Role of Protein Kinase C–Mediated Pathway in the Pathogenesis of Coronary Artery Spasm in a Swine Model

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Background The intracellular mechanism of coronary artery spasm is still unknown. The pathway mediated by protein kinase C (PKC) is an important intracellular process of various cellular responses, including vascular smooth muscle contraction. Thus, we examined the role of the PKC-mediated pathway in the pathogenesis of coronary artery spasm in our in vivo swine model.

Methods and Results Seven Göttingen miniature pigs underwent coronary balloon injury and x-ray irradiation to induce atherosclerotic lesion. After 6 to 18 months, intracoronary serotonin (3 μg/kg) or histamine (3 μg/kg) repeatedly induced coronary artery spasm at the atherosclerotic site. At the spastic site, intracoronary administration of phorbol-12,13-dibutyrate (PDBu) (10⁻⁹ mol/kg), a PKC-activating phorbol ester, also induced coronary artery spasm, which was completely blocked by pretreatment with intracoronary staurosporine (10 μg/kg), a PKC inhibitor. Intracoronary administration of an inactive phorbol ester, phorbol-12,13-didecanoate (10⁻⁵ mol/kg), did not induce coronary vasoconstriction. Coronary artery spasm induced by the autacoids was significantly augmented by pretreatment with intracoronary PDBu and partially inhibited by staurosporine. Intracoronary administration of Bay K 8644 (10 μg/kg), a dihydropyridine-sensitive L-type calcium channel agonist, also induced coronary artery spasm at the spastic site, which was significantly inhibited by pretreatment with intracoronary staurosporine or nifedipine (0.1 mg/kg).

Conclusions These results suggest (1) the PKC-mediated pathway is importantly involved in the pathogenesis of coronary artery spasm, (2) activation of the PKC-mediated pathway partially accounts for serotonin- and histamine-induced coronary artery spasm, and (3) at the spastic site, calcium influx through dihydropyridine-sensitive L-type calcium channel and/or calcium sensitivity of the contractile proteins may be augmented by the PKC-mediated pathway. (Circulation. 1994;90:2425-2431.)

Key Words • calcium channels • atherosclerosis • phorbol ester • vasoconstriction

Coronary artery spasm plays an important role in the pathogenesis of a wide variety of ischemic heart diseases, not only in variant angina but also in unstable angina, myocardial infarction, ventricular arrhythmias, and sudden death. However, the pathogenesis of coronary spasm is still unknown, and the elucidation of its mechanism remains an important clinical issue. We have developed a swine model of coronary spasm and revealed several pathogenetic aspects of the spasm. In our swine model, smooth muscle contraction to autacoids (serotonin and histamine) is augmented, endothelium-dependent relaxations to the autacoids are reduced, and calcium sensitivity of the contractile proteins in smooth muscle per se is not augmented at the spastic site. Thus, the key mechanism(s) for smooth muscle hypercontraction appears to exist in the signal transduction pathway at a level between receptors and the contractile proteins.

The pathway mediated by protein kinase C (PKC) is an important intracellular process of various cellular responses, including vascular smooth muscle contraction. Previous in vitro experiments have demonstrated that phorbol-12,13-dibutyrate (PDBu), a PKC-activating phorbol ester, induces sustained vasoconstriction, and augments the sensitivity of contractile proteins to calcium ions (Ca²⁺), suppresses the release and the actions of endothelium-derived relaxing factor (EDRF), and stimulates the release of endothelium-derived contracting factor (EDCF). However, little is known about the role of the PKC-mediated pathway in the pathogenesis of coronary artery spasm. Thus, the present study was designed to examine the role of the PKC-mediated pathway in the pathogenesis of coronary artery spasm in our in vivo swine model.

