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Interaction of Calmodulin- and PKC-Dependent Contractile Pathways In Cat Lower Esophageal Sphincter (LES)

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We have previously shown that, in circular muscle cells of the lower esophageal sphincter (LES) isolated by enzymatic digestion, contraction in response to maximally effective doses of acetylcholine (ACh) or Inositol Triphosphate (IP_3) depends on the release of Ca^{2+} from intracellular stores and activation of a Ca^{2+}-calmodulin (CaM)-dependent pathway. On the contrary, maintenance of LES tone, and response to low doses of ACh or IP_3 depend on a protein kinase C (PKC) mediated pathway. In the present investigation, we have examined requirements for Ca^{2+} regulation of the interaction between CaM- and PKC-dependent pathways in LES contraction. Thapsigargin (TG) treatment for 30 min dose dependently reduced ACh-induced contraction of permeable LES cells in free Ca^{2+} medium. ACh-induced contraction following the low level of reduction of Ca^{2+} stores by a low dose of TG (10^{-9} M) was blocked by the CaM antagonist, CGS9343B but not by the PKC antagonists chelerythrine or H7, indicating that the contraction is CaM-dependent. After maximal reduction in intracellular Ca^{2+} from Ca^{2+} stores by TG (10^{-6} M), ACh-induced contraction was blocked by chelerythrine or H7, but not by CGS9343B, indicating that it is PKC-dependent. In normal Ca^{2+} medium, the contraction by ACh after TG (10^{-9} M) treatment was also CaM-dependent, whereas the contraction by ACh after TG (10^{-9} M) treatment was PKC-dependent. We examined whether PKC activation was inhibited by activated CaM. CGS 9343B inhibited the CaM-induced contraction, but did not inhibit the DAG-induced contraction. CaM inhibited the DAG-induced contraction in the presence of CGS 9343B. This inhibition by CaM was Ca^{2+} dependent. These data are consistent with the view that the switch from a PKC-dependent pathway to a CaM dependent pathway can occur and can be regulated by cytosolic Ca^{2+} in the LES.

Key words: Calmodulin, Lower esophageal sphincter (LES), Inositol triphosphate (IP3), CaM antiagonist, PKC-dependent

INTRODUCTION

In smooth muscle cells isolated from the circular layer of lower esophageal sphincter (LES), contraction in response to a maximally effective dose of ACh is mediated through a calmodulin (CaM)-dependent pathway (Biancani *et al.*, 1994), but the tone or low dose of ACh is mediated througha PKC-dependent pathway. Both CaM and MLCK play a role in the ACh-induced contraction of LES but not of esophagus. This is PKC-dependent, but not regulated by CaM-MLCK (Sohn *et al.*, 2001).

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PKC- and Ca^{2+}/CaM-dependent pathways are very important for the intracellular regulation of signal transduction in smooth muscle. PKC displays synergistic action with Ca^{2+}. PKC can inhibit cellular response produced by receptor-induced Ca^{2+}-mobilization. CaM has also been studied as also an inhibitor of PKC (Pribilla *et al.*, 1988), since high CaM (1-2 μM) can inhibit PKC activity. The interaction of CaM with calponin and caldesmone might play a role in the regulation of PKC phosphorylation (Naka *et al.*, 1990). Although the mechanism underlying PKC and Ca/CaM action is still not known well, LES tone or ACH-induced submaximal contraction of LES is associated with low level phospholipase C activity, resulting in formation of IP_3, release of low Ca^{2+} concentrations from intracellular stores and the potentiation of diacylglycerol (DAG) to activate a PKC-

dependent pathway. In contrast, maximal cholinergic stimulation activates a calmodulin-dependent pathway (Biancani *et al.*, 1994; Hillemeier *et al.*, 1996; Sohn *et al.*, 2001). It may be of possible interest to examine that the switch from a PKC-dependent to a calmodulin-dependent pathway may result from the different calcium requirements of these pathways.

