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Involvement of Calmodulin Kinase II in the Action of Sulphur Mustard on the Contraction of Vascular Smooth Muscle

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Abstract: Sulphur mustard (SM) is an alkylating agent whose mechanism is not fully understood. To investigate the early action of SM, we examined the effect of SM on contraction of vascular smooth muscles. Phenylephrine (PE)-induced contraction was reduced by SM, but only marginally by 70 mM KCl^-. Additional reduction was induced by nifedipine in SM-treated arteries. In the absence of extracellular Ca^{2+}, contraction of arteries by PE was reduced, which was fully recovered by addition of 2 mM Ca^{2+}. However, recovery was attenuated by pre-treatment with SM. The effect of SM on contraction by PE was not influenced by pre- and post-treatment with Phorbol 12, 13-dibutyrate. Calmodulin kinase II (CaMKII) was implicated as being responsible for the action of SM, because the contractile mechanisms of vascular smooth muscle via both Ca^{2+}-calmodulin-myosin light chain kinase axis and protein kinase C-proline-rich tyrosine kinase axis were not related to the action of SM. Elevation of phosphorylated CaMKII level by Ionomycin or PE was attenuated by treatment of SM on western blot. CaMKII may be a candidate target molecule of SM in early stage contraction of vascular smooth muscle.

Sulphur mustard (SM) is a vesicant with cytotoxic and carcinogenic effects. First synthesized over a century ago, SM has been used as a weapon in many conflicts, notably the Iran–Iraq War of the 1980s, in which tens of thousands of people were affected [1]. The main target organs of SM are the skin, eyes and the respiratory system. At a low dose, its effects are restricted mainly to the skin in the form of erythema, itching and an aversion to touch. At higher levels of SM, skin damage is severe and includes blistering, necrosis and inflammation, which can increase the risk of infection [1,2]. Exposure to very high concentrations of SM can cause irreparable skin damage and lethal respiratory failure. Treatments are only supportive.

SM is a strong alkylating agent that causes DNA double-strand breakage and carcinogenesis by mechanisms that are as yet not fully resolved. To repair SM-induced DNA damage, poly (ADP-ribose) polymerase (PARP) is activated, which leads to NAD+ depletion. Replenishment of NAD+ requires the consumption of ATP; exhaustion of whole ATP is lethal for cells [2,3]. Furthermore, proteolytic enzymes such as matrix metalloproteinase (MMP) are increased. MMP-2 and -9 are thought to be important in the blistering of skin because of their collagenase activity and because they both produce dermal-epidermal separation [4]. Increasing mitogen-activated protein kinase (MAPK) also has been reported; in particular, p38 is increased after SM exposure [3,5]. SM induces apoptosis through increased FasL expression and Ca^{2+}level [3,6]. SM adducts various molecules such as DNA, RNA and protein, and stimulates production of reactive oxygen species and nitrogen oxygen species [3]. Accordingly, some antioxidants are candidates of treatment designed to relieve SM toxicity. SM-induced toxicity also involves inflammation, perhaps via the stimulated production of inflammatory mediators [5,7].

Several studies have indicated roles for $Ca^{2+}$and calmodulin in SM toxicity [3,8]. Calmodulin's role in Ca^{2+}homeostasis has been convincingly established, and it is reported to play a critical role in SM-induced toxicity. Especially calmodulin-calcineurin inhibition reduces SM-induced toxicity but calmodulin- calmodulin kinase II (CaMKII) inhibition does not [3]. The current consensus is that the calmodulin-calcineurin pathway contributes fundamentally to SM-induced apoptosis. The responsible mechanism has remained elusive, with several SM candidate target molecules proposed [2,3]. In particular, the early stage action of SM is unclear. Presently, the effect of SM in the contraction of vascular smooth muscle was examined to investigate the early stage action of SM.

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Materials and Methods

Cell lines and culture. Vascular smooth muscle cells (VSMCs) were obtained from the aortic media of male Sprague-Dawley rats (6 weeks old) using an enzymatic dissociation method. VSMCs were cultured Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated foetal bovine serum (FBS), 100 μg/mL streptomycin and 100 μg/mL penicillin. Cultures were maintained at 37°C in a humidified 95% air and 5% CO_2 atmosphere. Cell between passage 2 and 4 was applied for the experiments.