Methods

Animal Preparation

Seven male Göttingen miniature pigs, 2 to 4 months old and weighing 20 to 30 kg, were sedated with an intramuscular administration of ketamine hydrochloride (12.5 mg/kg) and anesthetized with an intravenous administration of sodium pentobarbital (25 mg/kg). The animals were then intubated and ventilated with room air, and oxygen was supplemented by a positive-pressure respirator (Shinano Inc.). Under asptic conditions, a preshaped Kifa catheter was inserted from the carotid artery into the orifice of the left coronary artery under the guidance of the fluoroscopy in a C-arm x-ray system (Toshiba). After pretreatment with heparin (3000 U IV), endothelial denudation about 3 cm long was performed on the left anterior descending coronary artery (LAD) using a bal-
Coronary Angiography

Six to 18 months after coronary denudation, the animals were again anesthetized and ventilated as described above, and selective coronary arteriography was performed. A pre-shaped Judkins catheter was inserted into the right or left femoral artery, and coronary arteriography in a left anterior oblique view was performed. ECGs (leads I, II, III, V1, and V2) and blood pressure were continuously recorded during the experiments. The angiographic study was performed twice at an interval of 1 to 2 weeks to examine the following two protocols.

Protocol 1

Coronary artery vasomotion in response to intracoronary administration of various vasoconstrictors was examined, including serotonin, histamine, phorbol-12,13-didecanoate (PDD), an inactive phorbol ester, and PDBu. Serotonin- and histamine-induced coronary vasoconstriction was also examined before and after intracoronary administration of PDBu or staurosporine, a PKC inhibitor. In three pigs, coronary artery vasomotion to prostaglandin F2α (PGF2α) was also examined. Coronary arteriography was performed 2 minutes after intracoronary administration of serotonin (1 and 3 μg/kg), 1 minute after that of histamine (1 and 3 μg/kg), and 5 minutes after that of PGF2α (50 μg/kg), when hemodynamic variables returned to the control level. To examine coronary artery vasomotion to phorbol esters, coronary arteriography was repeated at an interval of 5 to 10 minutes for 60 minutes after intracoronary administration of PDBu (10^-10, 3×10^-9, and 10^-8 mol/kg) and for 20 minutes after that of PDD (10^-9 mol/kg). These doses of PDBu were determined in the preliminary study so that the estimated maximum concentration of PDBu (10^-9 mol/kg) was 10^-7 mol/L when the phorbol ester was injected into the left coronary arteries for 3 minutes. The same dose (10^-8 mol/kg) was chosen for PDD. The different durations of the coronary arteriographic study for PDBu (60 minutes) and PDD (20 minutes) were determined based on the preliminary findings that the vasoconstrictor effect of PDBu (10^-9 mol/kg) lasts for 60 minutes and that PDD (10^-8 mol/kg) causes no coronary vasoconstriction. We also confirmed in the preliminary study that the vasoconstrictor effect of PDBu was reproducible. Care was taken so that the injected volume of PDD and PDBu (dissolved in physiological salt solution containing less than 5% dimethyl sulfoxide) was 6 mL and the injection time was 3 minutes. We confirmed in the preliminary study that the dimethyl sulfoxide solution alone did not affect the coronary diameter. Intracoronary staurosporine (10 μg/kg) was given 5 minutes before intracoronary administration of PDBu at 10^-9 mol/kg, and coronary arteriography was repeated after that. To examine the effect of activation of the PKC-mediated pathway on serotonin- and histamine-induced coronary vasoconstriction, coronary arteriography after intracoronary administration of those autacoids was repeated 5 minutes after staurosporine (10 μg/kg) and more than 60 minutes after PDBu (10^-8 mol/kg) when the coronary diameters almost returned to the control level. At the end of the experiments, coronary arteriography was repeated after intracoronary administration of nitroglycerin (10 μg/kg).

Protocol 2

Coronary artery vasmotion was examined in response to intracoronary administration of Bay K 8644, a dihydropyridine-sensitive L-type Ca^{2+} channel agonist, before and after intracoronary administration of staurosporine and nifedipine. Coronary arteriography was performed 2 minutes after intracoronary administration of Bay K 8644 (1, 3, and 10 μg/kg). Intracoronary staurosporine (10 μg/kg) or nifedipine (0.1 mg/kg) was given 5 minutes before intracoronary administration of Bay K 8644 (10 μg/kg).

Coronary Diameter Measurement

Cineangiograms at end diastole were chosen and printed, and the coronary luminal diameters were measured with calipers. With this technique, excellent correlations between repeated measurements (r = .99) and between different observers (r = .98) were confirmed in the range of the coronary diameter from 0.98 to 5.58 mm. The degree of constrictive response was expressed as the percent decrease in the luminal diameter from the control level. The coronary diameter was measured at the segment of the LAD where a focal excessive constriction evoked with drugs was maximal and at the segment of the left circumflex coronary artery (LCx) where the diameter was similar to that of the spastic segment of the LAD under control conditions.