The purpose of the present study was to answer questions from the following two findings: 1) the switch from a PKC-dependent to a CaM-dependent pathway may result from variations in Ca^{2+} sensitivity. 2) CaM may inhibit PKC-mediated contraction.

METERIALS AND METHODS

Animals

Adult cats of either sex weighing between 2.5 and 5 kilograms were used. The animals were initially anesthetized with ketamine (Aveco Fort Dodge IA), then euthanized with an overdose of phenobarbital (Schering Corp., Kennilworth, NJ). The chest and abdomen were opened with a mid-line incision exposing the esophagus and stomach. The esophagus and stomach were removed together, opened along the lesser curvature and pinned on a wax block at their *in vivo* dimensions. The location of the squamo-columnar junction was identified and the mucosa was peeled. The high pressure zone of the LES is characterized by a visible thickening of the circular muscle layer in corresponding to the squamo-columnar junction, and is immediately proximal to the sling fibers of the stomach.

Tissue dissection and dispersion of smooth muscle cells

The esophagus and LES smooth muscle squares were prepared as previously described (Biancani *et al.*, 1987; Sohn *et al.*, 1993). Tissue squares were chopped with a fine scissor, and digested in HEPES buffer, containing 0.1% collagenase type II to isolate smooth muscle cells. The HEPES solution contained: NaC1 115 mM, KC1 5.8 mM, KH_2PO_4 12 mM, glucose 2.5 mM, (N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]) sodium salt (HEPES) 25 mM, $CaC1_2$ 2 mM, $MgC1_2$ 0.6 mM, BME amino acid supplement 0.3 mg/ml, soybean trypsin inhibitor 0.09 mg/ml. The solution was gently gassed with 100% O2. At the end of the digestion period, the tissue was poured out over a 450 μm nylon mesh (Tetko Inc., Elmsford, NY), rinsed in collagenase-free HEPES buffer to remove any trace of collagenase, and then incubated in this solution at 31°C, and gassed with 100% O_2. The cells were allowed to dissociate freely for 10-20 min.

The cells were permeabilized, when required, to control intracellular Ca^{2+} concentration and to allow the use of agents such as PKC antibodies, which do not diffuse across the intact cell membrane. After completion of the enzymatic phase of the digestion process, the partly digested muscle tissue was washed with an enzyme-free cytosolic buffer of the following composition (mM): NaCl 20, KCl 100, $MgSO_4$ 5.0, NaH_2PO_4 0.96, EGTA 1.0, $CaCl_2$ 0.48, and 2% bovine serum albumin. The cytosolic buffer was equilibrated with a 95% O_2/5% CO_2 mixture to maintain a pH of 7.2 at 31°C. The muscle cells dispersed spontaneously in this medium, and were then permeabilized by incubation for 3 min in cytosolic buffer containing saponin 75 μg/ml. After exposure to saponin the cell suspension was spun at 500 g and the resulting pellet was resuspended in saponin-free modified cytosolic buffer containing antimycin A 10 μM, ATP 1.5 mM, and an ATP-regenerating system consisting of creatine phosphate 5 mM and creatine phosphokinase 10 units/ml. After the cells were washed free of saponin they were resuspended in modified cytosolic buffer.

Agonist-induced contraction of isolated muscle cells

The cells were contracted by exposure for 30 sec to ACh (5 sec). When antagonists (chelerythrine, H7, CGS9343B) were used the cells were incubated in each concentrations of the antagonists for 1 min prior to agonist addition. To deplete the Ca^{2+} stores, the cells were treated with thapsigargin (TG, 3 μM) for 30 min in permeable cells. After exposure to agonists the cells were fixed in acrolein at a final concentration of 0.6%. A drop of the cell-containing medium was placed on a glass slide, covered by a cover slip, and the edges were sealed with nail enamel to prevent evaporation.

