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Effect of Ceramide on K_{Ca} Channel Activity and Vascular Tone in Coronary Arteries

Pin-Lan Li, David X. Zhang, Ai-Ping Zou, William B. Campbell

Abstract—A sphingomyelin metabolite, ceramide, serves as a second messenger in a variety of mammalian cells. Little is known regarding the production and actions of this novel intracellular signaling lipid molecule in the vasculature. The present study was designed to test the hypothesis that a ceramide-mediated signaling pathway is present in coronary arterial smooth muscle and that ceramide serves as an inhibitor of the large-conductance Ca^{2+}-activated potassium (K_{Ca}) channels and mediates vasoconstriction in coronary circulation. We found that C_2-ceramide produced a concentration-dependent decrease in K_{Ca} channel activity in vascular smooth muscle cells from small bovine coronary arteries. The average channel activity of the K_{Ca} channels in cell-attached patches decreased from 0.046±0.01 to 0.008±0.001 at a C_2-ceramide concentration of 10 μmol/L. In inside-out patches, C_2-ceramide (1 μmol/L) reduced the average channel activity of the K_{Ca} channels from 0.06±0.007 to 0.016±0.004. Dithiothreitol, an inhibitor of acidic sphingomyelinase (1 mmol/L), increased the average channel activity of the K_{Ca} channels in cell-attached patches from 0.05±0.02 of control to 0.26±0.04, a 5-fold increase that was reversed by addition of 1 μmol/L ceramide. Glutathione, an inhibitor of neutral sphingomyelinase, was without effect. C_2-ceramide significantly reduced the diameter of isolated perfused small coronary arteries in a concentration-dependent manner. Addition of 1 μmol/L C_2-ceramide decreased average arterial diameter by 28%. When ^{14}C-sphingomyelin was incubated with coronary arterial homogenates at pH 7.4 and pH 5.0, ^{14}C–choline phosphate and ceramide were produced. The conversion rates of ^{14}C-sphingomyelin into ^{14}C–choline phosphate and ceramide were 65.1±1.0 fmol/min per milligram protein at pH 7.4 and 114.6±8.3 fmol/min per milligram protein at pH 5.0. We conclude that both acidic and neutral sphingomyelinases are present in the bovine coronary arteries and that ceramide inactivates the K_{Ca} channel in arterial smooth muscle cells and hence exerts a tonic vasoconstrictor action in coronary microcirculation. (***Hypertension*. 1999;33:1441-1446.**)

Key Words: sphingomyelinase ■ sphingolipid ■ muscle, smooth, vascular ■ potassium channels ■ heart

Sphingomyelin (SM), a membrane phosphosphingolipid, can be hydrolyzed into ceramide and choline phosphate through sphingomyelinase (SMase) in a variety of mammalian tissues and cells.[1,2] Ceramide serves as an intracellular messenger mediating the effects of a number of extracellular agents or hormones, such as 1α,25-dihydroxyvitamin D_3, tumor necrosis factor-α (TNF-α), interferon-γ, interleukin-1, arachidonate, and brefeldin A.[3–6] Ceramide may play an important role in cell differentiation, apoptosis, inflammation, and eukaryotic stress responses.[1,2,6] A recent study demonstrated that ceramide and SMase cause concentration-dependent relaxation in phenylephrine-contracted endothelium-denuded rat thoracic aortic rings, suggesting that a ceramide-mediated signaling pathway represents a novel mechanism for vasodilation.[7] This ceramide signaling pathway has been proposed to mediate the endothelium-independent vasodilator effect of TNF-α or other cytokines. However, the mechanism of ceramide-induced vasorelaxation remains unknown. More recently, Ferreri et al[8] reported that TNF-α may alter K^+ transport in renal medullary thick ascending limbs, thereby mediating the effects of angiotensin II on tubular intracellular K^+ concentrations. Given that TNF-α markedly stimulates the production of ceramide, the effects of TNF-α on intracellular K^+ concentrations may be associated with the production of ceramide, and the K^+ channel activity on the cell membrane may be influenced by ceramide. With the use of patch clamp technique, ceramide has been demonstrated to inhibit voltage-gated K^+ channel activity through a tyrosine kinase–mediated mechanism.[9] It is unknown whether this ceramide-mediated inhibition of the K^+ channel activity occurs in the vascular smooth muscle cells. If that is the case, ceramide should produce vasoconstriction.