Preparation of arterial rings and measurement of tension. New Zealand white rabbits (2–3 kg) of either sex were killed by exsanguination after anaesthesia with pentobarbital sodium (30 mg/kg intravenously). The carotid artery was quickly excised and placed in a cold physiological salt solution (normal Tyrode solution: 135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES and 5 mM glucose). The pH was adjusted to 7.4 using NaOH. Tyrode solution was equilibrated with a saturation gas mixture of 95% O_2–5% CO_2. The vessels were cut into 2 mm-wide ring segments and were placed into 20 ml tissue baths on L-shaped hooks, one of which was attached to a force transducer for isometric measurement of tension. The tension of the vessel was continuously recorded with the use of a computerized automated isometric transducer system (LabChart & Scope v6; AD Instruments, Heidelberg, Germany). The baths were thermostatically kept at 37o(C. A resting tension of 1.5 g was maintained throughout the experiments. Tissues were allowed to equilibrate for 90 90 min. before each experiment. Relaxations were studied in preparations contracted by 70 mM KCl solution (70K) of the following composition: 70 70 mM NaCl, 70 70 mM KCl, 2 2 mM CaCl2, 1 1 mM MgCl2, 10 10 mM HEPES and 5 mM glucose. When stable contractions were obtained, acetylcholine (ACh) was added cumulatively to determine the concentration-–response relationship. The function of the endothelium was checked at the beginning of each experiment with 10 μM ACh.

Western blotting analysis. Cellular lysates were prepared by suspending cells in 30 μL of lysis buffer (20 mM Tris–Cl (pH 7.9), 137 mM NaCl, 5 mM EDTA disodium salt, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 10 mM sodium fluoride, 1mM phenylmethanesulfonylfluoride, 1 mM sodium orthovanadate) and protease inhibitor cocktail (Roche, Indianapolis, IN, USA). The cells were disrupted by sonication and extracted at 4°C for 30 min.

Sprague–Dawley rats were killed in the same manner as the rabbits. The aorta was quickly excised and placed in a cold physiological salt solution. Contraction of aorta was measured by aforementioned rabbit carotid artery protocol. Each sample was removed when the reaction was stabilized. Aorta was homogenized in lysis buffer as mentioned above and protease inhibitor cocktail (Roche) using a model T10 ULTRA TURRAX homogenizer (Ika Werke GmbH, Staufen, Germany). Homogenized aorta was centrifuged at $10{,}956 \times g$, 15 min., 4°C and the supernatant was collected into another tube.

The total protein concentration in each sample was measured in duplicate by a BCA Protein Assay Kit (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard. Fifty micrograms of protein was loaded in each lane and proteins were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The resolved proteins were transferred from the gel to a BioTrace™ polyvinylidene fluoride membrane (Pall Life Science, Port Washington, NY, USA). The membrane was blocked using 5% skim milk in Tris-buffered saline containing 0.5% Tween-20 (TBST). Western blot was performed with a 1:1000 dilution in 1% skim milk TBST of p-CaMKII antibody followed by a 1:5000 dilution in 1% skim milk TBST of peroxidase-conjugated secondary antibody. Chemiluminescence was done using the ECL Plus Western Blotting Detection System (Amersham, Franklin Lakes, NJ, USA). Densitometric analyses were performed using the ImageJ programme.