The length of thirty consecutive, randomly encountered, intact cells in each slide were measured with a phase-contrast microscope (model CK2, Olympus, Tokyo, Japan), and a closed-circuit video camera (model IK-642K, Toshiba, Tokyo, Japan) connected to a Macintosh Computer (Apple Inc., Cupertino, CA, USA) with an image analysis software program (Image 1.59, National Institute of Health, Bethesda, MD, USA). Contraction was expressed as the average percentage shortening of the 30 consecutive cells compared with control.

Protein determination

Protein content was obtained after hydrolysis by 0.1 N NaOH at 80°C to solubilize the protein, followed by neutralization with HCl. The amount of protein present was determined by colorimetric analysis (Bio Rad Laboratories, Richmond CA) according to Bradfords method (Bradford, 1976).

Drugs and chemicals

Collagenase type II and soybean trypsin inhibitor was purchased from Worthington Biochemicals (Freehold,

NJ); H7 (1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride) from Seikagaku America Inc. (St. Petersberg, FL); calmodulin from Calbiochem (San Diego, CA); L-a-1,2-dioctanoyl glycerol from Avanti Polar Lipids (Alabaster, AL); chelerythrine from Research Biochemicals (Natick, MA). Cam antagonist CGS 9343B was a gift from Dr. Rchard A. Lovell of Ciba-Geigy Corp. (Summit, NJ) Acetylcholine, saponin, thapsigargin, BME amino acid supplement, ethylene glycol-bis (b-amino ethyl ether) n, n,n',n'-tetra-acetic acid (EGTA), guanosine 5'-0-(3-thiotriphosphate) tetralithium salt (GTPgS), (N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]) sodium salt (HEPES), creatine phosphate, creatine phosphokinase, ATP, antimycin A, and other reagents were purchased from Sigma (St. Louis, MO)

Data analysis

Data are expressed as the mean ± S.E.M. Statistical differences between means were determined by the Student's *t*-test. Differences between multiple groups were tested using analysis of variance for repeated measures and checked for significance using the Scheffe F-test.

RESULTS

ACh-induced contraction after depletion of intracellular Ca^{2+} by thapsigargin

In LES circular muscle cells isolated by enzymatic digestion, contraction in response to maximally effective doses of ACh or IP_3 depends on release of Ca^{2+} from intracellular stores and activation of a Ca^{2+}-CaM dependent pathway (Biancani *et al.*, 1994). On the contrary, maintenance of LES tone, and response to low doses of ACh or IP_3 depend on a PKC mediated pathway. We have therefore proposed that the switch between the PKC dependent and the Ca^{2+}-CaM dependent pathways depends on different Ca^{2+} concentrations released by various stimuli.

Cells were maintained in Ca^{2+} free physiological salt solution to examine contraction mediated exclusively by intracellular Ca^{2+} release. In the absence of extracellular Ca^{2+}, thapsigargin (TG) incubation for 30 min dose dependently reduced ACh -induced contraction of permeable LES cells (Fig. 1A). ACh-induced maximum contraction following low level reduction of Ca^{2+} stores by low dose of thapsigargin (TG, 10^{-9} M) in Ca^{2+} free medium was blocked by the CaM antagonist CGS9343B but not by the PKC antagonists chelerythrine or H7 (Fig. 1A). In contrast, after maximal depletion of Ca^{2+} stores by TG (10^{-6} M) in 0 Ca^{2+} medium, ACh-induced contraction was small, and blocked by chelerythrine or H7 ($P < 0.01$ by ANOVA), but not by CGS9343B (Fig. 1A). These data are consistent with the results regarding the low dose effects of IP_3 or ACh already presented in Biancanis finding (Biancani *et al.*, 1994). Low intracellular Ca^{2+} ($[Ca^{2+}]i$) activates PKC-dependent pathway, whereas a high dose of IP_3 or ACh , and high $[Ca^{2+}]i$ activates CaM-dependent pathway. Additionally, TG was incubated for 30 min in normal Ca^{2+} medium. Even in conditions of high depletion of Ca^{2+} store with high TG, the degree of ACh-induced contraction was still over 70% when compared to maximum contraction (Fig. 2A). ACh-induced contraction following reduction of Ca^{2+} stores by TG (10^{-8} M) was partially blocked by CGS9343B (10^{-5} M) but not by chelerythrine (10^{-5} M) or H7 (10^{-5} M) (Fig. 2A). In contrast, the ACh-induced contraction following reduction of Ca^{2+} stores by TG (10^{-6} M) was blocked by chelerythrine or H7, but not by CGS9343B (Fig. 2B). These data suggest that Ca^{2+} influx might be related to the PKC-dependent

