotinin, β-mercaptoethanol, EGTA, EDTA, and other reagents were purchased from Sigma (St Louis, MO). Syntheic ceramide analogues were obtained from the Department of Medicine and Organic chemistry, College of Pharmacy, Chung-Ang University.

Preparation of dispersed single cells

Single muscle cells were isolated as previously described (Sohn et al., 1995). Muscle strips were incubated in Krebs solution containing 10 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES), 0.09 mg/ml soybean trypsin inhibitor, 10% bovine serum albumin (BSA) and 0.1% collagenase (Type II, Worthington Biochemicals. Freehold, NJ) and equilibrated with 95% O_2～5% CO_2 to maintain pH 7.45±0.05 at 31°C. The composition of the solution was as follow : (in mM) 118 NaCl, 4.8 KCl, 1.2 $MgSO_4$, 24 $NaHCO_3$, 1.2 KH_2PO_4, 11 glucose, and 2.5 $CaCl_2$. After incubation, the tissue was washed with 50 ml enzyme-free solution on 360 μm Nitex filter and reincubated in enzyme-free solution at 31°C and gassed with 95% O_2/5% CO_2 and the cells were allowed disperse spontaneously for 10～20 min. Suspensions of single muscle cells were harvested by filtration through 500 μm Nitex mesh. Throughout the entire procedure, it was optimally agitated the fluid to avoid cell contraction in response to mechanical stress.

Measurement of contraction

Contraction of isolated muscle cells was measured by scanning micrometry. An aliquot of cell suspension containing 10^4 muscle cells/ml was added to Krebs solution containing the test agents. The reaction was terminated by addition of formalin to final concentration of 5%. A drop of the cell-suspension was placed on a glass slide and covered by a cover slip and the edges were sealed with clear nail enamel to prevent evaporation. The length of muscle cells treated with a contractile agent was measured at random by scanning micrometry, phase contrast microscope (model ULWCD 0.30 Olympus, Japan) and digital closed-circuit video camera (CCD color camera, Toshiba, Japan) connected to a Macintosh computer (Apple, Cupertino, CA) with a software program, NIH Image 1.57 (National Institutes of Health, Bethesda, MD) compared with length of untreated cells (control length of smooth muscle cells, 65～75 μm). Contraction was expressed as the percent decrease in mean cell length from control. The time course of contraction with agonists consists of peak contraction followed by a lower sustained plateau.

Phospho-MAPK western blots

Previously frozen samples were homogenized in a buffer a containing 20 mM Tris, 0.5 mM EDTA, 0.5 mM EGTA, 10 (g/ml leupeptin, 10 μg/ml aprotinin, 10 mM β-mercaptoethanol (pH 7.5). Samples homogenates were then centrifuged for 10 min at 4°C, and the supernatants were collected. Aliquots were subjected to electrophoresis on 10% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane. Membranes were blocked in a PBS containing 5% dry milk for 2h before an overnight incubation in a PBS solution containing 0.1% BSA and a phosphospecific p44/p42 MAPK (Tyr-202/Tyr-204) or p38 MAPK antibody. Membranes were washed using PBS containing 0.05% tween 20 and then incubated with horseradish peroxidase-conjugated secondary antibody (dilution 1 : 2000) for 1h. Immunoreactive bands were visualized by enhanced chemiluminescence. Developed films from enhanced chemiluminescence were scanned and analyzed using NIH Image.

Data analysis

The data are expressed as the means±S.E.M. and the statistical differences between means were determined by Student's t test. Differences between multiple groups were tested using anlysis of variance (ANOVA) for repeated measures.

RESULTS

Inhibition of C_2-ceramide and ACh-induced contraction of feline esophagus smooth muscle cells by CY3523, CY3525 and CY3723

Our studies have shown that CY3523, CY3525 and CY3723 (10^{-5} M) had an inhibitory effect on C_2-ceramide induced contraction of feline esophageal smooth muscle cells. But other analogues, CY3125, CY3225 and CY3325 had not. CY3523, CY3525 and CY3723 (10^{-5} M) inhibited the contraction induced by C_2-ceramide, which was peaked at 5 min (27.39±3.5% inhibition) and the inhibition was sustained at a plateau (26.87±7.2%) at 15 min (Fig. 1).

