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Protein Methylation Activates Reconstituted Ryanodine Receptor-Ca^{2+} Release Channels from Coronary Artery Myocytes

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Key Words
Posttranslational modification · Ca^{2+} mobilization · Sarcoplasmic reticulum · Vascular smooth muscle · Coronary artery

Abstract
Ryanodine receptors (RyR) play an important role in the regulation of intracellular Ca^{2+} concentration and in the control of vascular tone. However, the mechanism regulating the activity of RyR is poorly understood. The present study determined whether protein methylation participates in the control of RyR activity. Using a planar lipid bilayer clamping system, S-adenosyl-*L*-methionine (SAM), a methyl donor, significantly increased the activity of a 245-pS reconstituted Ca^{2+} release channel from coronary arterial smooth muscle (CASM) in a concentration-dependent manner. Addition of the protein methylation blockers, 3-deazaadenosine, S-adenosylhomocysteine or sinefungin into the *cis* solution markedly attenuated SAM-induced activation of RyR/Ca^{2+} release channels. By Western blot analysis, arginine N-methyltransferase (PRMT1) and FK506 binding protein (FKBP) were detected in the SR used for reconstitution of RyR. In the presence of anti-PRMT1 antibody (1:100), SAM-induced activation of RyR/Ca^{2+} channel was completely abolished. In addition, this SAM-induced increase in RyR/Ca^{2+} channel activity was blocked by 30 μM ryanodine and by FK506 (100 μM), a ligand for the RyR accessory protein. These results suggest that protein methylation activates RyR/Ca^{2+} release channels and may participate in the control of intracellular Ca^{2+} mobilization in CASM cells by transferring a methyl group to the arginine moiety of the RyR accessory protein, FKBP 12.

Many different posttranslational modifications have been reported to play an important role in the regulation of protein activity, such as phosphorylation, ADP-ribosylation, glycolation, nitrosylation and others. Recently, there has been increasing evidence that methylation also importantly contributes to the regulation of the function or activity of many cellular proteins [1–3]. This methylation occurs by addition of a methyl group to different amino acid residues of proteins in a reversible manner [4–7]. It has been demonstrated that highly specific methyltransferases promote the methylation of proteins at specific consensus sites. They use S-adenosyl-*L*-methionine (SAM) as a methyl donor to transfer a methyl group onto a nucleophilic oxygen, nitrogen, or sulfur in a polypeptide or protein. These methyltransferases are classified into two major groups, including those that methylate carboxyl groups and those that methylate sulfur and nitrogen groups [8]. Recent studies have implicated arginine methylation that uses nitrogen as an acceptor of methyl trans-

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fer in a variety of cellular processes from signaling to protein transport [2, 9]. This arginine methylation of cellular protein has been considered as an important signaling mechanism similar to protein phosphorylation [10]. In this regard, protein methylation has been reported to mediate the effects of hormones such as aldosterone on sodium channel activity in renal collecting duct cells [8, 11], suggesting that ion channel methylation may be one of the important mechanisms regulating their activity. Moreover, protein methylation has been reported to participate in the regulation of cell growth and proliferation, immunological response, and different agonist responses.

Despite the increasing evidence that protein methylation plays an important role in numerous cellular activities [12], little is known regarding the role of protein methylation in regulating the function of vascular myocytes. Recent studies in our laboratory have indicated that ryanodine receptor (RyR) on the sarcoplasmic reticulum (SR) of vascular myocytes mediates Ca^{2+} mobilization [13, 14] and participates in the control of vascular tone [15–17]. It has also been reported that the functional activity of RyR as a Ca^{2+} release channel is regulated by protein posttranslational modifications such as phosphorylation, nitrosylation and protein-protein interactions [18, 19]. Given that protein methylation is considered as an important regulatory mechanism of ion channel activity and Ca^{2+} release response in different cells [10, 12, 20] and that methyltransferase inhibitor, 3-deazaadenosine (3-DZA) induces vasodilation through an unknown mechanism [21], we hypothesized that RyRs may be an acceptor of methyl transfer by a methyltransferase and that the methylation of these SR receptors may activate Ca^{2+} release from the SR and consequently induce the contraction of vascular myocytes. To test this hypothesis, we determined the effects of the methyl donor SAM on RyR activity in coronary artery myocytes and the effects of three methylation inhibitors and an anti-methyltransferase antibody on the SAM-induced activation of these SR receptors using a planar lipid bilayer clamping technique. Using Western blot analysis, we confirmed the presence of an arginine N-methyltransferase (PRMT1) and FK506 binding protein 12 (FKBP12) in the cytoplasm and SR of coronary artery smooth muscle (CASM). PRMT1 as a methyl transferase is responsible for the methylation of RyR related protein, FKBP12, thereby increasing the activity of RyR. These studies indicate that the arginine methylation of FKBP12 importantly participates in the regulation on RyR-induced Ca^{2+} release in vascular myocytes.

Materials and Methods

Preparation of the SR Membrane and Removal of FKBP 12 from Bovine Coronary Arteries

Coronary arteries were dissected from bovine heart, and the SR-enriched microsomes (SR membrane) of these arteries were prepared as described previously [13, 14]. Briefly, the dissected coronary arteries were cut into very small pieces and homogenized with a Tenbroeck tissue grinder in ice-cold 3-(N-morpholino)-propanesulfonic acid (MOPS) buffer containing 0.9 % NaCl, 10 m*M* MOPS (pH 7.0), 2 μ*M* leupeptin, and 0.8 μ*M* benzamidine. The homogenate was centrifuged at 4,000 *g* for 20 min at 4°C, and the supernatant was further centrifuged at 8,000 *g* for 20 min at 4°C. Then, the supernatant was collected and centrifuged at 40,000 *g* for 30 min, and the pellet, termed SR membrane was resuspended in buffer A containing 0.9% NaCl, 0.3 *M* sucrose, and 0.1 μ*M* PMSF. The supernatant was collected as cytosol. These cytosol and SR membrane preparations were aliquoted, frozen in liquid N_2, and stored at –80°C until used. FKBP 12 was removed from the RyRs as described previously [22, 23]. Briefly, the SR membranes were incubated with FK506 (10 μ*M*) in buffer A at 37°C for 15 min. Then, FK 506 incubation mixtures were centrifuged at 24,000 *g* for 15 min to remove the soluble FK 506-FKBP 12 complex in the supernatant from the pellet containing the RyRs complex. The pellet was washed once again by recentrifugation using buffer A and then resuspended in buffer A at a protein concentration of 2 mg/ml, termed FKBP 12-stripped SR. In these stripped SR, no FKBP12 band was detected using specific antibody for this protein as we reported previously, which indicates that FK506 (10 μ*M*) can completely dissociate FKBP 12 from the SR [13].