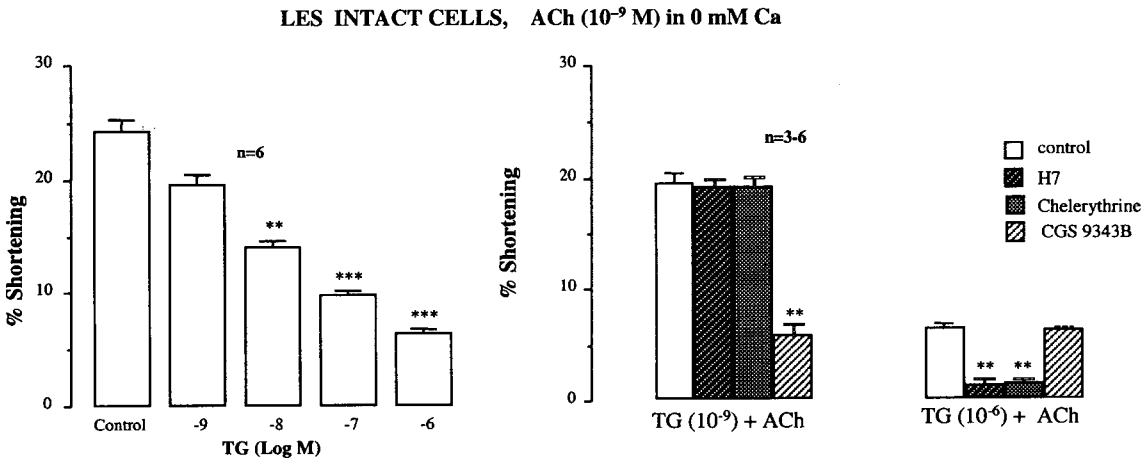

Fig. 1. Thapsigargin (TG) incubation on ACh -induced contraction of permeable LES cells in 0 mM Ca^{2+}. TG incubation for 30 min dose dependently reduced ACh -induced contraction of permeable LES cells (Fig. 1A). ACh-induced maximum contraction after small reduction of Ca^{2+} stores by low dose of thapsigargin (TG, 10^{-9} M) was blocked by the CaM antagonist CGS9343B but not by the PKC antagonists chelerythrine or H7 (Fig 1B). **; $P < 0.01$, ***; $P < 0.001$ by ANOVA.