Histological Examination

After the angiographic experiments, hearts were removed and the left coronary arteries were perfused by a constant-pressure perfusion system (120 cm H2O) with saline (500 mL) and subsequently with 6% formaldehyde (1000 mL). After the fixation, both the LAD and the LCx were cut transversely into segments at a 5-mm interval along the main trunk with small surrounded tissues. These segments were stained with hematoxylin-eosin and van Gieson’s elastic staining for photomicroscopy.

Drugs

The following drugs were used: 5-hydroxytryptamine (serotonin), histamine, PGF2α, PDD, PDBu, staurosporine (Sigma Chemical Co), Bay K 8644 (Wako Junyaku Co), and nifedipine (Bayer Pharmaceutical Co). PDD, PDBu, and staurosporine were prepared as stock solutions in dimethyl sulfoxide. Dilution was made with a physiological salt solution. Nifedipine was prepared by diluting the content of a nifedipine capsule (10 mg) in 10 mL of physiological salt solution.

Statistical Analysis

The results were expressed as mean±SEM. Multiple comparisons were made by a two-way ANOVA followed by a post hoc test, and unpaired data were analyzed by Student’s t test. A value of P < .05 was considered statistically significant.

Results

At the angiographic study, pigs weighed 35 to 47 kg. Intracoronary serotonin (3 μg/kg) or histamine (3 μg/kg) repeatedly induced coronary spasm at the previously denuded and x-irradiated LAD with ischemic ECG changes (Fig 1). In contrast, PGF2α (50 μg/kg) induced comparable vasoconstriction at the spastic LAD (29.2±7.9%, n = 3) and at the control LCx (29.4±6.4%, n = 3). At those spastic sites, PDBu (10^-9 mol/kg) also induced coronary spasm with ischemic ECG changes (Fig 1). The time course of phorbol ester-induced coronary vasoconstriction is shown in Fig 2. At the spastic LAD, PDBu (10^-8 mol/kg) induced marked coronary vasoconstriction that peaked at 20 minutes. On the other hand, at the control LCx, PDBu induced only mild coronary vasoconstriction. The PDBu-induced spasm at the LAD and the mild vasoconstriction at the LCx was completely blocked by pretreatment with intracoronary staurosporine (10 μg/
kg) (Fig 2). PDD (10⁻⁹ mol/kg) did not induce coronary vasoconstriction at either site. PDBu-induced coronary vasoconstriction was dose dependent and was significantly greater at the spastic LAD than at the control LCx (Fig 3). Intracoronary administration of nitroglycerin (10 μg/kg) caused coronary vasodilatation at the spastic LAD (27.6±11.7%) and at the control LCx (7.8±3.6%, n=6, NS). Intracoronary administration of staurosporine (10 μg/kg) also caused coronary vasodilatation at the spastic LAD (14.3±4.7%) and at the control LCx (9.2±5.5%, n=5, NS).

At the spastic site, serotonin- and histamine-induced coronary spasm was significantly augmented by pretreatment with intracoronary PDBu (10⁻⁹ mol/kg) (Figs 4 and 5), whereas it was partially inhibited by pretreatment with intracoronary staurosporine (10 μg/kg) (Figs 4 and 5). However, even after the treatment with staurosporine, vasoconstricting responses to the autacoids were still greater at the spastic LAD than at the control LCx. In contrast, at the control LCx, this PDBu-induced augmentation of coronary vasoconstriction was not observed in response to either serotonin or histamine, although the inhibition by staurosporine was noted in response to serotonin (Figs 4 and 5).

Bay K 8644 (10 μg/kg) also induced coronary spasm at the spastic LAD (Fig 6). Bay K 8644–induced coronary vasoconstriction was dose dependent and significantly inhibited by pretreatment with intracoronary PDBu (10⁻⁹ mol/kg) (Figs 4 and 5), whereas it was partially inhibited by pretreatment with intracoronary staurosporine (10 μg/kg) (Figs 4 and 5). However, even after the treatment with staurosporine, vasoconstricting responses to the autacoids were still greater at the spastic LAD than at the control LCx. In contrast, at the control LCx, this PDBu-induced augmentation of coronary vasoconstriction was not observed in response to either serotonin or histamine, although the inhibition by staurosporine was noted in response to serotonin (Figs 4 and 5).