Reconstitution of RyR/Ca^{2+} into Planar Lipid Bilayer

The coronary arterial SR membranes containing RyR/Ca^{2+} release channels were reconstituted into planar lipid bilayer as we described previously [13, 14]. In brief, phosphatidylethanolamine and phosphatidylserine (1:1) were dissolved in decane (25 mg/ml) and used to form a planar lipid bilayer in a 250-μm aperture between two chambers filled with *cis* and *trans* solutions, respectively. The SR membranes (50–100 μg protein) were added into the *cis* solution, which corresponded to the cytosolic side of the SR RyR/Ca^{2+} release channels. The *trans* solution represented the lumenal side of these SR RyR/Ca^{2+} release channels. The recording solution in the *cis* chamber was 300 m*M* cesium (Cs^+) methanesulfonate and 10 m*M* MOPS (pH 7.2). The *trans* solution was the same as the *cis* solution except that Cs^+ methanesulfonate was 50 m*M* before fusion and 300 m*M* after fusion. In this configuration, Cs^+ flows from the lumenal *(trans)* to the cytosolic *(cis)* side at negative holding potentials. Cs^+ was chosen instead of Ca^{2+} as the charge carrier to precisely control $[Ca^{2+}]_i$ around the channel, to increase the channel conductance ($g_{Cs^+}/g_{Ca^{2+}} = 2$) and to avoid interference from K^+ channels present in the SR membrane [24]. Cl^- channels were blocked by replacing chloride with the impermeant anion methanesulfonate. RyR/Ca^{2+} release channel activity was detected in a symmetrical cesium methanesulfonate solution (300 m*M*) in all experiments. To increase the channel activity, 1 μ*M* free Ca^{2+} in the *cis* solution was adjusted by adding Ca^{2+} standard solution containing $CaCl_2$ and EGTA as described previously [14, 24].

Recordings of RyR/Ca^{2+} Release Channel Currents

An Integrating Bilayer Clamp Amplifier (Model BC-525C, Warner Ins. CT) was used to record single-channel currents in the bilayer.

The amplifier output signals were filtered at 1 kHz with an eight-pole Bessel filter (Frequency Devices Inc., Haverhill, Mass., USA). Currents were digitized at a sampling rate of 10 kHz and stored on a Micron Pentium III computer for off-line analysis. Data acquisition and analysis were performed with pClamp software (version 8, Axon Instruments, Union City, Calif., USA). The open probability (NP_o) of the channels in the lipid bilayer was determined from recordings of 3–5 min as described previously in our patch clamp studies [25]. All lipid bilayer experiments were performed at room temperature about 20°C.

Using this bilayer preparation, the effects of SAM on the activity of RyR/Ca^{2+} release channels were first determined. After a 3-min control-recording period at holding potentials of –40 mV, a series of concentrations of SAM were added into the bath solution, and RyR/Ca^{2+} release channel currents were recorded at each 3-min intervals in the presence or absence of methylation blockers. In these experiments, the SR and other proteins were consistently present in the *cis*-solution. Therefore, ryanodine, FK506 and anti-FKBP were used to confirm that SAM-induced channel activity is associated with the methylation of RyR complex, rather than other proteins. These compounds were added into the *cis* solution to determine their effects on SAM-induced activation of RyR/Ca^{2+} release channels. In another series of experiments, anti-arginine N-methyltransferase (anti-PRMT1) antibody (1:100) was used to determine whether PRMT1 is involved in this SAM-dependent RyR protein methylation. In addition, a rabbit IgG was used as a negative control to rule out the potential effect of nonspecific binding of the antibody. This antibody-negative experiment was described in our previous study [13]. A 1:100 concentration of these antibodies or IgG was based on preliminary experiments indicating that anti-PRMT-1 antibody at this concentration could substantially block the SR RyR/Ca^{2+} channels.

To further address whether SAM-induced activation of the RyR/Ca^{2+} release channels is associated with FKBP 12 or occurs on the channel protein, the effects of SAM on RyR/Ca^{2+} release channel activity were examined in the presence of FK 506 (100 μ*M*), ryanodine (30 μ*M*) or anti-FKBP12 antibody (1:200), respectively. All these compounds used in these experiments were added into the *cis* solution, and currents were recorded at holding potentials at –40 mV. The concentrations of FK 506, ryanodine or anti-FKBP12 antibody (ABR) and other compounds were chosen based on previous studies showing that they effectively altered the RyR activity [13, 14, 26, 27].

Western Blot Analysis

Western blot analysis was performed as described in our previous studies [13, 28, 29]. Briefly, 60 μg proteins of homogenate, cytosol or SR from BCASM were subjected to SDS-PAGE (12% running gel) after heating at 100°C for 5 min. The proteins were electrophoretically transferred at 100 V for 1.5 h onto nitrocellulose membrane, which was blocked by 5% non-fat dry milk solution overnight at 4°C. The membrane was incubated with a monoclonal antibody (1:300) against the protein arginine N-methyl transferase (anti-PRMT1, Abcam Ltd.) or a polyclonal antibody against the synthetic FKBP12 peptide for 4 h at room temperature. After washing 3 times with TBS-T, the membrane was incubated for 2 h with 1:2,000 horseradish peroxidase-labeled anti-mouse IgG or anti-rabbit Ig G. To detect immunoreactive bands, 4 ml of enhanced chemiluminescence detection solution 1 and 2 (1:1) (ECL, Pierce, Rockford, Ill., USA) were added directly to the blots on the surface carrying proteins, and the membrane was wrapped in Saran wrap and then exposed to Kodak Omat film.

Determination of FKBP Methylation in the SR

To provide direct evidence that FKBP in the SR preparations can be methylated, an in vitro analysis of FKBP methylation was performed by a modification of the method previously described [8, 11, 20]. The SR from bovine CASM (20–100 μg protein) were incubated at 37°C for 60 min with 5 μCi [methyl-^{3}H]-S-adenosylmethionine ([^{3}H]-SAM) (15 Ci/mmol from Sigma Biochem, Inc.). This incubation allowed the enzyme to transfer the ^{3}H-methyl group from SAM into SR proteins. To ascertain that FKBP is methylated, we used FK506 to dissociate this protein from RyR complex and then quantify the amount of methyl-containing FKBP. This FK506 dissociation was described in our previous studies for characterization of the SR RyR/Ca^{2+} channels [13]. In brief, the reaction mixtures were incubated with FK506 (10 μ*M*) for 15 min and then centrifuged at 24,000 *g* for 15 min. As stated above, the FKBP with bound FK506 remained in the supernatant. This supernatant was used to quantify methylated FKBP. To remove free [^{3}H]-SAM, the supernatant was filtered using Whatman GF/C glass fiber filters, and the filters were washed twice with cold distilled water using a Brandel M-24R harvester (Gaithersburg, Md., USA). Then, the filters were placed in a liquid scintillation cocktail and counted in a Beckman LS 6000IC β-counter. In this assay, negative controls including nonradioactive reaction mixtures and non-SR protein reaction mixtures were performed in parallel. The scintillation counts (cpm) in experimental groups were normalized by subtracting negative controls and then converted to the amount of [^{3}H]-SAM (fmol) consumed in the reaction or bound to the FK506-dissociated proteins. Although FK506 may dissociate other FKBPs in the supernatant, our previous results demonstrated that the primary form of FKBP was FKBP 12 in coronary arterial preparations. Therefore, we used the radioactivity of FK506-bound protein in the supernatant to represent methylated FKBP 12, which was expressed as femtomoles ^{3}H-labeled methyl per microgram protein.