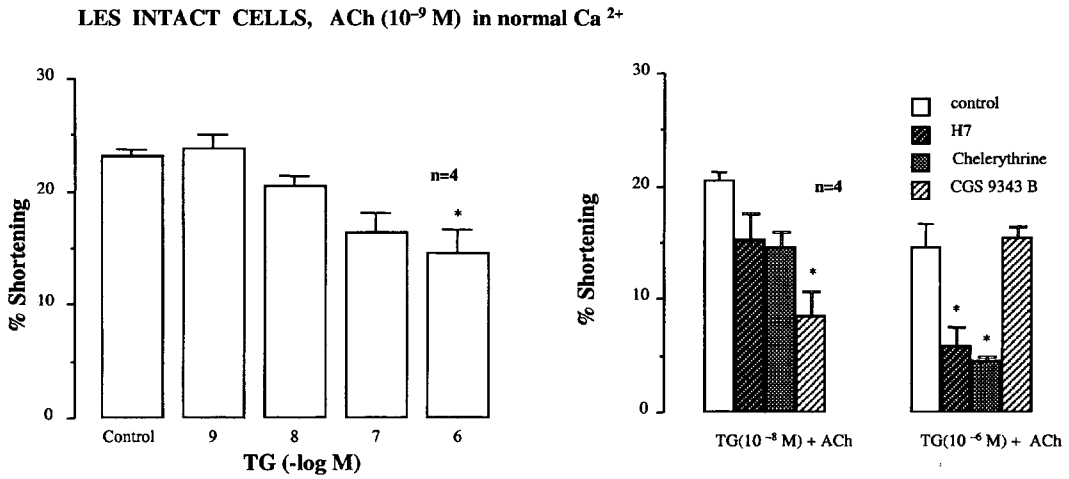

Fig. 2. Thapsigargin (TG) incubation on ACh -induced contraction of permeable LES cells in normal Ca^{2+}. The ACh-induced contraction after reduction of Ca^{2+} stores by TG (10^{-8} M) in normal Ca^{2+} medium was partially blocked by CGS9343B (10^{-5} M) but not by chelerythrine (10^{-5} M) or H7 (10^{-5} M) (Fig. 2A). In contrast, ACh-induced contraction after reduction of Ca^{2+} stores by TG (10^{-6} M) was blocked by chelerythrine or H7, but not by CGS9343B (Fig. 2 B). *; $P<0.05$ by ANOVA.

contraction.

CaM inhibit PKC dependent pathway

We proposed DAG induced contraction requires lower Ca^{2+} concentrations than CaM. The normal 0.18 μM Ca^{2+} cytosolic medium does not support the CaM-induced contraction (Biancani *et al.*, 1994; Sohn *et al.*, 1994). To test if CaM could inhibit the effect of PKC, we examined the effect of CaM on the DAG-induced contraction. The contraction by maximal dose of CaM (10^{-6} M) in presence of 1.0 μM Ca^{2+} was inhibited by CGS9343B (***; $P<0.001$ by ANOVA), but the DAG induced contraction was not affected. DAG induced contraction was inhibited by CaM in presence of CGS9343B (***; $P<0.001$ by ANOVA). We also found that CaM dose dependently inhibited DAG-induced contraction at 1.0 mM $[Ca^{2+}]i$ ($P<0.01$ by two factors ANOVA), but that this inhibition was not manifested at either 0.18 or 0.76 μM $[Ca^{2+}]i$ (Fig. 4).

DISCUSSION

LES maintains basal resting tone and contracts in response to agonists such as ACh. The ACh-induced contraction of LES smooth muscle results from a change grom phospholipase C-induced metabolism of PIP_2 to IP_3 and DAG (Sohn *et al.*, 1993; Sohn *et al.*, 1994). On the contrary, maintenance of LES tone, and response to low doses of ACh or IP_3 depend on a PKC mediated pathway. We have also shown that the PKC and the Ca^{2+}-CaM dependent pathways have different Ca^{2+} requirements (Biancani *et al.*, 1994). It was aimed to investigate whether