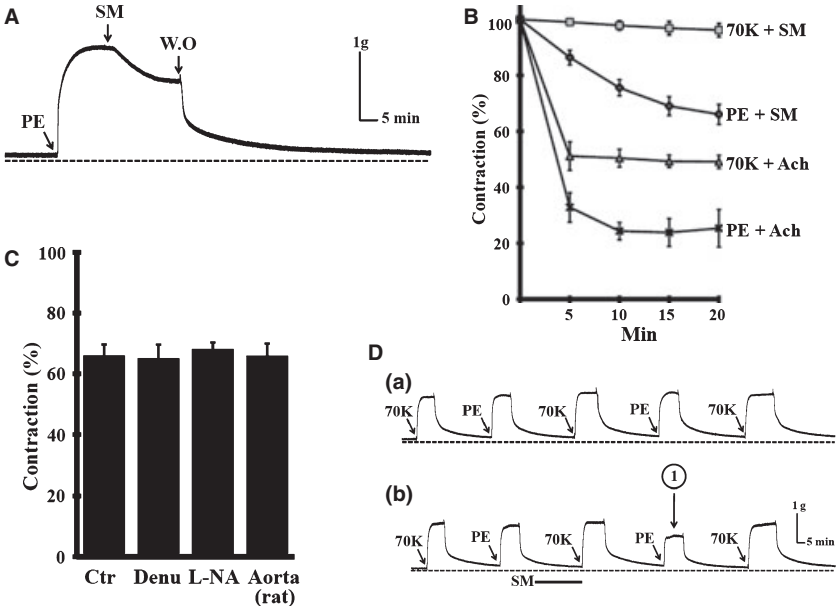

Fig. 1. Effect of sulphur mustard (SM) in several conditions. (A) Original recording of relaxation of isolated carotid arterial smooth muscle strips of rabbit treated by 100 μM SM in the presence of 3 μM PE. (B) Summarized data show relaxation effect of 100 μM SM and 10 μM ACh on 70K- and 3 μM PE-induced contraction of rabbit carotid arteries. Each point represents mean ± S.E.M. (n = 12). (C) SM-induced relaxation of normal rabbit carotid arteries (control) compared to arteries prior to treatment of 100 μM L-NA, endothelium denuded and normal smooth muscle strips of rat aorta. Each contraction was not significantly different from that in control (n = 10). (D) Contraction of rabbit carotid arteries by 70K and 3 μM PE in the absence (a) and presence (b) of 100 μM SM. The 70K-induced contraction did not change, but PE-induced contraction was significantly decreased (①) after SM pre-treatment (n = 7). Contraction %: Contractile percentage from maximal contraction to maximal relaxation of muscle strip. Min: minute.

Drugs and materials. SM was obtained from the Agency for Defense Development in the Republic of Korea. Phenylephrine (PE), acetylcholine (Ach), N^G-nitro-L-arginine methyl ester (L-NA), nifedipine, phorbol 12, 13-dibutyrate (PDBu), ionomycin calcium salt and KN-93 were purchased from Sigma-Aldrich (St Louis, MO, USA). Anti-p-CaMKII and anti-CaMKII antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

Statistical analyses. The results of the experiments are expressed as mean ± S.E.M. SAS ver. 9.0 (SAS Institute Inc., Cary, NC, USA) was used for statistical analysis of the results and the number of preparations taken from separate animals was indicated by n. The comparison of densitometric results was accessed by the Wilcoxon rank sum test. p values < 0.05 were considered significant.

Results and Discussion

Contraction induced by 3 μM PE was 1.35 ± 0.26 g tension and contraction induced by 70K was 1.39 ± 0.18 g tension in rabbit carotid arteries (n = 30). Maximal tension of induced contraction was measured after 10–15 min. When maximal contraction induced by PE stabilized, treatment with 100 μM SM was carried out in an organ bath. PE-induced contraction was reduced by SM within 1 min. When SM-induced dilation stabilized after 20 min., washing out was done (fig. 1A). PE-induced contraction was reduced by 66.09 ± 3.59% by SM, but 70K-induced contraction was only marginally changed. ACh at a concentration of 10 μM also reduced both PE- and 70K-induced contraction, but both the percentage and velocity of relaxation were different from SM-induced relaxation (fig. 1B, n = 12). Accordingly, it was observed that SM-induced dilation was not associated with Ach-induced dilation via the nitric oxide-mediated pathway [9]. The patterns of SM-induced relaxation in rabbit carotid artery and rat aorta were similar regardless of treatment with 100 μM L-NA and endothelium-denuded condition. Furthermore, the maximal percentage of relaxation from PE-induced contraction was not significantly different (fig. 1C, n = 10). These results were consistent with the suggestion that the influence of SM on smooth muscle is independent of endothelial nitric oxide synthase and endothelium.

Contractions induced by 70K and PE were similar in rabbit carotid arteries (fig. 1D, a). PE-induced contraction was