Statistics

Data are presented as mean ± SEM. The significance of the differences in mean values between and within multiple groups was examined using an analysis of variance for repeated measures followed by a Duncan multiple range test. $p < 0.05$ was considered statistically significant.

Results

SAM-Induced Activation of RyR/Ca^{2+} Release Channels

With symmetrical cesium in *cis* and *trans* solutions, a unitary Cs^+ current through the reconstituted receptor/channel complex in the lipid bilayer was detected at holding potentials from –40 to +40 mV. Mean slope conductance for these SR Cs^+ currents was 245 ± 4.5 pS with a reversal potential of ~0 mV, and the channel activity is dependent on the magnitude of holding potentials rather than the polarity of clamp voltage, which is consistent with our previous results [14]. Figure 1a depicts the representative recordings of single channel Cs^+ currents before

and after the addition of SAM into the *cis* solution. SAM significantly increased the channel openings. In addition, in the presence of SAM, a small conductance of Cs^+ with about 25 pS could be detected. However, these small conductance channels were not characterized in the present study. In figure 1b, SAM at concentrations of 0.01–1.0 m*M* increased the NP_o of the 245 pS channels in a concentration-dependent manner. The NP_O was increased from 0.0033 ± 0.0006 in control to 0.0189 ± 0.0039 in the presence of 1.0 m*M* SAM.

Effects of Protein Methylation Blockers on SAM-Induced Activation of Reconstituted RyR/Ca^{2+} Release Channels from BCASM

As shown in figure 1, the protein methylation blocker, 3-DZA substantially blocked SAM-induced activation of RyR/Ca^{2+} release channels. In the presence of 3-DZA, SAM-induced increase in the NP_o of RyR/Ca^{2+} release channels was completely blocked. Figure 2a presents representative recordings of RyR/Ca^{2+} release channels in the presence of SAM and two other specific protein methylation inhibitors. Both SAH and sinefungin inhibited SAM-induced activation of RyR/Ca^{2+} release channels. As shown in figure 2b, the NP_o of these RyR/Ca^{2+} release channels was increased by SAM by 1.9- to 6.3-fold at 0.1 and 1 m*M*, respectively. Pretreatment of the SR membrane with the methylation blockers, SAH or sinefungin substantially inhibited SAM-induced increase in the NP_o of RyR/Ca^{2+} release channels.

Identification of PRMT1 in the SR of BCASM

Western blot analysis was performed using the homogenate, cytosol or SR from bovine coronary arterial smooth muscle (BCASM) to confirm the presence of PRMT1 and FKBP12. As shown in figure, an immunoreactive band was recognized by anti-PRMT1 or anti-FKBP12 antibodies, respectively. Both PRMT1 and FKBP12 were higher in the SR fraction than in cytosol. The densitometry analysis was performed. Since most loading markers such as β-actin and GAPDH exist in different amounts in the cytosol compared to the SR, there was no good loading control that could be used to show an equal loading amount of SR and cytosol. In these experiments, therefore, care must be taken to measure and calculate protein concentrations for each lane on gel before loading. After requisition of the intensity of each immunoreactive band, a relative intensity (normalized to that detected in homogenates) was calculated to present results from these experiments. As shown in figure 3b, the relative intensity was significantly higher in the SR preparations than in cytosol

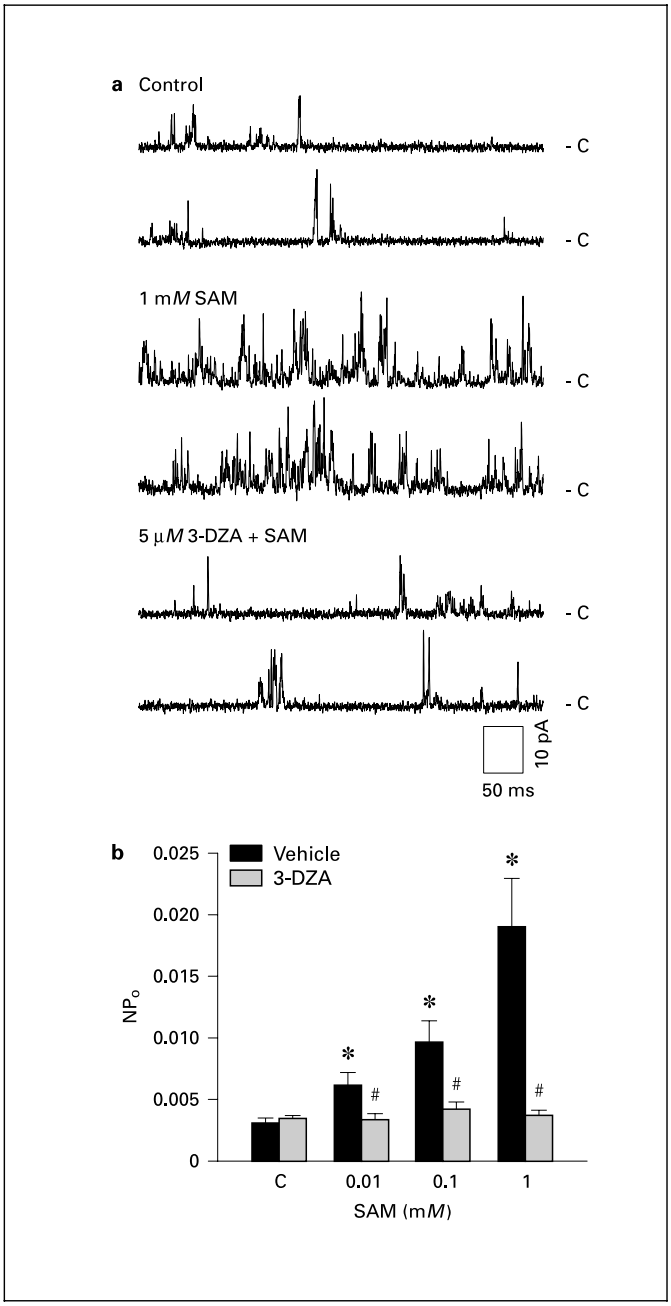
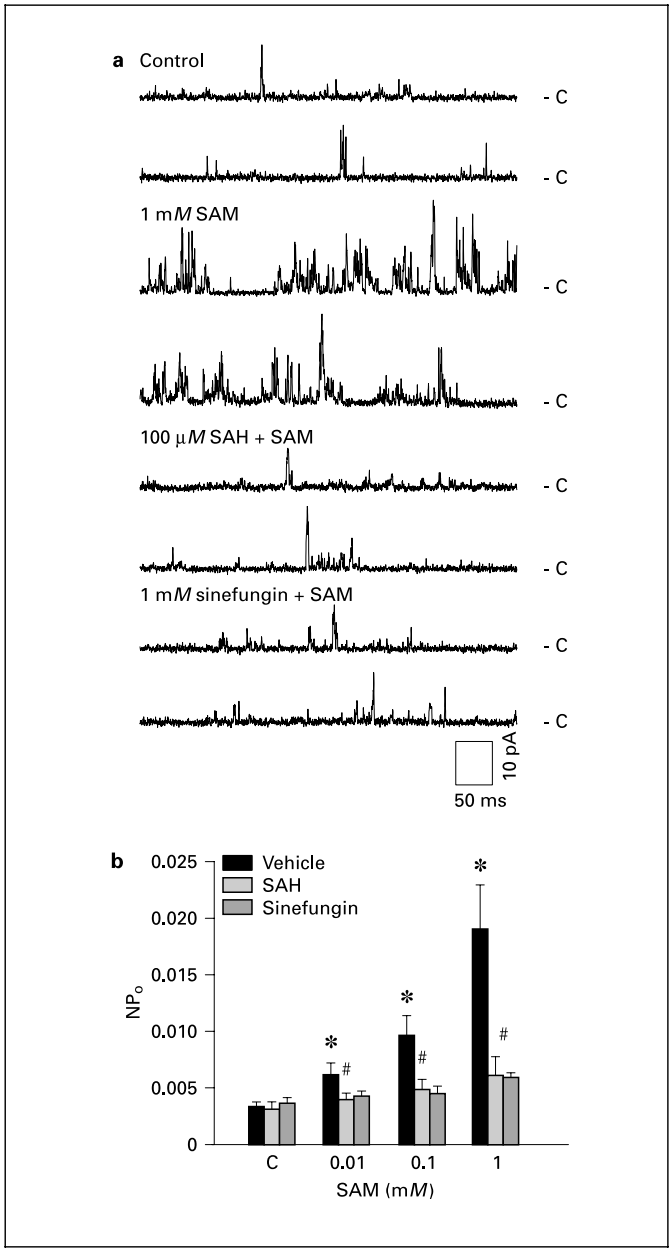