We hypothesize that a ceramide-mediated signaling pathway is present in coronary vascular smooth muscle and that ceramide serves as an inhibitor of the large-conductance Ca^{2+}-activated potassium (K_{Ca}) channels and mediates vasoconstriction in coronary circulation. To test this hypothesis, we determined the effects of exogenous and endogenous ceramide on K_{Ca} channel activity in vascular smooth muscle

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cells isolated from small bovine coronary arteries. Patch clamp studies were performed to determine the effect of C_2-ceramide, a cell-permeant analogue of ceramide, on K_{Ca} channel activity in cell-attached and inside-out patches. The effect of ceramide on vascular tone was determined by using isolated perfused small bovine coronary arteries. The activities of both acidic and neutral SMases in coronary arteries were characterized, and the effects of SMase inhibitors on K_{Ca} channel activity were examined.

Methods

Patch Clamp Study

Bovine hearts were obtained from a local slaughterhouse. Patch clamp recording of the K_{Ca} channel currents was performed as we described previously.[10] The effects of C_2-ceramide on K^+ channel activity were examined in the cell-attached and inside-out patch modes. In these experiments, a 3-minute control recording was obtained at a membrane potential of +40 mV, and the solution in the bath was exchanged with the solution containing C_2-ceramide (0.01 to 10 μmol/L) (n=18 cells from 7 hearts in the cell-attached mode and n=11 cells from 5 hearts in the inside-out mode), and then a second successive 3-minute recording was obtained. To determine the role of endogenous ceramide on K_{Ca} channel activity in coronary arterial smooth muscle cells, dithiothreitol, an inhibitor of acidic SMase (0.01 to 1 mmol/L, n=13 cells from 6 hearts), and glutathione, an inhibitor of neutral SMase (1 to 10 mmol/L, n=8 cells from 7 hearts), were added to the bath solution in cell-attached patches, respectively, after a 3-minute control recording. Then a second successive 3-minute recording was obtained. In some experiments, C_2-ceramide was added to the bath after application of the highest dose of dithiothreitol (1 mmol/L, n=6 cells from 6 hearts). These experiments were designed to examine whether exogenous ceramide reversed the effects of SMase inhibitors. In another group of cells, SM and SMase alone or in combination were added to the bath solution in the inside-out patch mode to determine whether the metabolite of SM by SMase alters K_{Ca} channel activity (n=7 cells from 6 hearts).

Isolated Small Coronary Artery Preparation

Small intramural coronary arteries of bovine hearts were carefully dissected and stored in ice-cold PSS. Segments of small arteries were mounted on glass pipettes in a water-jacketed perfusion chamber as we described previously.[11] The arteries were perfused and bathed with PSS that was equilibrated with 95% O_2/5% CO_2 and maintained at 37°C. This arterial preparation has been shown to have an intact endothelium.[11,12] After the artery was mounted, the outflow cannula was clamped, and the artery was pressurized to 70 mm Hg and equilibrated for 1.5 hours. Internal diameter of the vessel was measured with the use of a video system composed of a stereomicroscope (Leica MZ8), a charge-coupled device camera (KP-MI AU, Hitachi), a video monitor (VM-1221, Hitachi), a video measuring apparatus (VIA-170, Boeckeler Instrument), and a video printer (UP890 MD, Sony). The arterial images were recorded continuously with a videocassette recorder (M-674, Toshiba). The effects of C_2-ceramide on arterial diameters were studied after the vessels were preconstricted by 25% compared with the resting diameter (from 312±18 to 232±28 μm) for 30 to 40 minutes with Bay K8644, a Ca^{2+} channel opener (10 nmol/L). After a sustained contraction by Bay K8644 was obtained, cumulative additions of C_2-ceramide (0.01 to 1 μmol/L) were made every 10 minutes, and the diameters of arteries were recorded and measured (n=8 arteries from 7 hearts).