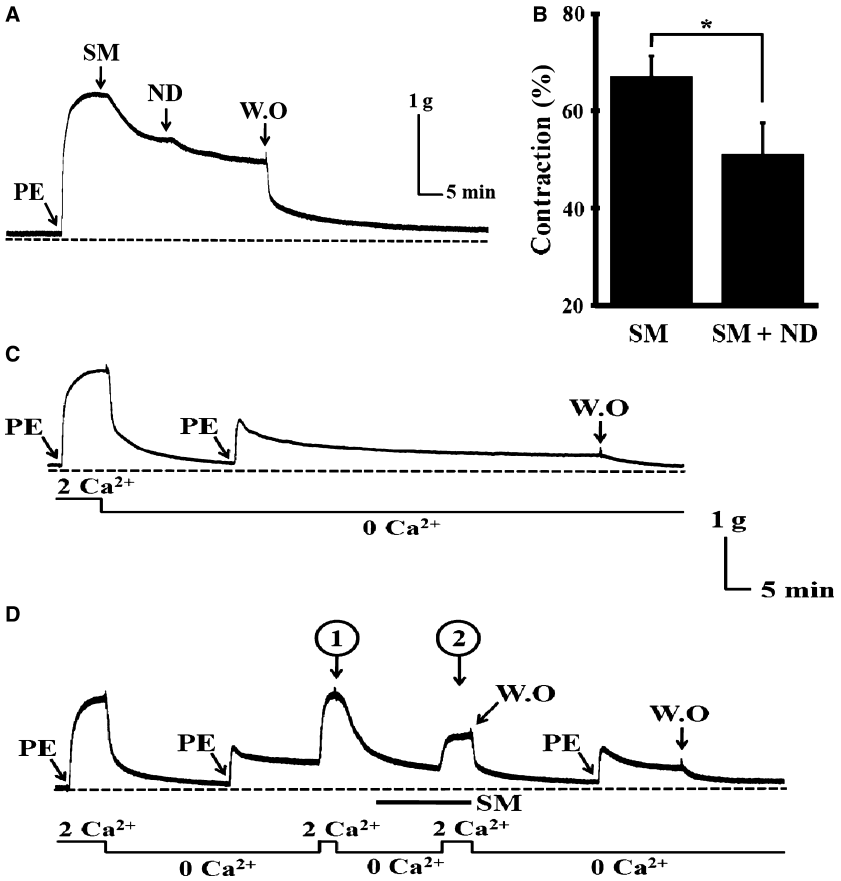

Fig. 2. Effect of nifedipine and extracellular Ca^{2+}concentration in rabbit carotid arteries relaxed by SM. (A) Traces showing the effect of 10 μM nifedipin after SM-induced relaxation in contracted rabbit carotid arteries by 3 μM PE. (B) Relaxation by additional nifedipin was compared with SM only (B, n = 10). (C) The effect of normal Tyrode solution containing 2 mM $Ca^{2+}$and Ca^{2+}-free Tyrode solution on 3 μM PE-induced contraction of rabbit carotid arteries. (D) When $Ca^{2+}$was applied to Ca^{2+}free Tyrode to a final concentration of 2 mM, normal contraction of rabbit carotid arteries was evoked. (①). After treatment with 100 μM SM using Ca^{2+}-free Tyrode solution, 2 mM Ca^{2+}-induced contraction was decreased (②). PE- induced contraction after SM treatment in Ca^{2+}-free Tyrode solution was not changed. * p < 0.05.

reduced by not only post-treatment but also pre-treatment of SM, but 70K-induced contraction was not. Even when washed out, the effect of SM continued and was irreversible in PE-induced contraction (fig. 1D, b, n = 7).

After relaxation by SM had stabilized, 10 μM nifedipine was added as a voltage-operated Ca^{2+}channel (VOCC) blocker. Artery contraction was further reduced from 67.20 ± 4.11% to 51.11 ± 6.41% by nifedipine (fig. 2A). The additional relaxation in the presence of nifedipine was significantly different from that observed in the presence of SM only (fig. 2B, n = 10). Contraction induced by 70 K was only marginally changed by SM but was attenuated by over 80% by nifedipine (data not shown).

In a previous study, the inhibitory effect of nifedipine was reported in agonist-induced constriction of human mesenteric and pulmonary arteries [10,11]. Contraction induced by 70K is mediated by activation of VOCC via membrane depolarization. Conversely, contraction induced by PE is related to both the release of $Ca^{2+}$from the sarcoplasmic reticulum (SR) and activation of receptor-operated Ca^{2+}channel (ROCC). For this reason, the inhibitory effect of nifedipine is significant in 70K-induced contraction, whereas PE-induced contraction is slightly inhibited by nifedipine [10,11]. Because the effects of SM and nifedipine were independent, we suggest that the pathway of relaxation by SM is different from voltage-operated Ca^{2+}channel (VOCC)-mediated pathway.