The inhibitory action of CY3523, CY3525 and CY3723 was concentration-dependent. Isolated smooth muscle cells were stimulated for 5 min with 10^{-8} M～10^{-5} M. The maximal response was seen at 10^{-5} M. CY3723 showed the strongest inhibition. C_2-ceramide induced smooth muscle cell contraction was inhibited to 36.9%, and ACh induced contraction was inhibited to 34.4% by CY3723 (Fig. 1). The effect of CY3723 was concentration dependent and maximal effect was showed at 10^{-5} M. CY3523 showed 35.1% inhibition to C_2-ceramide induced contraction and 26.8%

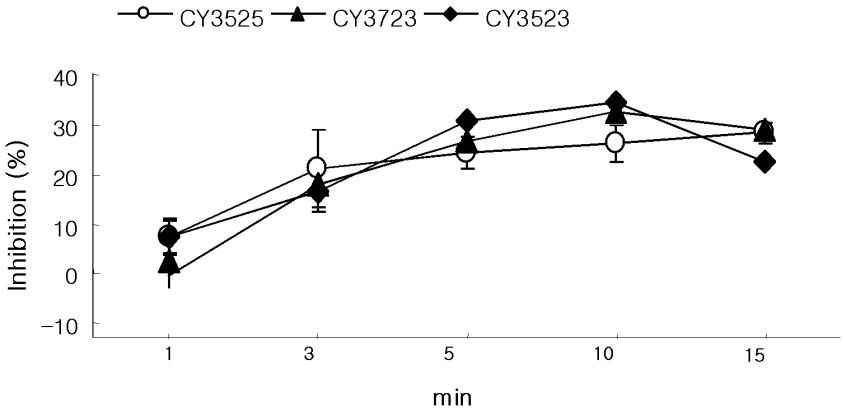

Fig. 1. CY3523, CY3525, CY3723 inhibited C2 ceramide induced contraction in a time-dependent manner. Isolated cells were incubated in ceramide amalogues (10^{-5} M) for indicated times. Data are means±S.E. of 4 experiments (ANOVA , $p<0.001$).