Preparation of Homogenate of Small Bovine Coronary Arteries

To determine the production of ceramide from coronary arteries, small bovine coronary arteries were dissected as described above. The dissected arteries were cut into very small pieces and homogenized with a glass homogenizer in ice-cold HEPES buffer containing the following (in mmol/L): Na-HEPES 25, EDTA 1, sucrose 255, phenylmethylsulfonyl fluoride 0.1. After centrifugation of the homogenate at 6000*g* for 5 minutes at 4°C, the supernatant containing membrane and cytosolic components, termed homogenates, was aliquoted, frozen in liquid N_2, and stored at −80°C until used.[13]

Assay of SMase Activity

The SMase activity in coronary arterial homogenates was detected as described previously by Liu and Hannun.[14] To determine the activity of neutral SMase, homogenate (100 μg) was added to 50 μL of reaction solution containing Tris-HCl 20 mmol/L (pH 7.5) and EDTA 1 mmol/L and then mixed with 0.01 μCi of [^{14}C-choline]SM in 50 μL of 100 mmol/L Tris-HCl solution (pH 7.4) containing 5 mmol/L $MgCl_2$ and 0.05% Triton X-100. The reaction was performed at 37°C for 60 minutes and terminated by addition of 1.5 mL chloroform/methanol (2:1, vol/vol) and then 0.2 mL H_2O. The reaction mixtures were mixed and centrifuged at 3000 rpm at 4°C (15 minutes) for phase separation. A portion of the upper aqueous phase containing ^{14}C–choline phosphate was collected, and the radioactivity was determined by liquid scintillation counting. The activity of acidic SMase was also determined with the use of [^{14}C-choline]SM as substrate, but the reactions were performed in 100 mmol/L sodium acetate buffer (pH 5.0).

Thin-Layer Chromatography Analysis of SMase Products

To confirm the identity of the SMase product ceramide, thin-layer chromatography (TLC) was performed.[14] Briefly, the lower organic phase of the lipid extraction was collected and separated with the use of silica gel G TLC plates (Whatman, LSD) and a solvent system of chloroform/methanol/25% ammonium hydroxide/water (50:50:1:2). Synthetic SM, C_{18}-ceramide, and sphingosine were used as standards. SM, ceramide, and sphingosine were visualized by iodine staining.

Statistical Analysis

Data are presented as mean±SEM. Significance of differences in mean values within and between multiple groups was examined with an AVOVA for repeated measures followed by a Duncan's post hoc test. Student's *t* test was used to examine significance of difference in 2 groups. $P<0.05$ was considered statistically significant.

Results

Effect of C_2-Ceramide on K_{Ca} Channel Activity of Coronary Arterial Smooth Muscle Cells in Cell-Attached and Inside-Out Patches

In cell-attached and inside-out patch clamp modes, synthetic C_2-ceramide produced a concentration-dependent decrease in K_{Ca} channel activity. Figure 1A and 1C represent an example depicting the effects of C_2-ceramide at a concentration of 10 μmol/L in cell-attached patches and at 1 μmol/L in inside-out patches. The results of these experiments are summarized in Figure 1B and 1D. The average channel activity (NP_O) of the K_{Ca} channels in cell-attached patches was decreased in a concentration-dependent manner. C_2-ceramide at a concentration of 10 μmol/L reduced the NP_O of the K_{Ca} channels in cell-attached patches from 0.046±0.01 to 0.008±0.001, which represents an 82% decrease (Figure 1B). In inside-out patches, 1 μmol/L C_2-ceramide produced a 74% decrease in the NP_O of the K_{Ca} channels.

Effects of Dithiothreitol on K_{Ca} Channel Activity in Cell-Attached Patches

Figure 2A shows representative recordings of single-channel K_{Ca} currents under control conditions, after the addition of

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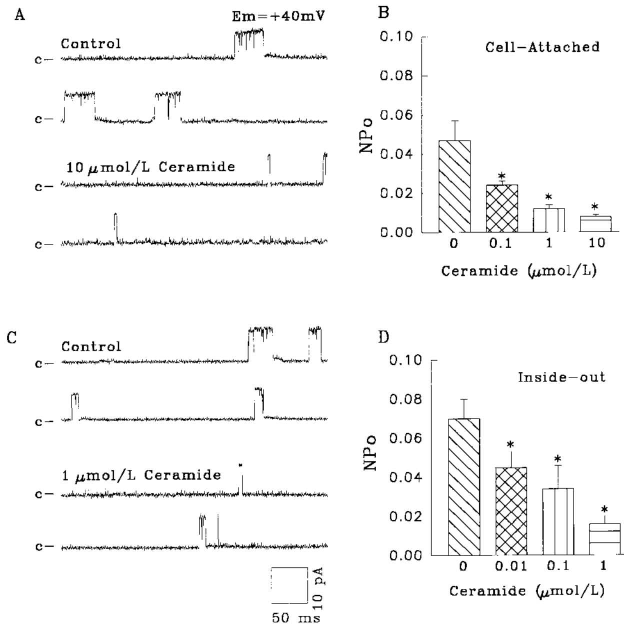