Activation of α1-adrenergic receptor by PE leads to an adrenergic receptor-induced release of $Ca^{2+}$from the SR through the G protein (q/11), phospholipase C, phosphatidylinositol 4,5-bisphosphate and inositol 1,4,5-triphosphate pathways [10,12]. Even if extracellular $Ca^{2+}$is absent, Ca^{2+} inside of the SR is released and can produce contraction by PE binding. Presently, contraction of rabbit carotid arteries induced by 3 μM PE was transient and was reduced in Ca^{2+}-free Tyrode solution (fig. 2C). The reduced arterial contraction noted in the absence of $Ca^{2+}$was fully recovered by 2 mM Ca^{2+}, likely because of Ca^{2+}influx through the PE-activated receptor-operated Ca^{2+}channel. But the recovery of contraction by addition of 2 mM $Ca^{2+}$was reduced by pre-treatment with 100 μM SM. After washing out, the PE-induced transient contraction of arteries in the absence of $Ca^{2+}$was unchanged by SM pre-treatment and was not different from that of the control group (fig. 2D, n = 8). Thus, we suggest that SM has no effects on the Ca^{2+}release pathway from SR but has effects on Ca^{2+}inflow through the ROCC.

After Ca^{2+}inflow, calmodulin binds with $Ca^{2+}$and activates both calmodulin kinase II (CaMKII) and myocin light chain kinase (MLCK). CaMKII additionally plays a critical role in ROCC opening and MLCK acts on actin, inducing contraction in vascular smooth muscle [13]. TRPC6 (classic or canonical transient receptor potential channel 6), a typical voltage independent Ca^{2+}channel that is enriched in vascular smooth muscle, may be related with CaMKII [14]. TRPC6 is activated by both α1-adrenergic receptor and muscarinic receptor (M2, M3), which activate G protein (q/11) [14]. The muscarinic receptor shares the same pathway with α1 adrenergic receptor [14]. Therefore, they also share the calmodulin, CaMKII and MLCK pathways. Consistent with this relationship, inhibition of CaMKII causes the inhibition of TRPC6 [14]. On the other hand, another TRPC group member, TRPC5, which is enriched in gastrointestinal smooth muscle cells, is not related with CaMKII but is related with MLCK [15].

Phosphorylation of CaMKII induces proline-rich tyrosine kinase (PYK2) activation [16]. Extracellular Ca^{2+}influx through voltage-independent Ca^{2+}channel plays an essential role in ET-1-induced PYK2 phosphorylation in vascular smooth muscle of rabbit [17]. PYK2 is activated by CaMKII and also protein kinase C (PKC) [16]. PKC activation can induce Ca^{2+}-independent contraction via extracellular-regulated kinase 1/2 and inhibition of myosin light chain phosphatase (MLCP) [18,19]. Presently, the contraction induced by 1μM of the PKC activator PDBu was slower than that induced by PE. The contraction was not reduced by SM (fig. 3A). Although PE-induced contraction was reduced by SM, contraction was fully recovered by PDBu (fig. 3B). We set the standard of maximal contraction induced by 3 μM PE as 100%. Contraction induced by PDBu with SM was 107.91 ± 4.93% and that of PDBu only was 106.66 ± 4.48%. Contraction induced by pre- and post-treatment of PDBu

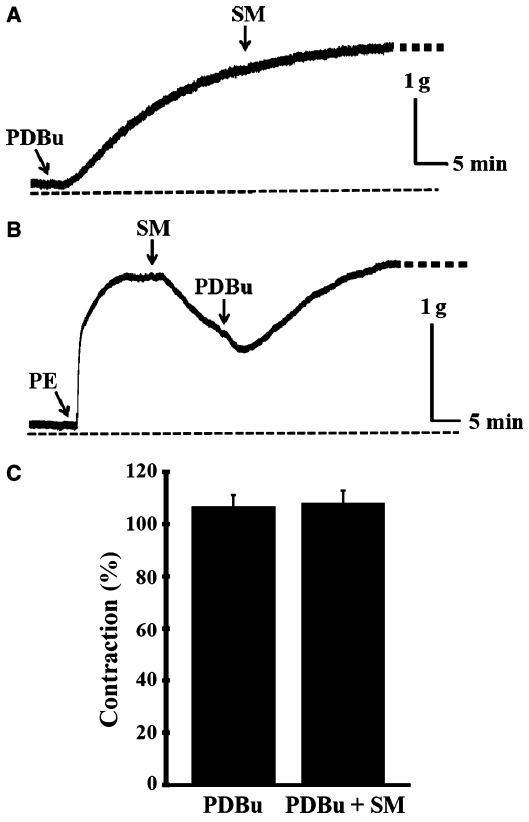

Fig. 3. Effect of PDBu on SM reaction. (A) Treatment with 100 μM SM did not inhibit 1 μM PDBu-induced contraction of rabbit carotid arteries. (B) After SM-induced relaxation in contracted arteries by 3 μM PE, contraction was fully recovered by PDBu treatment. (C) Contraction of PDBu with SM and that of PDBu only were not significantly different.