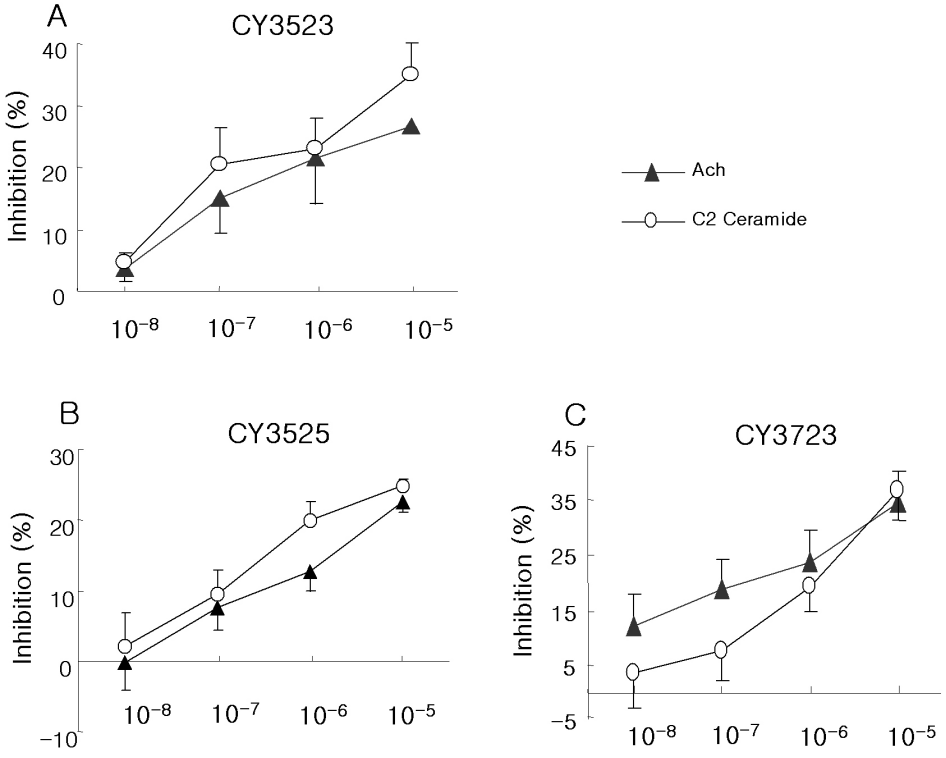

Fig. 2. Dose-dependent contractile inhibition response of smooth muscle cells from feline esophagus to CY3523 (A), CY3525 (B), or CY3723 (C). Isolated smooth muscle cells were incubated in CY3523 for 5min for indicated dose, then stimulated for 30 s with ACh (10^{-9} M) and C2 ceramide (10^{-7} M). Data are means± S.E. of 4 experiments (ANOVA, $p<0.001$).

inhibition to ACh induced contraction (Fig. 2). CY3525 showed 24.9% inhibition to C_2- ceramide induced contractio n, and 22.7% inhibition to ACh (Fig. 2).

CY3523, CY3525, and CY3723 had no effect on phorbol 12 myristate 13-acetate (PMA) induced contraction

Phorbol 12 myristate 13-acetate (PMA), is known as a PKC activator. In this study, PMA induced 10～14% contraction on feline esophageal smooth muscle cell (100 nM, incubated for 10 min). Isolated smooth muscle cells were incubated with CY3523, CY3525 and CY3723 (10^{-5} M) for 5 min (Fig. 3). CY3523 had no effect on PMA induced contraction. CY3525 or CY3723 showed only 5.4% or 5.8% inhibition, respectively.

Anisomycin induces smooth muscle contraction and the effect was reduced by CY3523, CY3525 and CY3723

Anisomycin is known to activate MAPK. In this study, anisomycin showed 10～12% smooth muscle cell contraction (500ng/ml, incubated for 1 min). CY3523 inhibited the anisomycin-induced contraction to 8.1%. CY3525 or CY3723 showed 15.8% or 13.4% inhibition, respectively (Fig. 4).

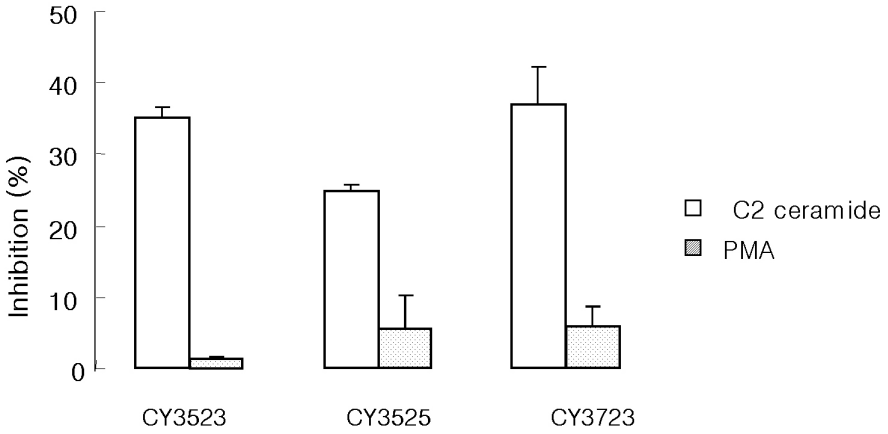

Fig. 3. Effect of CY3523, CY3525, and CY3723 on PMA induced contraction. Isolated smooth muscle cells were treated with CY3523, CY3525 and CY3723 (10^{-5} M, incubated 5 min). No inhibitory effect was shown with phorbol 12 myristate 13-acetate (PMA), PKC activator.