Fig. 1. a Representative recordings showing the effects of SAM (0.01–1 m*M*) on the activity of reconstituted Ca^{2+} release channels from coronary arterial smooth muscle in the absence or presence of 3-DZA. The traces were chosen from the same bilayer preparation if the experiments were paired (before and after drug treatment) or from two recordings using the same SR if the experiments were unpaired. **b** Summarized data depicting the effect of SAM on the NP_o of reconstituted RyR/Ca^{2+} channels in the absence or presence of 3-DZA (5 μ*M*) (n = 8–12 membrane preparations from 6 hearts). Vehicle indicates addition of the solvent for 3-DZA (1% ethanol in *cis*-solution). * Indicates significant difference from control ($p < 0.05$). # indicates significant difference from the values obtained from vehicle-treated group.



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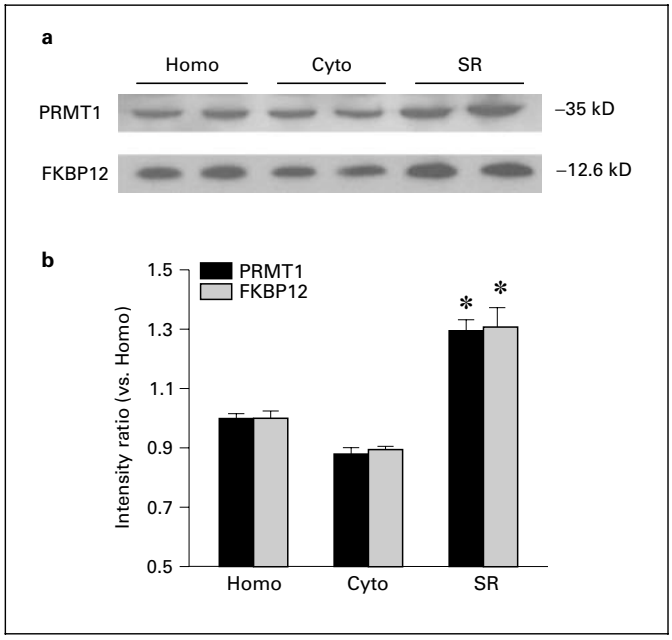

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Fig. 2. a Representative recordings showing the effects of SAM on the activity of reconstituted Ca^{2+} release channels from coronary arterial smooth muscle in the absence or presence of SAH (100 μ*M*) or sinefungin (1 m*M*). **b** Summarized data depicting the effect of SAM on the NP_o of reconstituted RyR/Ca^{2+} channels in the absence or presence of SAH or sinefungin (n = 6 hearts). Vehicle indicates addition of the solvent for SAH or sinefungin (1% methanol in *cis*-solution). * Indicates significant difference from control ($p < 0.05$). # Indicates significant difference from the values obtained from the vehicle-treated group.

Fig. 3. Western blot analysis of PRMT1 and FKBP 12. **a** Representative gel document showing the expression of PRMT1 and FKBP 12 in the homogenate (Homo), cytosol and SR prepared from bovine coronary arterial smooth muscle. **b** Summarized data depicting the distribution of PRMT1 and FKBP (n = 4). * $p < 0.05$ compared with the value obtained from cytosol.

(n = 4). It should be noted that since the major goal of these experiments was to confirm the coexistence of these proteins, the comparison of their amounts in the SR and cytosol was not critical. From the results, it is clear that both PRMT1 and FKBP are present in the SR of BCASM and are recognized by specific antibodies. Thus, these antibodies can be used in the bilayer experiments to determine the role of PRMT1 or FKBP in SAM-induced activity of RyR/Ca^{2+} release channels.

Effects of Anti-PRMT Antibody on SAM-Induced Activation of Reconstituted RyR/Ca^{2+} Release Channels from BCASM

As shown in figure 4a, SAM increased the activity of RyR/Ca^{2+} release channels in the absence of the antibody against PRMT1 (control) or in the presence of rabbit IgG (IgG + SAM). Pretreatment of the SR membrane with an anti-PRMT1 (1:100) markedly inhibited SAM-induced activation of RyR/Ca^{2+} release channels. Figure 4b sum-

marizes the effects of anti-PRMT1 antibody and rabbit IgG on SAM-induced increase in the NP_o of RyR/Ca^{2+} release channels. The anti-PRMT1 antibody substantially blocked the stimulatory effect of SAM on RyR/Ca^{2+} release channels, but IgG alone had no effect on SAM-induced increase of the NP_o.