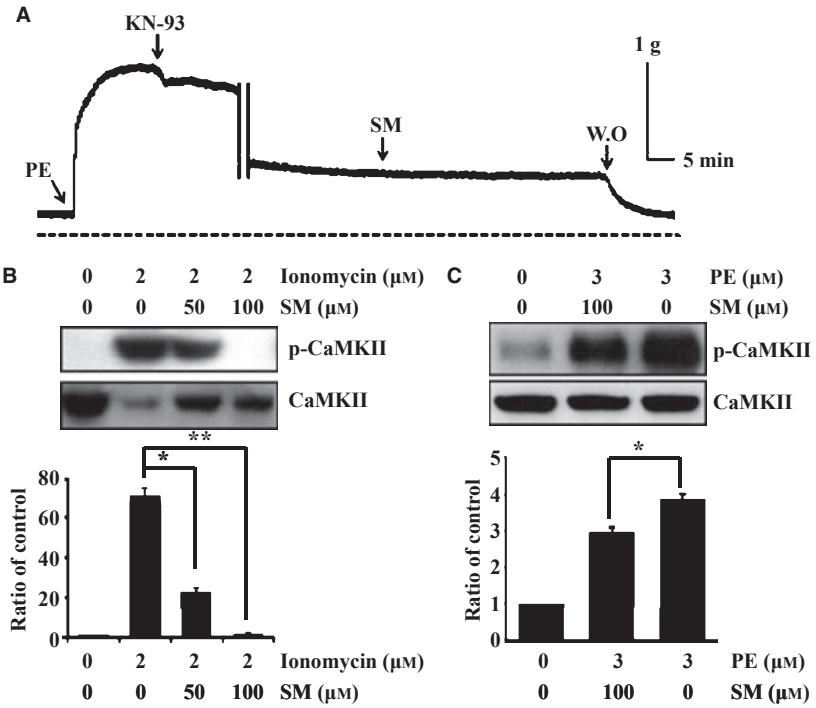

Fig. 4. Effect of SM on CaMKII. (A) Traces showing the effect of 100 μM SM after 10 μM KN-93-induced relaxation in contracted rabbit carotid arteries by 3 μM PE. (B) Phosphorylation of CaMKII induced by ionomycin was reduced by SM in VSMCs. Cells were treated with 2 μM Ionomycin or SM for 20 min. Equal amounts of cell lysates (30 μg) were subjected to electrophoresis and analysed by western blot for p-CaMKII and CaMKII antibodies. CaMKII antibody was used as control for the loading of protein level. Relative density value of band from densitometry calculation was set on the standard of control as 1; relative density value for Ionomycin with SM 50 μM treatment was 22.19 ± 2.10, Ionomycin with SM 100 μM treatment was 1.46 ± 0.36 and Ionomycin treatment only was 71.56 ± 3.54. Treatment of Ionomycin with SM and Ionomycin significantly differed from each other. * $p < 0.05$, ** $p < 0.01$ (C) Phosphorylation of CaMKII in rat aortas was reduced by 100 μM SM. Density value of band from densitometry calculation was set on the standard of control as 1; density value for 3 μM PE after SM treatment was 2.96 ± 0.15, and PE treatment only was 3.88 ± 0.16. Treatment of PE after SM and PE significantly differed from each other. * $p < 0.05$.

with SM was not significantly different from that of PDBu only (fig. 3C; n = 5). Therefore, we assumed that SM does not affect both PKC-PYK2-ROCC-Ca^{2+}influx axis and PKC-PYK2-MLCP axis in the contractile mechanism of vascular smooth muscle.

The present results show that SM has no relation to both the pathways of contraction caused by Ca^{2+}release from SR and downstream of PYK2. Moreover, SM negligibly affected contraction via the VOCC. Furthermore, our result from treatment of PDBu was similar to that of the specific CaMKII inhibitor KN-93 [20]. The effecter molecule of CaMKII is PYK2, and CaMKII is one of the important molecules in control of ROCC through PYK2 (fig. 5).