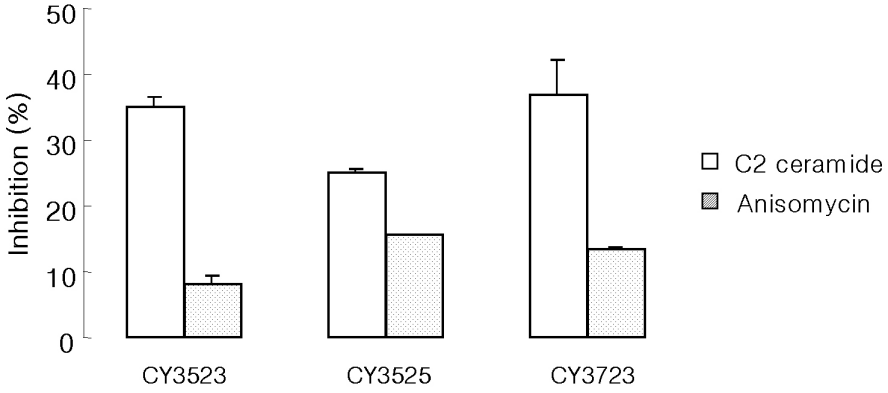

Fig. 4. Effect of CY3523, CY3525, and CY3723 on anisomycin induced contraction. Anisomycin (p38 MAPK activator) produced contraction. CY3523, CY3525 and CY3723 had a little contractile inhibition of anisomycin induced contraction.

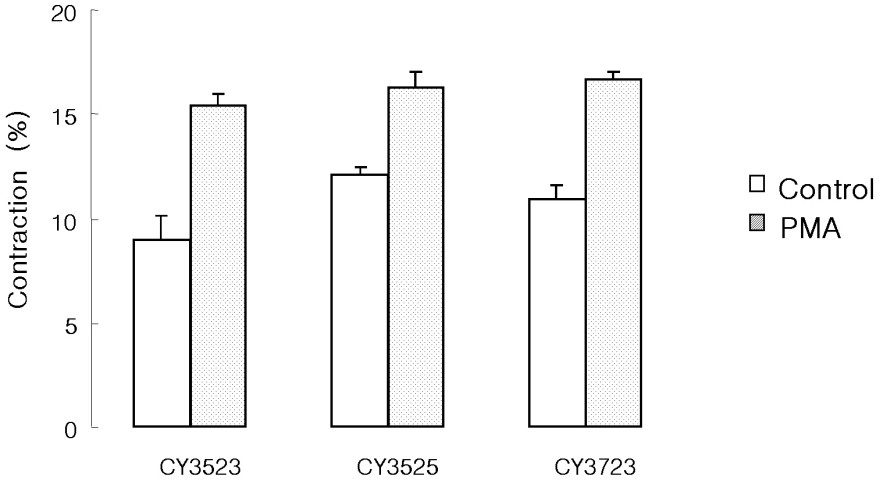

Fig. 5. The effect of phorbol 12 myristate 13-acetate (PMA) on CY3523, CY3525, CY3723. Inhibition of C_2-ceramide induced contraction by ceramide anaogues was recovered with PMA (100 nM, PKC activator) treatment. Data are means±S.E. of 4 experiments (student *t*-test, $p < 0.01$).

C_2-ceramide induced contraction was recovered by the treatment with PMA.

Isolated cells were incubated with CY3523, CY3525 and CY3723, 10^{-5} M, for 5 min and C_2-ceramide was added to these cells. Then PMA was treated and incubated for 5 min. The inhibition of C_2-ceramide induced contraction was recovered by PMA (Fig. 5). The contraction rate reduced to 10% was raised to 16% and this rate is similar to the contraction rate induced by PMA only.