Effects of Ryanodine on SAM-Induced Activation of Reconstituted RyR/Ca^{2+} Release Channels from BCASM

To explore the mechanism by which protein methylation enhances the activity of RyR/Ca^{2+} release channels, we performed a series of experiments to determine the possible activating site of SAM-induced protein methylation. First, we examined whether SAM-induced activation of these channels is blockable by ryanodine to further confirm the dependence of the SAM effect on RyR. As shown in figure 5a, SAM increased the activity of RyR/Ca^{2+} release channels from the SR in a concentration-related manner. Pretreatment of the SR membrane with ryanodine markedly inhibited SAM-induced activation of RyR/Ca^{2+} release channels at a concentration of 30 μ*M* that inhibited RyR/Ca^{2+} channel activity without subconductance formation [14]. Figure 5b summarizes the effects of SAM-induced increase in the NP_o of RyR/Ca^{2+} release channels before and after the SR preparations were pretreated with ryanodine. In the presence of ryanodine, basal NP_o of RyR/Ca^{2+} release channels was reduced and the SAM-induced increase in the NP_o of these channels was markedly blocked.

Effects of FK506 on SAM-Induced Activation of Reconstituted SR Ca^{2+} Release Channels from BCASM

FK506 is a binding ligand of a RyR accessory protein, FKBP, which can lead to a dissociation of FKBP from RyR, thereby activating RyR/Ca^{2+} release. These experiments determined whether protein methylation affects the function of this accessory protein. Figure 6 shows the effects of SAM on the SR RyR/Ca^{2+} release channels in the presence or absence of FK 506 (100 μ*M*). SAM significantly increased the activity of these channels in a concentration-dependent manner in the absence of FK506. FK506 (100 μg) alone significantly increased the channel activity. In the presence of FK506, SAM did not further increase the channel activity at 0.01 and 1 m*M*.

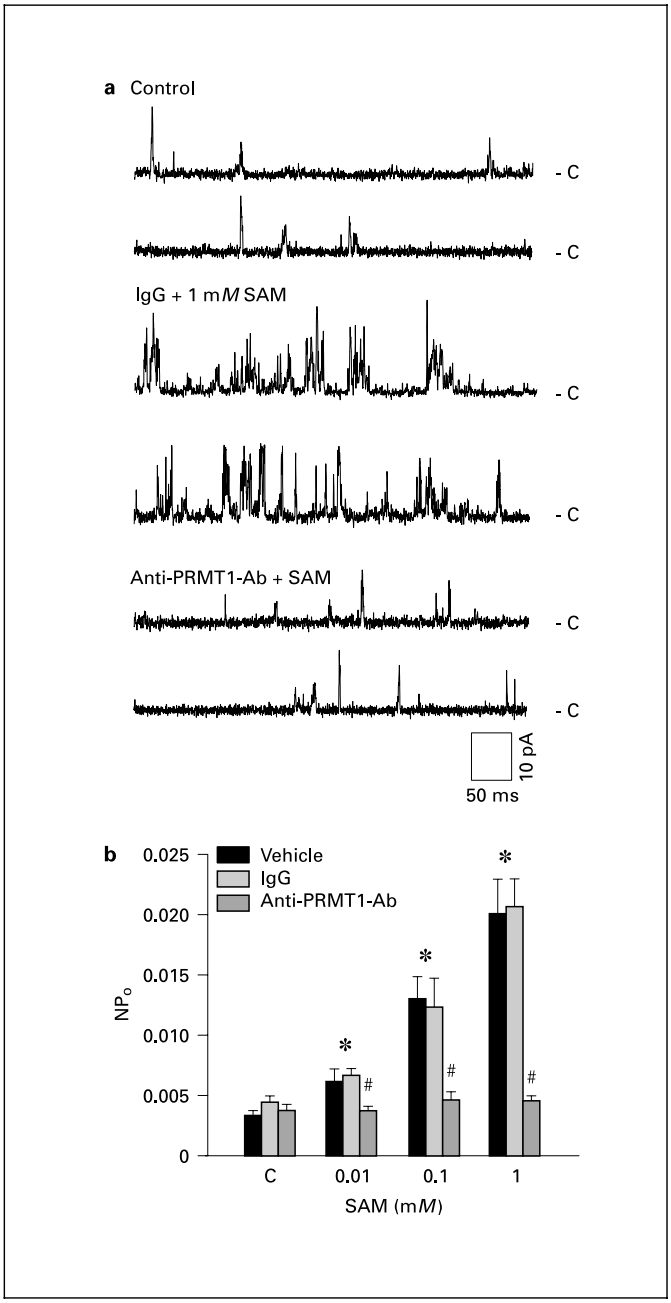

Fig. 4. a Representative recordings showing the effects of SAM on the activity of reconstituted Ca^{2+} release channels from coronary arterial smooth muscle in the absence or presence of anti-PRMT antibody. **b** Summarized data depicting the effect of SAM on the NP_o of reconstituted RyR/Ca^{2+} channels in the absence or presence of anti-PRMT antibody (n = 6 hearts). Pretreatment of the SR membrane with IgG served as negative control. Vehicle indicates addition of the dilution solution for antibodies (*cis*-solution). * Indicates significant difference from control ($p < 0.05$). # Indicates significant difference from the values obtained from vehicle-treated group.