Summing up, the candidate target molecule of SM is CaMKII (fig. 5). Therefore, western blotting was performed using samples which were treated with SM in rat aorta. Although the physiographical trace was achieved in rabbit carotid artery, antibody of rabbit is non-existent because rabbits are usually the antigen host.

With the selective CaMKII inhibitor, KN-93, we investigated the involvement of CaMKII in the response of SM by physiographical trace. When PE-induced maximal contraction had stabilized, 10 μM KN-93 was applied. PE-induced contraction was reduced by KN-93 for about 2 hr. The dilation response by KN-93 was slower than that by SM. After 2 hr, SM was applied successively. However, the KN-93-induced dilation of arteries was unchanged by addition of SM (fig. 4A, n = 4). When only SM was applied for 2 hr, the dilation response was increased more than application for just 20 min. The relative dilation value by only KN-93 addition after 2 hr was almost the same as that by SM addition after 2 hr (data not shown). Thus, we suggest that SM exerts the inhibitory effects of CaMKII.

Recent studies have usually used ionomycin calcium salt for elevation of intracellular calcium level. Moreover, elevation of intracellular calcium by ionomycin evoked phosphorylation of CaMKII (p-CaMKII) [16]. In the present studies, elevation of p-CaMKII level by 2 μM Ionomycin was attenuated by treatment of VSMCs lysate with 50 μM SM and was completely abolished by 100 μM SM. Loss of CaMKII from the soluble pool after 20 min. of stimulation was observed with ionomycin treatment [21]. Immunoblotting and crude subcellular fractionation revealed that the loss in total CaMKII activity was coincident with its redistribution from a detergent soluble fraction to an insoluble fraction [21]. Densitometry results showed that the relative density value of band induced by Ionomycin was about 72-fold greater than that of control, which was decreased to about 22-fold by 50 μM SM and 1-fold of control by 100 μM SM (fig. 4B, n = 5).

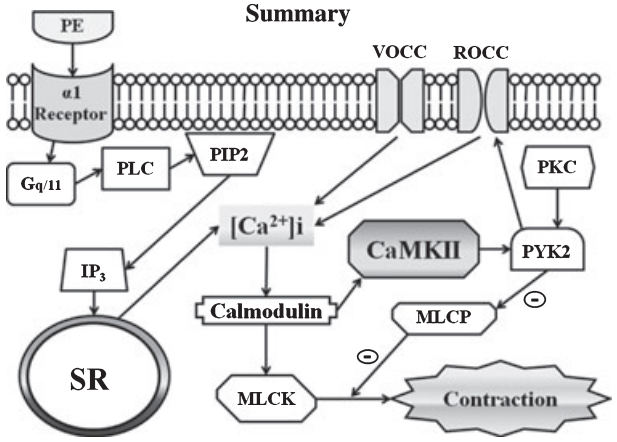

Fig. 5. Summary diagram of α1 adrenergic receptor pathway in vascular smooth muscle. The contractile mechanisms of vascular smooth muscle via both Ca^{2+}-calmodulin-MLCK axis and downstream of PKC-PYK2 axis are not related to the action of SM. Therefore, we suggest that the target molecule of SM is CaMKII. PE, phenylephrine; Gq/11, G protein (q/11 group); PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; IP3, inositol 1,4,5-triphosphate; SR, sarcoplasmic reticulum; $[Ca^{2+}]_i$, intracellular calcium ion; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphotase; CaMKII, calmodulin kinase II; PYK2, proline-rich tyrosine kinase; PKC, protein kinase C; VOCC, voltage-operated calcium channel; ROCC, receptor-operated calcium channel.

Elevation of p-CaMKII level by 3μM PE was attenuated by treatment of rat aorta lysate with 100 μM SM. Densitometry results showed that the density value of band induced by PE was about fourfold greater than that of control, which was decreased to about threefold by SM (Fig. 4C, n = 3).

Thus, SM reduced the level of p-CaMKII. It has been reported that in early reaction of SM, there is no elevation of calmodulin expression, while expression becomes elevated after 8 hr [8]. The present and previous observations prompt us to cautiously suggest that SM induces the expression of calmodulin through feedback inhibition of CaMKII phosphorylation. The elevated calmodulin levels may influence the calcineurin pathway and apoptosis.

In conclusion, CaMKII is the candidate target molecule of SM in contraction of vascular smooth muscle.

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