Figure 1. Effect of C_2-ceramide on K_{Ca} channel activity of coronary arterial smooth muscle cells. A, Representative recordings of K_{Ca} channel currents under control conditions and after addition of C_2-ceramide to the bath at a membrane potential (Em) of +40 mV in cell-attached patches. B, Effect of C_2-ceramide on NP_O of K_{Ca} channels in smooth muscle cells. C, Representative recordings of K_{Ca} channel currents in inside-out patches. D, Effect of C_2-ceramide on NP_O of K_{Ca} channels in smooth muscle cells. *Significant difference from control ($P<0.05$).

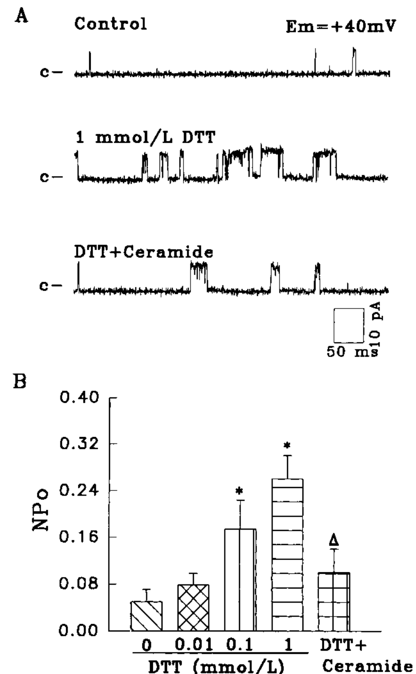

Figure 2. Effect of dithiothreitol (DTT) on K_{Ca} channel activity in cell-attached patches of smooth muscle cells. A, Representative recordings of K_{Ca} channel currents under control conditions and after addition of DTT or C_2-ceramide in the presence of DTT. B, Effect of DTT on NP_O of K_{Ca} channels in smooth muscle cells. *Significant difference from control ($P<0.05$). △Significant difference from the values obtained in the presence of 1 mmol/L DTT.

dithiothreitol, an acidic SMase inhibitor,[14] or after dithiothreitol followed by ceramide. Dithiothreitol markedly increased opening of the K_{Ca} channels. Dithiothreitol (0.01, 0.1, and 1 mmol/L) produced a concentration-dependent increase in the NP_O of the K_{Ca} channels (Figure 2B). A 5-fold increase in K_{Ca} channel activity was observed when 1 mmol/L dithiothreitol was added to the bath. C_2-ceramide (10 μmol/L) significantly attenuated the effects of dithiothreitol (1 mmol/L) on K_{Ca} channel activity. C_2-ceramide (10 μmol/L) decreased the NP_O of the K_{Ca} channels from 0.26±0.05 to 0.1±0.04 in the presence of dithiothreitol.

Effects of Glutathione on K_{Ca} Channel Activity in Cell-Attached Patches

In contrast to the marked effect of dithiothreitol in cell-attached patches, glutathione, a neutral SMase inhibitor, had no effect on K_{Ca} channel activity (Figure 3). The NP_O of these K channels was not significantly altered when even a high concentration of glutathione (10 mmol/L) was added to the bath solution (Figure 3B).

Effects of SM and SMase on K_{Ca} Channel Activity in Inside-Out Patches

Addition of SM to the bath solution slightly but not significantly increased the activity of the K_{Ca} channels in inside-out patches (Figure 4). The NP_O was 0.06±0.014 under control conditions and 0.091±0.012 in the presence of SM (10 μmol/L). However, the activity of the K_{Ca} channels was significantly decreased by SM in the presence of 10 μmol/L SMase. The NP_O of these K channels was reduced from 0.091±0.012 to 0.023±0.006 (Figure 4B).