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Fig 3. Plot shows dose-response relation of percent luminal narrowing in response to PDBu. PDBu-induced coronary vasoconstriction was dose dependent and was significantly greater at the spastic left anterior descending coronary artery (LAD) (closed circles) than at the control left circumflex coronary artery (LCx) (open circles).

Staurosporine (10 μg/kg) or nifedipine (0.1 mg/kg) (P < .01) (Fig 6).

Histologically, intimal and medial thickening, which was compatible with atherosclerotic changes, was noted at the spastic LAD but not at the control LCx (Fig 7). Full lining of regenerated endothelial cells at the spastic site was confirmed by electron microscopy, as we reported in the previous study.20

**Discussion**

The major findings of this study were (1) PDBu, a PKC-activating phorbol ester, induced coronary spasm at the atherosclerotic site, where serotonin and histamine also induced the spasm, (2) coronary spasm induced by serotonin and histamine was attenuated by pretreatment with staurosporine, a PKC inhibitor, but vasoconstricting responses to the autacoids were still greater at the spastic than at the control site even after pretreatment with staurosporine at a concentration that abolished the PDBu-induced coronary spasm, and (3) Bay K 8644, a dihydropyridine-sensitive L-type Ca2+ channel agonist, also induced coronary spasm at the spastic site, which was inhibited by pretreatment with staurosporine or nifedipine. To our knowledge, this is the first report that demonstrated the important role of PKC-mediated pathway in the pathogenesis of coronary artery spasm in vivo.

**PDBu-Induced Coronary Artery Spasm**

The previous in vitro experiments have shown that PKC-activating phorbol esters induce sustained coronary contraction.13 However, the mechanism of phorbol ester–induced vasocontraction is unknown. Fish et al21 reported that 12-O-tetradecanoyl phorbol-13-acetate, an active phorbol ester, increased the dihydropyridine-sensitive calcium conductance in the A7r5 rat aortic smooth muscle cell line. Other investigators have reported that phorbol-ester–induced vasocontraction is not accompanied by a change in intracellular calcium level,22,23 which implies that calcium sensitivity of the contractile proteins may be augmented by activating PKC with phorbol esters. Furthermore, PDBu inhibits endothelium-dependent relaxations in canine coronary arteries by inhibiting both synthesis/release and actions of EDHF.14,15 PDBu also stimulates the release of EDCF from canine femoral arteries.16 Despite these in vitro findings, little is known about the in vivo effects of phorbol esters on vascular responses, especially in relation to the pathogenesis of coronary spasm.

The present study demonstrates that PDBu, a PKC-activating phorbol ester, induced vasoconstriction in porcine coronary arteries in vivo and that the vasoconstriction was significantly greater at the spastic than at the control site in our swine model. The marked coronary vasoconstriction induced by PDBu (10−6 mol/kg) at the spastic site was accompanied by ischemic ECG changes. Thus, PDBu caused coronary spasm with myocardial ischemia, as did serotonin and histamine. Interestingly, however, PDBu-induced spasm lasted for a longer duration (response peaked at 20 minutes and was sustained for 50 to 60 minutes) than that induced by serotonin (response peaked at 2 to 3 minutes and was sustained for 5 to 7 minutes) or histamine (response peaked at 1 minute and was sustained for 3 to 4 minutes). These results are consistent with the in vitro finding that PDBu induces slowly developing contraction of isolated porcine coronary artery.24 PDBu-induced coronary spasm was completely blocked by pretreatment with intracoronary staurosporine. An inactive phorbol ester, PDD, did not induce coronary vasoconstriction. These results suggest that the PKC-mediated pathway in vascular smooth muscle is importantly involved in the pathogenesis of coronary spasm in our swine model. We also confirmed in the subsequent in vitro studies that responsiveness of vascular smooth muscle to PDBu was greater in vessels taken from the spastic site than in those from the control site (unpublished observations).