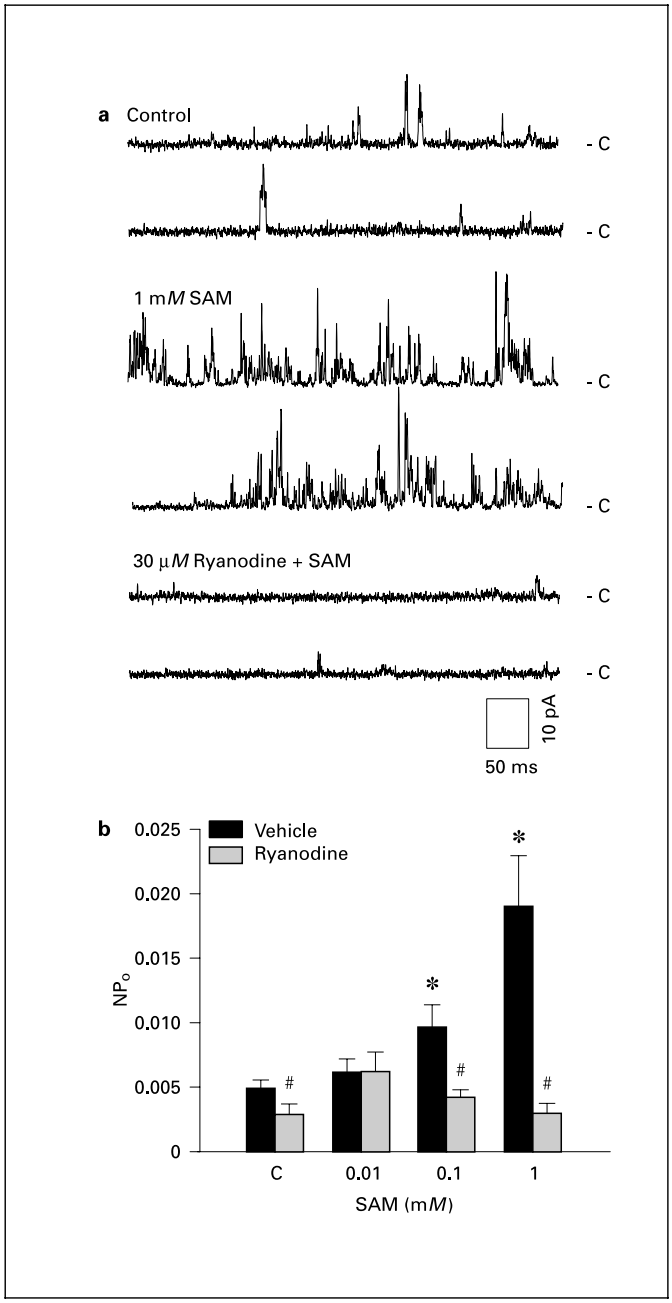

Fig. 5. a Representative recordings showing the effects of SAM on the activity of reconstituted Ca^{2+} release channels from coronary arterial smooth muscle in the absence or presence of ryanodine (30 μ*M*). **b** Summarized data depicting the effect of SAM on the NP_o of reconstituted RyR/Ca^{2+} channels in the absence or presence of ryanodine (n = 6 hearts). Vehicle indicates addition of the solvent for ryanodine (*cis*-solution). * Indicates significant difference from control ($p < 0.05$). # Indicates significant difference from the values obtained from vehicle-treated group.

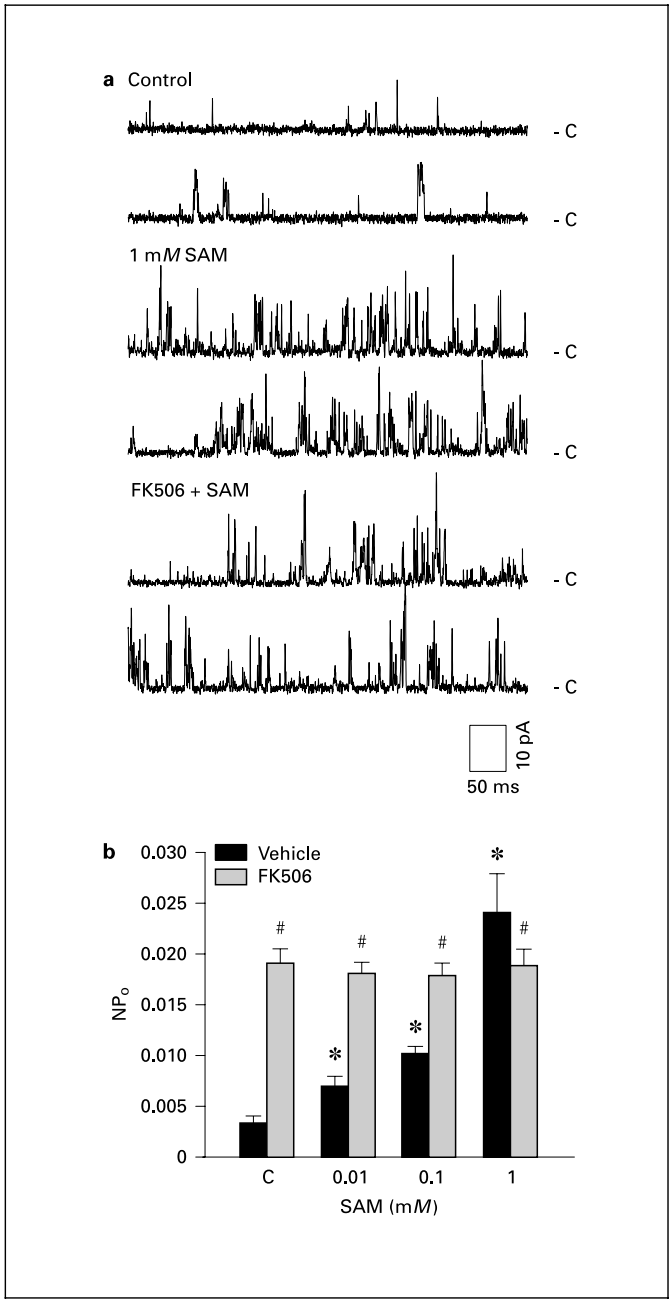

Fig. 6. a Representative recordings showing the effects of SAM on the activity of reconstituted Ca^{2+} release channels from coronary arterial smooth muscle in the absence or presence of FK 506 (10 μ*M*). **b** Summarized data depicting the effect of SAM on the NP_o of reconstituted RyR/Ca^{2+} channels in the absence or presence of FK 506 (n = 6 hearts). Vehicle indicates addition of the solvent for FK 506 (*cis*-solution). * Indicates significant difference from control ($p < 0.05$).