Effect of C_2-Ceramide on the Diameter of Small Coronary Arteries

Resting diameter of 8 perfused and pressurized coronary arteries averaged 312±18 μm, and Bay K8644 at a concentration of 10 nmol/L produced a 25% sustained contraction with arterial diameter decreasing to 232±28 μm. Under these conditions, we can determine whether ceramide dilates or constricts coronary arteries. The representative video prints of a small coronary artery before and after addition of ceramide are presented in Figure 5A. Ceramide, at a concentration of 1 μmol/L, markedly reduced the diameter of coronary artery. Figure 5B summarizes the effects of different concentrations of ceramide on arterial diameter. At the highest concentration of C_2-ceramide (1 μmol/L), the diameter of the vessels fell to 168±11 μm, a 28% reduction relative to that before the addition of ceramide. Unlike small coronary arteries, large epicardial coronary arteries (2 mm) had no vasoconstrictor or vasodilator response to C_2-ceramide even at the highest concentration tested (data not shown).

Activity of Acidic SMase and Neutral SMase in Coronary Homogenates

The homogenate was incubated with [^{14}C-choline]SM, and the production of ^{14}C–choline phosphate and ceramide was measured as SMase activity. As shown in Figure 6A, the activities of both acidic SMase (pH 5) and neutral SMase (pH 7.4) were detected in coronary arterial homogenates. The

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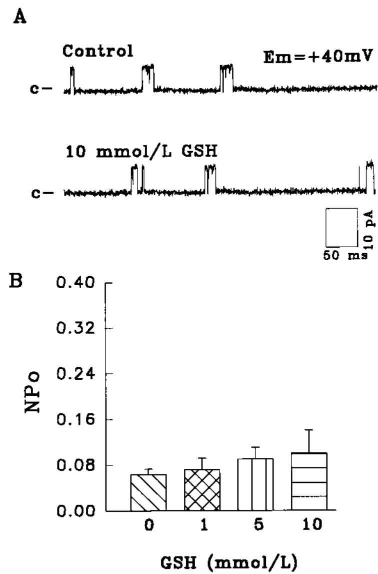

Figure 3. Effect of glutathione (GSH) on K_{Ca} channel activity in cell-attached patches of smooth muscle cells. A, Representative recordings of K_{Ca} channel currents under control conditions and after addition of GSH. B, Effect of GSH on NP_O of K_{Ca} channels in smooth muscle cells.

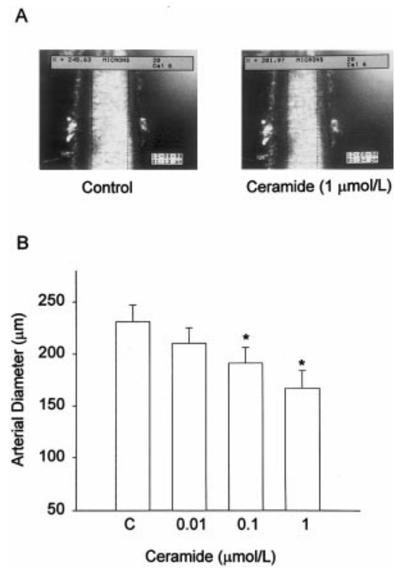

Figure 5. Effect of C_2-ceramide on the diameter of small coronary arteries. A, Representative video prints of a coronary artery before and after addition of C_2-ceramide. B, Effects of C_2-ceramide on the diameter of coronary arteries.

^{14}C–choline phosphate conversion rate of acidic SMase was 114.6±8.3 fmol/min per milligram coronary arterial homogenate protein, which was significantly greater than that of neutral SMase (65.1±1.0 fmol/min per milligram protein). Dithiothreitol at 0.01 to 1 mmol/L produced a concentration-dependent decrease in acidic SMase activity and was without effect on neutral SMase activity. Figure 6B summarizes the effect of glutathione on the conversion rate of [^{14}C-choline]SM into ^{14}C–choline phosphate. Glutathione partially inhibited neutral SMase activity and had no effect on acidic SMase activity. With the use of TLC, SM and its lipid metabolites, ceramide and sphingosine, were identified. When the homogenate was incubated with [^{14}C-choline]SM, the lipid metabolites had a pattern of migration on TLC similar to that with incubation of [^{14}C-choline]SM with purified SMase. The bands with R_f (ratio to front) of 0.1, 0.45, and 0.82 comigrated with standard SM, sphingosine, and C_{18}-ceramide, respectively.