In the present study, PDBu was administered intracoronarily at an estimated concentration of 10−7 mol/L only for 3 minutes. The vasoconstrictor effect of PDBu was reproducible. These results suggest that the intra-

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**Fig 4.** Line plots: Serotonin-induced coronary vasoconstriction at the spastic left anterior descending coronary artery (LAD) (left) and the control left circumflex coronary artery (LCx) (right). Serotonin-induced spasm (open circles) was significantly augmented by pretreatment with intracoronary PDBu (10−6 mol/kg) (closed circle), whereas this spasm was partially inhibited by pretreatment with intracoronary staurosporine (10 μg/kg) (closed square) (left). Note that this augmentation was not observed at the control site, although the inhibition by staurosporine was observed in response to serotonin (right).
coronary administration of PDBu did not cause a sustained, intense activation of PKC that might lead to a downregulation of the enzyme.\textsuperscript{25}

If PKC is tonically activated at the atherosclerotic and spastic sites under basal conditions, a smaller response to PDBu (because of the reduced availability of PKC) and a greater vasodilation to staurosporine would be expected at the spastic site, which was not the case in the present study. PKC-mediated sustained vasoconstriction may be operative in the cerebral vasospasm after subarachnoid hemorrhage, where PKC is tonically activated under basal conditions and the spontaneous, diffuse vasospasm lasts for days to weeks.\textsuperscript{26} This type of vasospasm is in contrast to the coronary spasm in our model, where PKC-mediated responses are augmented only upon stimulation. Thus, we interpret our data as that the mass of and/or the substrate for PKC may be increased at the atherosclerotic and spastic sites and that PKC-mediated responses are augmented only upon stimulation but not under basal conditions. Although this interpretation should be examined in future studies, we have recently demonstrated in vitro that the PKC-mediated pathway is also importantly involved in the hyperconstriction to serotonin of isolated vascular smooth muscle from Watanabe heritable hyperlipidemic rabbits.\textsuperscript{27}

**PKC-Mediated Pathway in Serotonin- and Histamine-Induced Coronary Artery Spasm**

In the present study, serotonin- and histamine-induced coronary vasoconstriction was augmented by pretreatment with PDBu at the spastic but not at the control site in vivo. Miller et al.\textsuperscript{28} examined the role of the PKC-mediated pathway in regulating the contractile responses of isolated coronary arterial smooth muscle to a variety of stimuli in both intact and skinned coronary artery strips. They demonstrated that 12-\textit{O-}tetradecanoyl phorbol-13-acetate produced a leftward shift of the concentration-response relation of K\textsuperscript{+}, histamine-, and norepinephrine-induced contraction. They concluded that the PKC-mediated pathway regulates contractile responses of coronary arterial smooth muscle to a variety of stimuli at least in part by increasing the sensitivity of the contractile proteins to Ca\textsuperscript{2+}.\textsuperscript{28} The results of the present study are consistent with those previous findings.

However, in the present study, serotonin- and histamine-induced coronary spasm was only partially inhibited by pretreatment with staurosporine at a concentration of 10 \textmu{}g/kg, which abolished the PDBu-induced vasoconstriction; hyperconstriction to those autacoids still existed even after the PKC inhibition by staurosporine. In our swine model, the blockers of serotonin (ketanserin) and histamine (diphenhydramine) completely prevented the coronary spasm induced by serotonin and histamine, respectively, indicating that the signals for the hyperconstriction enter into the vascular smooth muscle exclusively through those receptors.\textsuperscript{5,6} Thus, these results may suggest that pathways other than the PKC-mediated one located downstream from the autacoid receptors are also involved in the pathogenesis of the spasm induced by the autacoids. The results may also suggest that the concentration of staurosporine used in the present study was relatively selective for PKC inhibition.