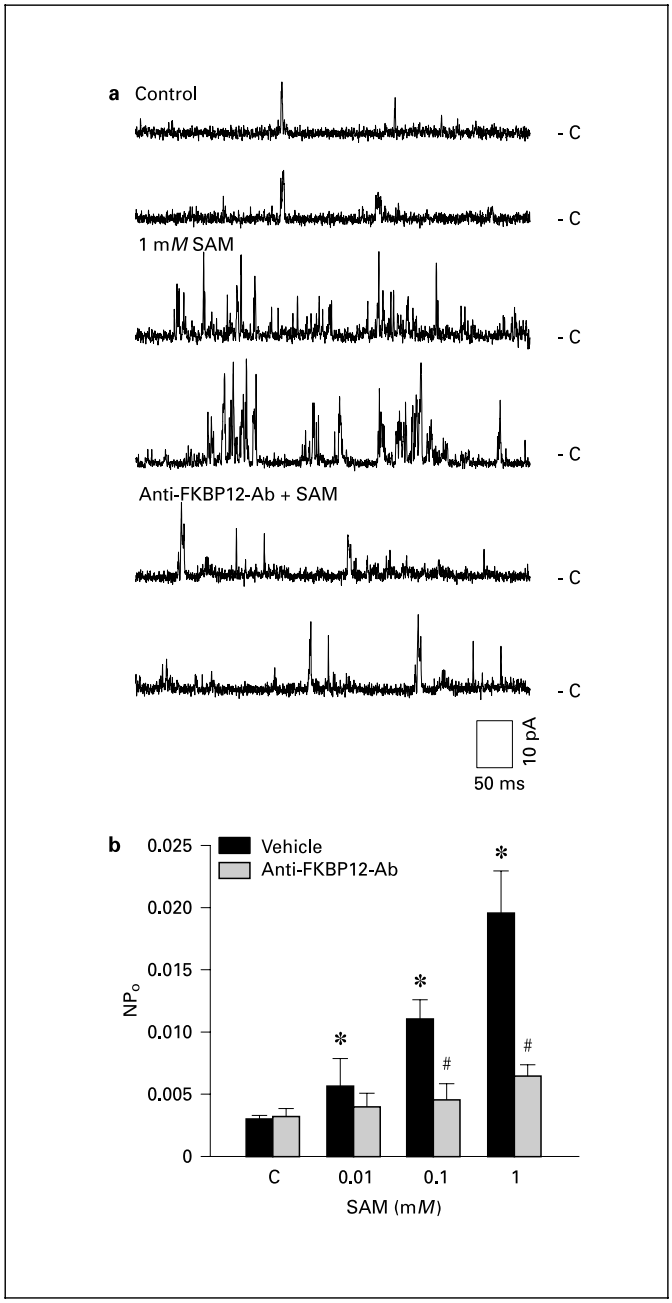

Fig. 7. a Representative recordings showing the effects of SAM on the activity of reconstituted Ca^{2+} release channels from coronary arterial smooth muscle in the absence or presence of anti-FKBP12 antibody. **b** Summarized data depicting the effect of SAM on the NP_o of reconstituted RyR/Ca^{2+} channels in the absence or presence of anti-FKBP12 antibody (n = 6 hearts). Vehicle indicates addition of the solvent for antibodies (*cis*-solution). * Indicates significant difference from control ($p < 0.05$). # Indicates significant difference from the values obtained from vehicle-treated group.

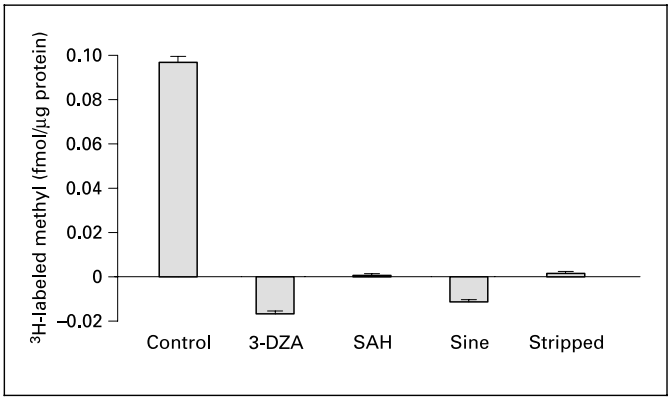

Fig. 8. Methylation of FK506-binding protein in the SR prepared from bovine coronary arterial smooth muscle. Transferred ^3H-labeled methyl moieties were detected by measuring the radioactivity of FK506 stripped protein from the SR incubated with [^3H]-SAM (n = 6). Sine indicates sinefungin and stripped designates the pre-stripped SR incubated with [^3H]-SAM.

Effects of Anti-FKBP12 Antibody on SAM-Induced Activation of Reconstituted RyR/Ca^{2+} Release Channels from BCASM

To determine if SAM-induced activation of RyR/Ca^{2+} release is associated with RyR accessory protein, FKBP12, a specific neutralizing anti-FKBP12 antibody, was used to examine the effect of blockade of FKBP12 on RyR/Ca^{2+} release channel activity [13]. As shown in figure 7a, SAM increased the activity of RyR/Ca^{2+} release channels (control). Pretreatment of the SR membrane with an anti-FKBP12 (1:200) markedly inhibited SAM-induced activation of RyR/Ca^{2+} release channels. Figure 7b summarizes the effects of this anti-FKBP12 antibody on SAM-induced increase in the NP_o of RyR/Ca^{2+} release channels from BCASM. The anti-FKBP12 antibody substantially blocked the stimulatory effect of SAM on RyR/ Ca^{2+} release channels.

Methylation of FKBP12 in the SR Prepared from BCASM

Rapid filtration technique and radiospectrometry were used to determine SAM-induced FKBP methylation and to examine the effects of protein methylation. As shown in figure 8, incubation of [^3H]-SAM with the SR from BCASM resulted in the accumulation of a methyl ^3H group on a fraction of FK506-bound protein, indicating the methylation of this FKBP. Under control conditions, 0.096 fmol ^3H methyl moieties were added per microgram SR protein. This incorporation was completely

blocked by mechanistically different protein methylation inhibitors, 3-DZA, SAH and sinefungin. This suggests that an enzyme-catalyzed methylation occurs. Importantly, when FKBP was stripped from SR proteins by FK506 incubation and centrifugation prior to addition of [^{3}H]-SAM, no methylation products could be detected in stripped SR preparations. This further suggests that the methylated protein in the SR used in this assay is FKBP.

Discussion

In addition to IP_3 receptors, RyR are present in the SR of vascular smooth muscle cells [26, 30–32]. Three subtypes of the RyR, RyR_1, RyR_2, and RyR_3, are described in different tissues or cells. RyR_3 is a predominant receptor subtype on the SR of vascular smooth muscle cells [33]. In recent studies, we have shown that RyR_3 is present in coronary resistance arterial smooth muscle, mediates Ca^{2+} release from the SR of these muscle cells, and participates in Ca^{2+}-induced Ca^{2+} release [13, 14] and the regulation of vascular tone [15–17]. Using a lipid bilayer reconstitution technique, this RyR is activated through dissociation of its accessory protein, FKBP 12, and an endogenously produced cyclic nucleotide, cyclic ADP-ribose promotes the dissociation of this protein and thereby activates RyR/Ca^{2+} release channels [13]. The present study determined the role of protein methylation in the regulation of this RyR/Ca^{2+} release channel activity from arterial smooth muscle. We provide the first functional evidence that enhanced methylation activates RyR to release Ca^{2+}, which may represent an important regulatory mechanism of intracellular Ca^{2+} mobilization and vascular tone.

SAM-dependent protein methylation has been reported to activate the purified renal Na^+ channels in reconstituted planar lipid bilayers or the Na^+ channels in cultured A6 cells [10, 20]. In the present study, a lipid bilayer channel reconstitution technique was also used to examine the role of protein methylation induced by SAM as a methyl donor in the activation of reconstituted RyR/Ca^{2+} release channels from the SR of BCASM. We found that SAM concentration-dependently increased the activity of RyR/Ca^{2+} release channels in BCASM, indicating that the methylation response may activate these channels. To confirm that the effects of SAM are specifically due to protein methylation, three selective mechanistically different inhibitors of methyltransferase were used to test whether SAM response could be blocked. 3-DZA, a membrane-permeable transmethylation inhibitor, specifically blocks S-adenosylhomocysteine hydrolase, promotes the accumulation of SAH and consequently inhibits its SAM-dependent methyltransferases [36]. It was found that 3-DZA substantially inhibited SAM-increased activity of reconstituted RyR/Ca^{2+} release channels, confirming that SAM-induced activation of RyR/Ca^{2+} release channels is associated with protein methylation. In an additional series of experiments, another two specific inhibitors of protein methylation, SAH and sinefungin, were used. SAH is a potent product-feedback inhibitor of the transmethylation reactions involving SAM as the methyl donor [37], and sinefungin is a structural analog of SAH [38, 39]. Consistently, both compounds significantly inhibited the SAM-induced increase in RyR/Ca^{2+} release channel activity. From these results, we conclude that a methylation modification may activate RyR/Ca^{2+} release channels from the SR in coronary smooth muscle.