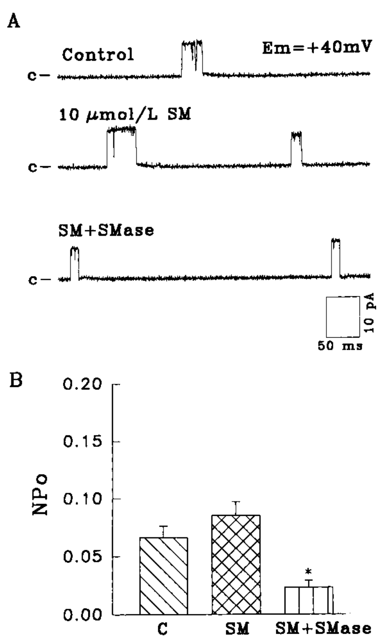

Figure 4. Effect of SM on K_{Ca} channel activity in inside-out patches of smooth muscle cells. A, Representative recordings of K_{Ca} channel currents under control conditions and after addition of SM in the absence or presence of purified SMase. B, Summary of the effect of SM on K_{Ca} channel activity. *Significant difference from control (C) ($P<0.05$).

Discussion

A previous study demonstrated that ceramide produces dose-dependent relaxation in phenylephrine-contracted endothelium-denuded rat aorta.[7] The mechanism of ceramide-induced vasorelaxation has yet to be determined. The present study determined whether ceramide-induced vasorelaxation is associated with the activation of the K_{Ca} channels in vascular smooth muscle cells. Unexpectedly, ceramide produced a concentration-dependent inhibition of K_{Ca} channel activity in arterial smooth muscle cells and vasoconstriction in small bovine coronary arteries. The effect of C_2-ceramide on K^+ channel activity is similar to the results obtained in lymphocytes, in which ceramide inactivates voltage-gated K^+ channels.[9] Inhibition of K_{Ca} channel activity would not contribute to ceramide-induced vasorelaxation, since it would depolarize cell membrane, leading to activation of Ca^{2+} channels, elevation of intracellular [Ca^{2+}], and vasoconstriction.[10] Despite the importance of K^+ channel activation in mediating the effects of a number of endogenous and exogenous vasodilators,[15] it seems that is not the case with ceramide.

Using isolated perfused small bovine coronary artery preparation, we demonstrated that C_2-ceramide produced a marked vasoconstriction in these small coronary arteries. This provides the first evidence that ceramide is a vasoconstrictor in coronary microcirculation. Inactivation of the K_{Ca} channels

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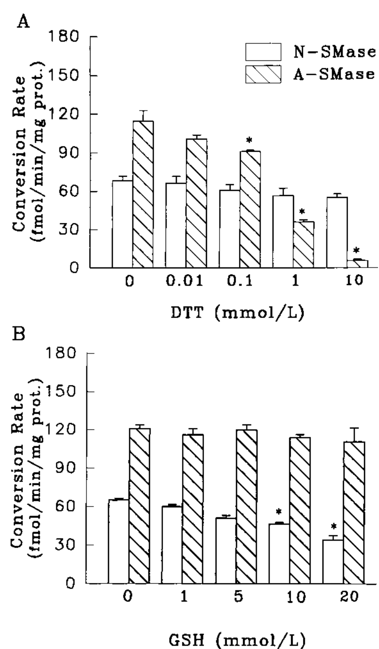

Figure 6. Effect of dithiothreitol (DTT) (A) and glutathione (GSH) (B) on acidic SMase and neutral SMase activity in coronary arterial homogenate. *Significant difference from control ($P<0.05$).

may mediate C_2-ceramide–induced coronary vasoconstriction. These findings are not in accordance with a previous report indicating that ceramide dilates rat aorta.[7] The reason for this discrepancy is unknown. It is likely that the action of C_2-ceramide on vascular tone varies with species, vascular beds, and artery sizes. In addition, we cannot exclude the possibility that C_2-ceramide may be converted to some vasoconstrictor metabolites or stimulate the production of some endogenous vasoconstrictors in coronary arteries. Further studies are needed to address these issues.