**Bay K 8644--Induced Coronary Artery Spasm**

Bay K 8644, a dihydropyridine-sensitive L-type Ca\textsuperscript{2+} channel agonist, also induced coronary spasm at the spastic site in our swine model. The finding that Bay K 8644--induced spasm was significantly inhibited by pretreatment with staurosporine suggests the important role of the PKC-mediated pathway in the Ca\textsuperscript{2+} channel agonist--induced spasm. A previous report from our institution demonstrated that Bay K 8644--induced coronary vasoconstriction was dose dependent and was significantly greater at the spastic left anterior descending coronary artery (LAD) than at the control left circumflex coronary artery (LCx) (open circles). Right, Bay K 8644 (10 \textmu{}g/kg)--induced spasm at the LAD (open bar) was significantly inhibited by pretreatment with intracoronary staurosporine (10 \textmu{}g/kg) (hatched bar) or nifedipine (0.1 mg/kg) (closed bar). These inhibitions were also observed at the control left circumflex coronary artery.
laboratory has shown that in our swine model there is no difference in the calcium-tension relation of the contractile proteins in the skinned arterial strips between the spastic and the control site, indicating that calcium sensitivity of the contractile proteins per se is not augmented at the spastic site. Fish et al demonstrated that activation of PKC increases the dihydropyridine-sensitive Ca\(^{2+}\) conductance in a vascular smooth muscle cell line. Thus, the results in the present study suggest that Ca\(^{2+}\) influx through the dihydropyridine-sensitive L-type Ca\(^{2+}\) channel and/or calcium sensitivity of the contractile proteins may be augmented by the PKC-mediated pathway.

**Role of PKC-Mediated Pathway in the Pathogenesis of Coronary Spasm**

As discussed earlier, we speculate that the mass of and/or the substrate for PKC may be increased at the atherosclerotic and spastic site. It is also possible that the signal transduction for vasoconstriction is augmented at a certain step of PKC-mediated pathway. Indeed, the role of the PKC-mediated pathway in the vascular smooth muscle hypercontraction may be complex, and multiple sites might be involved in addition to PKC itself. Those include the dihydropyridine-sensitive L-type Ca\(^{2+}\) channel, sarcolemmal receptor-operated Ca\(^{2+}\) channels and the sarcoplasmic reticulum (SR) inositol triphosphate (IP\(_3\))/Ca\(^{2+}\) release channel (mechanisms allowing entry of Ca\(^{2+}\) into the cytosol upon activation), and the sarcolemmal and SR Ca\(^{2+}\) pumps and the sarcolemmal Na\(^+\)/Ca\(^{2+}\) exchanger (mechanisms removing Ca\(^{2+}\) from the cytosol). Our data suggest that among those possible mechanisms, at least the interaction between the PKC-mediated pathway and the dihydropyridine-sensitive L-type Ca\(^{2+}\) channel may be augmented at the spastic site.

Although it was beyond the scope of this study to examine all of the possible mechanisms, another important clue was that PGF\(_{2\alpha}\) induced comparable coronary vasoconstriction at the spastic and control sites. This finding also has been repeatedly observed in our previous studies. In our swine model, phenylephrine and a thromboxane analogue also caused comparable coronary vasoconstriction at the spastic and control sites. These results may not only exclude the major contribution of the geometric theory but also suggest that coronary hyperreactivity is developed selectively to some agonists but not to others, probably due to the altered intracellular signal transduction mechanisms. Recently, it has been reported that in the rat basilar artery PKC mediates vasoconstrictor responses to serotonin but not to prostaglandin F\(_{2\alpha}\) in vivo. Frequently, also we have observed that the vasospastic responses of porcine coronary arteries to serotonin and histamine but not the vasoconstriction to prostaglandin F\(_{2\alpha}\) were significantly inhibited by staurosporine in vivo. In contrast, ryanodine, which inhibits the Ca\(^{2+}\)-induced Ca\(^{2+}\) release from the SR store, significantly inhibited the vasoconstriction of porcine coronary arteries to prostaglandin F\(_{2\alpha}\) but not the vasospastic responses to serotonin or histamine in vivo. These results suggest that the PKC-mediated pathway is important for the vasospastic responses induced by serotonin and histamine, whereas ryanodine-mediated calcium release may be important for the vasoconstriction induced by prostaglandin F\(_{2\alpha}\). Further studies are needed to clarify the mechanisms for the selective hyperreactivity at the spastic site.

**Clinical Implications**

An intimate relation between coronary spasm and atherosclerotic changes has been repeatedly confirmed in our swine model. Atherosclerosis is the inflammatory/proliferative process in which the PKC-mediated pathway may play an important role. The present study suggests that the PKC-mediated pathway is im-
portantly involved in the pathogenesis of coronary spasm in our swine model. If this is also the case for human coronary artery spasm, the present findings may have important clinical implications in understanding the pathogenesis of the spasm and in developing newer therapeutic interventions to prevent and manage it.

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