Next, we examined which enzyme is responsible for the effects of protein methylation on RyR activity. There are several types of protein methyltransferases, which use SAM as a donor to transfer a methyl group to a nucleophilic oxygen, nitrogen, or sulfur acceptor in a polypeptide chain [1, 3, 8]. These methyltransferases that methylate amino acids of protein such as arginine have been implicated in the processes of signal transduction [40–42]. Recently, a protein arginine methyltransferase family (PRMT) has been characterized and shown to participate in a variety of cellular processes [1]. PRMT1 transfers a methyl moiety to arginine and interacts with a variety of proteins of interest, many of which are involved in cell signaling [9, 40]. To explore the possibility that PRMT1 is involved in the regulation of RyR/Ca^{2+} activity, we first confirmed the presence of PRMT1 in the SR preparation. By Western blot analysis with an anti-PRMT1 monoclonal antibody, a 35 kDa immunoreactive band was detected in coronary arterial homogenate, cytosol and SR. This provides direct evidence that PRMT1 is present in BCASM, and it may mediate protein methylation of the RyR. In lipid bilayer SR channel reconstitution experiments, the anti-PRMT1 antibody was added into the *cis* solution to examine if SAM-induced activation of RyR/Ca^{2+} release channels could be blocked. As predicted, this anti-PRMT1 antibody substantially blocked SAM-induced activation of RyR/Ca^{2+} release channels, whereas an unspecific rabbit IgG had no effect on the SAM-induced activation of these channels. Together, these results suggest that PRMT1 is present in the SR preparation and contributes to SAM-induced protein methylation, which increases the activity of RyR/Ca^{2+} release channels in BCASM.

To explore the mechanism of protein methylation-activating RyR/Ca^{2+} release channels, we determined the possible site of the methylation on the RyR of the SR from BCASM. The RyR complex is a tetramer complex comprised of four RyR monomers [13]. Each of these monomers binds to one FK506-binding protein (FKBP12) to function as a RyR/Ca^{2+} release channel [43]. These binding proteins or monomers in the RyR complex are regulated by different mechanisms. Recent studies have demonstrated that protein kinase A (PKA)-induced phosphorylation dissociates FKBP 12 from the RyR_2 monomers, resulting in an increased open probability (NP_o) of these Ca^{2+} release channels [44, 45]. More recently, we have shown that cyclic ADP-ribose dissociates FKBP 12 from RyR_3, leading to Ca^{2+} release from the SR of vascular smooth muscle [13]. In the present study, a series of experiments were performed to determine whether protein methylation occurs on the RyR or FKBP 12. First, we examined the effects of a high concentration of ryanodine on SAM-induced activation of RyR/Ca^{2+} release channels. Since ryanodine directly binds to RyR and inhibits the activity of RyR, these experiments would further confirm that the effect of SAM is associated with the activation of the RyR. Ryanodine indeed completely blocked the SAM-induced increase in the NP_o of RyR/Ca^{2+} release channels. Although these results do not imply the direct action of SAM-induced methylation on RyR activity, it seems that a normal RyR activity is important and permissive for its methylation activation. Next, we tested whether protein methylation occurs on FKBP 12 of the RyR, since these accessory proteins are the important regulatory elements for its Ca^{2+} release activity in the SR of BCASM. Western blot analysis demonstrated that both PRMT1 and FKBP12 are present in our SR preparation, and therefore the methyl group of SAM could be transferred to FKBP12 by PRMT1. To test this hypothesis, we performed lipid bilayer channel reconstitution experiments. When FK506, a binding ligand of FKBP 12 was incubated with the SR bilayer preparations, the RyR/Ca^{2+} release channel activity was markedly increased. In the presence of FK 506, SAM could not further increase the NP_o of these RyR/Ca^{2+} release channels, suggesting that SAM and FK506 may share the same mechanism to activate these channels, which may be related to the dissociation of FKBP from RyRs. Furthermore, a neutralizing anti-FKBP12 antibody was used to test whether SAM-induced activation is associated with the functional activity of this RyR accessory protein. Addition of anti-FKBP 12 antibody in *cis* solution did not significantly alter the basal activity of the RyR/Ca^{2+} release channels, but substantially blocked SAM-induced activation of RyR/Ca^{2+} release channels. It appears that the neutralizing anti-FKBP 12 antibody itself does not lead to dissociation of FKBP from the RyR, whereby the basal activity of the RyR/Ca^{2+} release channels is not changed. In the presence of this antibody, however, FKBP dissociation induced by SAM methylation could be blocked. These results further provide evidence indicating that the effect of SAM on the activity RyR/Ca^{2+} release channels may be attributed to enhancement of FKBP dissociation from RyRs due to its methylation.

To provide direct evidence that SAM can methylate FKBP, we incubated [^{3}H]-SAM with the SR prepared from BCASM and then separated ^{3}H-bound FKBP by FK506 dissociation and centrifugation. It was found that [^{3}H-methyl] was transferred to FKBP and all inhibitors of protein methylation, 3-DZA, SAH and sinefungin completely blocked this FKBP methylation. In FK506 pre-stripped SR, however, there was no detectable ^{3}H-methyl transfer.These results further support the view that SAM-induced activation of RyR/Ca^{2+}channels is associated with the FKBP methylation. It should be noted that these biochemical analyses did not rule out other FKBPs possibly methylated in the reaction mixture since FK506 might also dissociate them. However, since our previous study demonstrated that FKBP 12 was a primary form of FKBP in coronary arterial smooth muscle and that this FKBP importantly participated in the control of RyR activity [13], we believe that at least FKBP 12 is a primary methylated protein that mediates the action of SAM on RyR activation. This view was strongly supported by the results obtained from our bilayer recording experiments using an anti-FKBP 12 antibody, as discussed above.

In summary, the present study demonstrated that a methyl donor, SAM, activated RyR/Ca^{2+} release channels on the SR of BCASM. This SAM-induced activation of the RyR/Ca^{2+} release channels was substantially blocked by the methylation inhibitors 3-DZA, SAH and sinefungin. PRMT1 was present in the SR from BCASM. Our results indicate that this PRMT1 contributes to the SAM-dependent protein methylation of RyR, since the anti-PRMT1 antibody blocked the effects of SAM, on the activity of RyR/Ca^{2+} channels. In addition, a high concentration of ryanodine, FK506 and anti-FKBP 12 antibody also blocked SAM-induced activation of RyR/Ca^{2+} release channels. Biochemical assays demonstrated that both PRMT1 and FKBP 12 were present in the SR prepared from BCASM and ^{3}H-methyl group was transferred to FKBP protein when [^{3}H]-SAM was incubated with the SR. Taken together, these results suggest that protein

methylation participates in the regulation of RyR/Ca^{2+} release channel activity on the SR of BCASM. This methylation may occur at an accessory FKBP of the RyR. It is proposed that protein methylation of RyR may serve as a novel signaling mechanism regulating intracellular Ca^{2+} mobilization in BCASM.

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