The effect on the 42/ 44 MAPK activity of the analogues

Western blot analysis of homogenates derived from esophageal smooth muscle cells using antibodies to p42/44 MAPK showed that ceramide analogues inhibited the activation of p42/44 MAPK. Cells were cultured with CY3523, CY3525, and CY3723, and then stimulated with ceramide,

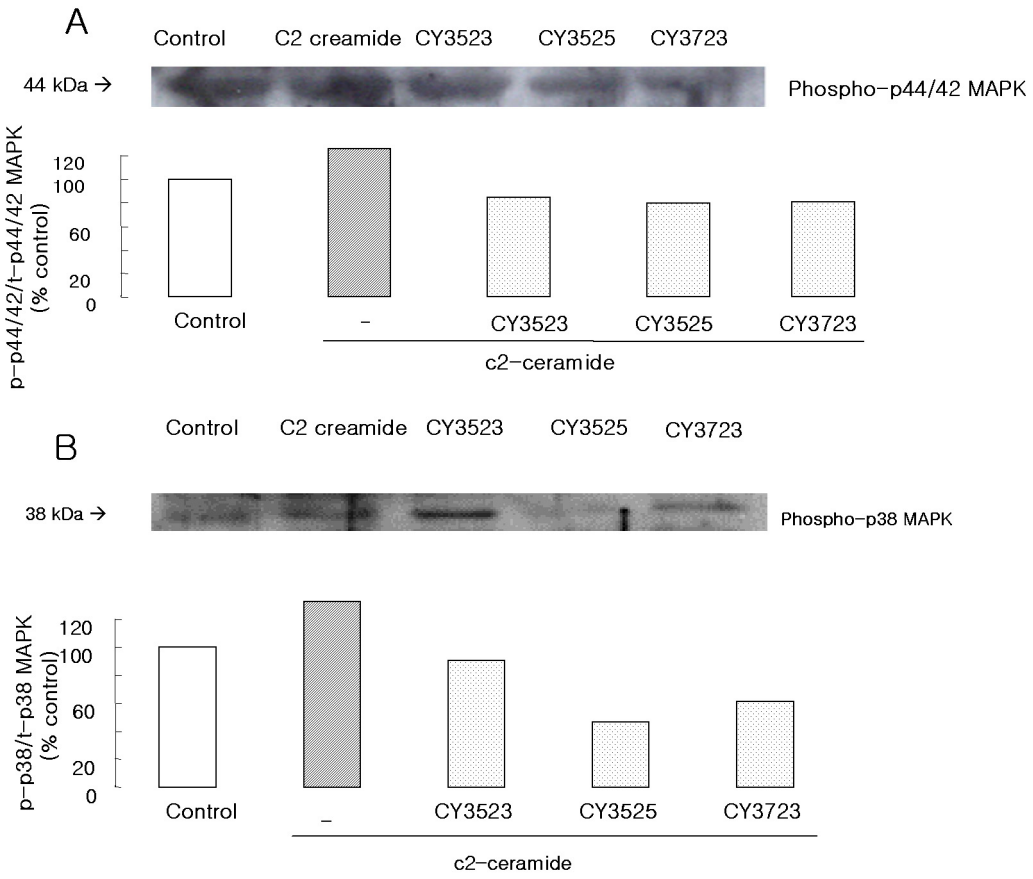

Fig. 6. The effects on the analogues of MAPK activation. (A) Phosphorylated p44/42 MAPK was detected with anti-phospho p44/42 MAPK antibody. In lower panel, the each level on the control displayed as the ratio of phospho- p44/42 divided by total p44/42 MAPK density (n=3). (B) p38 MAPK was inhibited by synthetic ceramide analogues. Phosphorylated p38 MAPK was detected with anti-phospho p38 MAPK antibody. In lower panel, the each level on the control displayed as the ratio of phospho-p38 density divided by total p38 MAPK density (n=3).

and immunodetection of p42/44 MAPK was performed. As shown in Fig 6A, the densitiy of detection band of p42/44 MAPK was decreased when treated with ceramide analogues.

C_2-ceramide induced contraction in cat esophageal smooth muscle cells was known to be mediated by PKC and MAPK activated pathway. It is possible that the inhibitory effect of ceramide analogues can occur by the inhibition of PKC and MAPK activated pathway.

Cells were precultured with CY3523, CY3525, and CY3723, and then stimulated with ceramide, and immunodetection of p38 MAPK was performed. As shown in Fig 6B, p38 MAPK band density was increased when stimulated with ceramide. CY3523, CY3525, or CY3723 had inhibitory effect on the band density of p38 MAPK. It is possible that p38 MAPK is responsible for the contraction induced by C_2-ceramide.