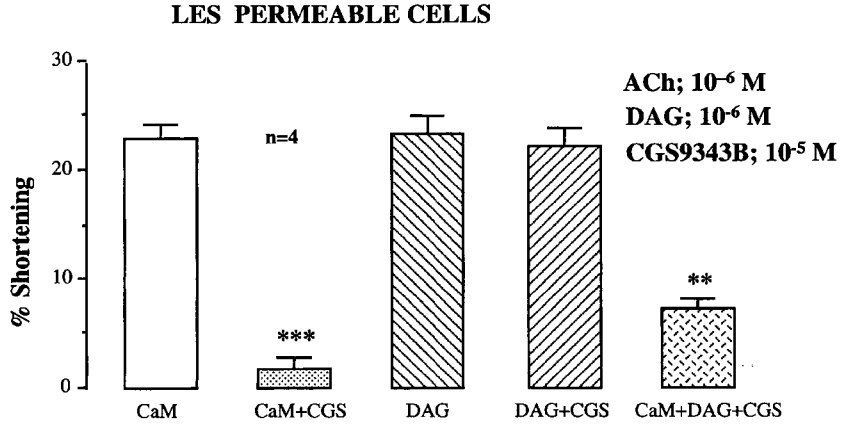

Fig. 3. CaM-dependent inhibition to DAG-induced contraction. The contraction by CaM (10^{-6} M) in presence of 1.0 μM Ca^{2+} was inhibited by CGS9343B (***; $P<0.001$), but DAG induced contraction was not affected. DAG induced contraction was inhibited by CaM in presence of CGS9343B (**; $P<0.01$ by ANOVA).

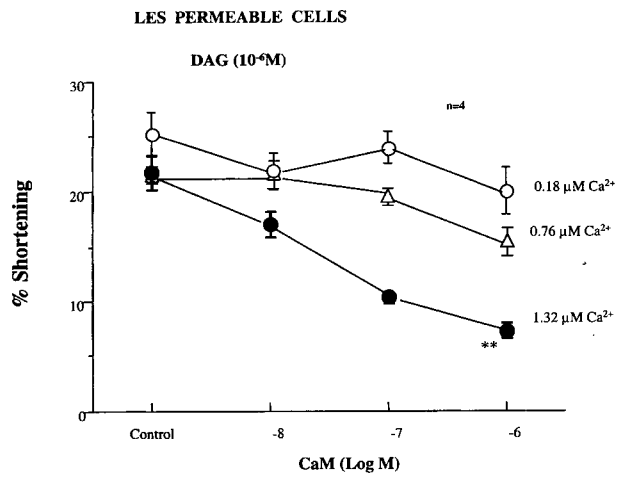

Fig. 4. The effect of Ca^{2+} on the inhibitory action of CaM in DAG-induced contraction. The CaM dose response curve on DAG-induced contraction in the presence of a normal Ca^{2+} (0.18 μM) was not different when compared the CaM dose response curve in presence of 0.76 μM Ca^{2+}. However, in presence of 1.0 μM [Ca^{2+}]i CaM dose dependently inhibited the DAG-induced contraction. **; P<0.01 by two factors ANOVA.

the switch between the PKC dependent and the Ca^{2+}-CaM dependent pathways depends on variations in Ca^{2+} concentrations that are regulated by TG treatment.

In attempting to investigate the role of endogenous Ca^{2+} on ACh-induced contraction, TG was incubated for 30 min to deplete intracellular Ca^{2+}. TG has been shown to enhance the release and / or prevent uptake of IP_3-sensitive and IP_3-insensitive Ca^{2+} (Bian *et al.*, 1991). As expected, ACh produced very little contraction after high TG treatment, which was PKC dependent. Such a result indicates that 70% of the Ca^{2+} source was derived from Ca^{2+} storage, a Ca^{2+} store which may be IP_3-sensitive. This suggestion was confirmed by the finding that the ACh-induced contraction was mediated by IP_3 receptor activation, a finding confirmed using IP_3 receptor antagonist heparin (Sohn *et al.*, 1993; Yang *et al.*, 2000). This is consistent with our own finding that low rates of PIP_2 hydrolysis with the formation of low dose of IP_3 and DAG may occur during the development of basal tone, since basal tone and IP_3 production were reduced by PLC inhibitor U73122 (Biancani *et al.*, 1994). On the other hand the maximal contraction induced by ACh may cause maximal activation of phospholipase C along with maximal production of IP_3, and a magnitude of Ca^{2+} release sufficient to activate CaM, indicating that the maximal Ca^{2+} release may activate CaM. We found that CaM has relatively low Ca^{2+} affinity and requires a high Ca^{2+} concentration for activation. Previous data showed that 1.3-3 μM Ca^{2+} is required to obtain CaM-induced contraction. Others have found that the dissociation constant for the Ca^{2+} binding sites on CaM, under ionic conditions approximating the intracellular medium, range from 10^{-6} M to 10^{-4} M (Stoclet *et al.*, 1987). These values are of the same order of magnitude as those used in our investigation.