To explore the mechanism of the effect of ceramide, we examined whether any second messengers are required for the action of ceramide on K_{Ca} channel activity in coronary arterial smooth muscle cells. The inside-out patch mode was used to detach a membrane patch from the cell. By the use of this patch mode, intracellular soluble factors were removed, and ceramide could be added to the cytosolic side of patches. Addition of ceramide to the cytosolic solution also markedly decreased K_{Ca} channel activity in these detached membrane patches. This suggests that the effect of ceramide does not require any soluble factors as second messengers. It appears that ceramide directly acts on K_{Ca} channels or some K_{Ca} channel–associated membrane proteins on the cytosolic side of the cell membrane. This view is supported by the finding that a much lower threshold concentration of ceramide was required to inhibit K_{Ca} channel activity in inside-out patches than cell-attached patches. A previous study indicated that tyrosine kinase may mediate ceramide-induced inhibition of voltage-gated K^+ channels.[9] However, our results do not support a role for tyrosine kinase, since ATP or other factors required for tyrosine kinase activity were not included in the cytosolic solution.

Another important aspect of the present study is the demonstration that a biochemical pathway for ceramide production is present in coronary arteries and that endogenously produced ceramide may play a tonic regulatory role in the control of K_{Ca} channel activity. Both acidic and neutral SMases were detected in coronary arterial homogenate, and the activity of acidic SMase was greater compared with neutral SMase. In patch clamp experiments, dithiothreitol, an acidic SMase inhibitor, produced a concentration-dependent increase in K_{Ca} channel activity. However, neutral SMase inhibitor, glutathione, was without effect on the activity of these K^+ channels even at 10 mmol/L, a concentration that inhibited the activity of neutral SMase by 50%. These results suggest that under resting or physiological conditions, acidic SMase exerts a tonic regulatory action on K_{Ca} channel activity and that neutral SMase may not participate in the control of K_{Ca} channel activity in coronary arterial smooth muscle. Previous studies have indicated that under resting and stable conditions, intracellular neutral SMase exists as an inactive enzyme because of a high concentration of glutathione in the cytoplasm.[14] Depletion of glutathione from the cell may relieve the inhibition and activate this enzyme. Moreover, neutral SMase constitutes only a small portion of the total SMase activity (≈30%). Therefore, inhibition of the activity of this enzyme by glutathione even by 50% may not be enough to alter K_{Ca} channel activity.

Since the inhibitors of SMases, dithiothreitol and glutathione, are well-known thiol-containing reducing agents, one concern is that their effects on K_{Ca} channel activity may be associated with alteration in the redox status of the cells rather than with the inhibition of ceramide production. The cellular redox status does influence K_{Ca} channel activity.[16,17] However, there is considerable evidence indicating that the effect of dithiothreitol on K_{Ca} channel activity is not a general characteristic of thiol-containing reducing agents. First, only dithiothreitol increased K_{Ca} channel activity in the present study. Glutathione, another thiol-containing reducing agent, had no effect on channel activity. Increased K_{Ca} channel activity by dithiothreitol can be reversed by ceramide. These results suggest that the effects of dithiothreitol on K_{Ca} channel activity in coronary vascular smooth muscle cells are not due to alteration of redox status in these cells. Second, recent studies indicated that the K_{Ca} channels in pulmonary arteries but not in other arteries are sensitive to alteration of cell redox status. A number of reducing agents did not change the activity of the K_{Ca} channels in vascular smooth muscle cells prepared from the vascular beds other than the lung, such as ear arteries.[17] The sensitivity of pulmonary arterial K_{Ca} channels to reducing agents may be related to the chronic exposure of these vessels in hypoxic circumstances.[17] Therefore, alteration of redox status does not appear to change K_{Ca} channel activity in coronary arteries. Third, biochemical analyses in the present study demonstrated that dithiothreitol, but not glutathione, inhibited the activity of acidic SMase, suggesting that the effect of dithiothreitol on acidic SMase is specific but not a general effect of reducing agents. Otherwise, glutathione should have had an inhibitory effect on this enzyme.

In conclusion, both acidic and neutral SMases are present in bovine coronary arteries. Endogenous ceramide is produced by acidic SMase, inactivates the K_{Ca} channel, and hence exerts a tonic vasoconstrictor action in coronary microcirculation.

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