DISCUSSION

In this experiments, the synthetic ceramide analogues showed the inhibition on the contraction induced by C_2- ceramide as well as ACh. In contrast, the analogues had no effect on phorbol 12 myristate 13-acetate (PMA) induced contraction. PMA is known as an allosteric PKC activator like DAG. The allosteric activator evokes this intramolecular control by inducing a conformational change in the molecule that might liberate the substrate binding domain from the pseudosubstrate, thereby activating the enzyme.

In this study, the inhibition of C_2-ceramide induced contraction was recovered by PMA. This result represents that the inhibitory effect of synthetic ceramide analogues to the C_2-ceramide induced contraction is evoked by inhibition of upstream pathway of PKC.

It is known that C_2-ceramide and ACh induced contraction is mediated via PKC dependent pathway, ACh-induced contraction of esophageal smooth muscle cell is independent on calmodulin and is mediated through a protein kinase C (PKC)-dependent pathway (Sohn et al, 1995), involving a calcium-insensitive PKC-ε. (Sohn et al, 1997). Activated CaM-dependent pathways inhibit PKC-dependent pathways, this switch mechanism might be regulated by Ca^{2+} in the LES (Sohn et al, 2003). In the esophageal smooth muscle cells, ACh stimulates receptor-medated activation of PLA_2, PLD, and phosphatidyl choline specifeic PLC, and produces AA and DAG. AA and DAG activate PKC-ε DAG synergistically and calcium-independently.

Western blot anlysis showed that p42/44 MAPK activity stimulated by C_2-ceramide was inhibited by treatment with CY3523, CY3525 and CY3723. PKC-dependent contraction in ESO and LES is mediated by the following two distinct MAPK pathways: ERK1/2 and HSP 27-linked p38 MAPK (Cao et al, 2003). p42/44 MAPK was known to induce contraction of smooth muscle cells via activation of PKC by ceramide. C2 ceramide produced the contraction of smooth muscle cells of cat oesophagus. The Contraction is mediated by PKC-epsilon, resulting in the activation of p42/44 MAPK (Shin et al, 2007).

It is possible that the inhibition of C_2 induced contraction in feline esophageal smooth muscle cells evoked by CY3523, CY3525 and CY 3723 was mediated by the inhibition of PKC and p42/44 MAPK dependent contraction pathway. Ceramidase is involved in the inactivation of ceramide. It is possible that CY3523, CY3525 and CY3723 have a positive effect on ceramidase activity, and activated ceramidase metabolize ceramide to be inactive. In result, ceramide induced smooth muscle cell contraction may is inhibited.

Anisomycin is known to activate MAPK. In this study, the each analogue inhibited the ansomycin induced contraction may suggest that the contraction is mediated by the increase of the MAPK activity.

Western bolt analysis showed that the density of p38MAPK was increased when stimulated with C_2- ceramide. It was known that the smooth muscle cells contraction induced by C_2-ceramide is mediated by MAPK. With this result, it is possible that p38 MAPK may be involved in this contractile pathway, but is not the main subtype of the enzyme acting in contractile response.

CY3523 had a different effect on the activation of p38 MAPK. CY3523 increased the band density of p38 MAPK in western blot anlysis. In contrast, ceramide analogues involving CY3523, had an inhibitory effect on the activation of p42/44 MAPK. The inhibition of p42/44 activation may be induced by the upstream inhibition of PKC activation involved in this contractile response. CY3523 activated p38 MAPK, but the enzyme is not involved in the main stream of C_2-ceramide induced contractile reponse, so it had no effect on the contraction of smooth muscle cells.

In summary, CY3523, CY3525 and CY3723 inhibited C_2-ceramide and ACh induced contraction, which is mediated by PKC and MAPK involved pathway. The inhibitory effect to C_2-ceramide induced contraction by CY3523, CY3525 and CY3723 is possibly induced by the activation of ceramidase by synthetic ceramide analogues.

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