CaM is a calcium-dependent regulatory protein that contains four domains of similar amino acid sequences corresponding to Ca^{2+} binding sites (Stoclet *et al.*, 1987). When CaM is bound to Ca^{2+} it undergoes a conformational change that exposes hydrophobic domains that are recognized by target proteins (LaPorte *et al.*, 1980). In smooth muscle this transformation allows the formation of a complex between Ca^{2+}-CaM and myosin light chain kinase, resulting in myosin light chain phosphorylation, cross bridge cycling and smooth muscle contraction.

There is evidence that CaM inhibits PKC activity. CaD and calponin are phosphorylated by PKC, whereas although CaM inhibited dose-dependently the phosphorylation of CaP by PKC to basal activity, it did not inhibit the phosphorylation of MLC by PKC (Naka *et al.*, 1990). CaM also inhibited dose-dependently the phosphorylation of p75 in heat-treated sonicated A10 cells by exogenous PKC in the presence of 1.0 μM Ca^{2+} but not in lower Ca^{2+} (Kruger *et al.*, 1990; Yu *et al.*, 1993; Zhao *et al.*, 1991).

Low rates of PIP_2 hydrolysis with the formation of low concentrations of IP_3 and DAG may occur during the development of LES basal tension. We also found that these two compounds potentiated each other to cause submaximal contraction, in a effect which was more additive (Biancani *et al.*, 1994). This potentiation effect appears to depend on intracellular Ca^{2+} release and on the activation of the PKC pathway although the effect is partly blocked by Sr^{2+} substitution for Ca^{2+} (to a level of DAG alone) and completely by H-7, but is unaffected by the CaM antagonist, similar to the tone in muscle strips (Biancani *et al.*, 1994). Previous studies have also shown that PKC could be activated by the synergistic action of an increase in Ca^{2+} concentration and formation of DAG, and that DAG dramatically increases the affinity of PKC for Ca^{2+} (Nishizuka, 1989, 1986).

It thus appears likely that there is switching phenomen between these two pathways, one that is dependent on the concentrations of IP_3, and that the key factor in modulating this switch might be their Ca^{2+} dependence, because different concentrations of IP_3 may release different concentrations of Ca^{2+} (Makhlouf, 1988; Murthy *et al.*, 1991). This assumption was supported by the following study in which the effect of activated CaM on muscle contraction mediated by the PKC pathway was tested. Exogenously activated CaM in 1.0 μM Ca^{2+} medium inhibited the PKC-mediated contraction. CGS-9343B was used in these experiments to block the activation of MLCK by CaM itself. Similar observations have been made in other tissues. It has also been shown that CaM binds the PKC substrates, i.e,. calponin in chicken gizzard smooth muscle, p75 in A10 smooth muscle cells from rat thoracic aorta, and the myristylated alanine-rich C kinase substrate in the brain, rather than the PKC itself and that CaM inhibits

phosphorylation of the substrates by PKC (Naka *et al.*, 1990; Zhao *et al.*, 1991).

It has been proposed that the site of interaction on CaM molecule with its antagonist must be different from the epitope that interacts with PKC (Kruger *et al.*, 1990), also tested the effects of two different CaM fragments on PKC activity. Each of the fragments contained an intact Ca^{2+}-binding domain with complete helix-loop-helix structure plus a few amino acids. Both fragments had the same potency for inhibiting PKC as intact CaM, but they did not stimulate cAMP phosphodiesterase even at concentrations hundreds of times greater than the CaM concentration needed for maximal stimulation. Furthermore, CaM can activate many different targets because of its unique structure (Kretsinger, 1992a, b). It is therefore likely that any conformational changes in the CaM molecule could alter its binding to specific targets.

These data are consistent with the view that the switch from a PKC-dependent pathway to a CaM dependent pathway can occur and be regulated by cytosolic Ca^{2+} in the LES. We found that CaM may inhibit the PKC-mediated contraction.

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