

Phorbol ester-induced protein phosphorylation and contraction in sphincter smooth muscle of rabbit iris

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Phorbol 12,13-dibutyrate (PDBu) was employed in studies designed to determine the role of C-kinase in muscle contraction in the iris sphincter. PDBu induced MLC phosphorylation and contraction in a dose- and time-dependent manner. Maximum responses exerted by PDBu were about 50–60% of that obtained with CCh, and were totally dependent on the presence of extracellular Ca^{2+} . PDBu had no effect on basal IP_3 levels, however it blocked the CCh-stimulated accumulation of IP_3 . PDBu-induced effects were potentiated by ionomycin, and inhibited by the C-kinase antagonist H-7. These results provide further evidence for the involvement of C-kinase in mediating the sustained phase of the contractile response in the iris sphincter.

1 Protein kinase; Phosphorylation; Myosin light chain; Muscle contraction; Phorbol ester; Ca^{2+} ;
(Iris sphincter smooth muscle)

1. INTRODUCTION

In smooth muscle, activation of Ca^{2+} -mobilizing receptors leads firstly to the rapid breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) to release inositol trisphosphate (IP_3) and 1,2-diacylglycerol (DG), the two putative second messengers [1,2], and secondly causes a contractile response which is mediated through an increase in the intracellular concentration of Ca^{2+} (review [3]). Further, there is good experimental evidence which suggests that synergism between IP_3 and DG could play an important role in regulating smooth

muscle contraction [3–6]. According to this the agonist-induced hydrolysis of PIP_2 may lead to the following events: (a) release of IP_3 , followed by Ca^{2+} mobilization from the sarcoplasmic reticulum, an increase in cytosolic free Ca^{2+} , activation of MLC kinase to phosphorylate the MLC proteins and consequently lead to contraction; (b) formation of DG, followed by activation of the Ca^{2+} -phospholipid dependent protein kinase (C-kinase), phosphorylation of MLC proteins, thus leading to contraction. In support of these concepts, in vascular smooth muscle IP_3 (0.5–30 μM) caused Ca^{2+} release and tension development in rabbit main pulmonary artery muscle permeabilized with saponin or digitonin [7], which has also been shown in other types of smooth muscle (review [3]). Furthermore, synergism between Ca^{2+} and activators of C-kinase, such as phorbol esters, has been reported to mediate contraction in various types of smooth muscle [4–6,8–13].

Previous work from this laboratory on the rabbit iris smooth muscle has suggested that agonist-stimulated hydrolysis of PIP_2 into IP_3 and DG may be involved in the coupling of Ca^{2+} -mobil-

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Abbreviations: PDBu, phorbol 12,13-dibutyrate; PMA, phorbol-12-myristate-13-acetate; PIP_2 , phosphatidylinositol 4,5-bisphosphate; DG, 1,2-diacylglycerol; IP_3 , inositol trisphosphate; MLC, myosin light chain; CCh, carbachol; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine

izing receptors to muscle response [5,14,15]. A close correlation between agonist-induced PIP_2 hydrolysis, MLC phosphorylation and contraction was demonstrated, and it was suggested that IP_3 and DG act synergistically to mediate muscle contraction. IP_3 , through intracellular Ca^{2+} mobilization and Ca^{2+} -calmodulin dependent MLC phosphorylation, may serve to initiate the phasic component of muscle response, while DG, acting through C-kinase and phosphorylation of MLC and/or other proteins, may mediate the sustained phase of the contractile response. In the present study we have employed agents which bypass receptor-mediated events and directly activate either the Ca^{2+} -calmodulin- or the Ca^{2+} -phospholipid dependent kinase in an attempt to determine whether or not the C-kinase branch of the Ca^{2+} -messenger system is involved in the sustained phase of muscle contraction in the iris sphincter. Our results show that the phorbol ester, PDBu, induces MLC phosphorylation and contraction in a dose- and time-dependent manner in this smooth muscle. These effects are dependent on extracellular Ca^{2+} , potentiated by the Ca^{2+} -ionophore, ionomycin, and inhibited by the C-kinase inhibitor H-7.

2. MATERIALS AND METHODS

2.1. Materials

Carbachol (CCh) and PMA were purchased from Sigma (St. Louis, MO). PDBu and 4α -PDBu were obtained from LC Service Corporation (Woburn, MA). Ionomycin was obtained from Calbiochem (San Diego, CA). [$^{32}\text{P}_i$]Orthophosphate, carrier-free, was from New England Nuclear (Boston, MA), and H-7 was purchased from Seikagaku America (St. Petersburg, FL).

2.2. Incubation of the sphincter muscle with $^{32}\text{P}_i$

Rabbit iris sphincter was isolated and incubated in a modified Krebs-Ringer bicarbonate buffer (pH 7.4) containing $50 \mu\text{Ci/ml}$ $^{32}\text{P}_i$ at 37°C for 90 min as described in [5]. The pH of the buffer was maintained at 7.4 with 97% O_2 /3% CO_2 . Following the 90 min preincubation, a period sufficient for equilibration of $^{32}\text{P}_i$ with intracellular ATP, CCh or phorbol esters were added and incubation continued for various time intervals. In experiments where H-7 was employed, the tissue

was incubated with the drug for 10 min prior to addition of the agonist. The reaction was stopped by immersion of the tissue in a methanol-dry ice slurry at -80°C . The tissue was then homogenized in 5% ice-cold trichloroacetic acid.

2.3. Measurement of $^{32}\text{P}_i$ incorporation into phosphoproteins

The trichloroacetic acid insoluble material was solubilized in $200 \mu\text{l}$ of SDS-sample buffer [16] by boiling for 30 min in sealed tubes. Protein was determined by the method of Lowry [17] using bovine serum albumin as standard. Aliquots containing $100 \mu\text{g}$ of protein were separated by SDS-polyacrylamide gel electrophoresis [16]. Gels were stained with Coomassie blue and dried onto filter paper. Results were analyzed by autoradiography on Kodak XAR-5 film. For quantitation the band corresponding to MLC was excised from the dried gel and counted in a Beckman liquid scintillation counter.

2.4. Measurement of the contractile response

Muscle contraction was as described in [5]. The sphincter muscles were mounted individually in separate organ baths (20 ml) containing the Krebs-Ringer buffer. A mixture of 97% O_2 and 3% CO_2 was bubbled continuously through the buffer maintained at 37°C . The tissue was allowed to equilibrate for 90 min under a resting tension of 50 mg. After equilibration, the agonist was added and changes in tension were monitored continuously using a Grass FT-03 force transducer connected to a Grass amplifier.

3. RESULTS

Although the phorbol ester PMA has been shown to induce contraction in certain types of smooth muscles [4,13], we have reported that it had no effect on MLC phosphorylation and muscle contraction in the iris sphincter ([5] and table 1). In the present studies we have investigated the effects of PDBu, instead of PMA, in the iris sphincter, and as can be seen from fig.1 the phorbol ester increased MLC phosphorylation and contraction in a dose-dependent manner, over a concentration range of 10–200 nM. These PDBu-induced responses were found to be completely

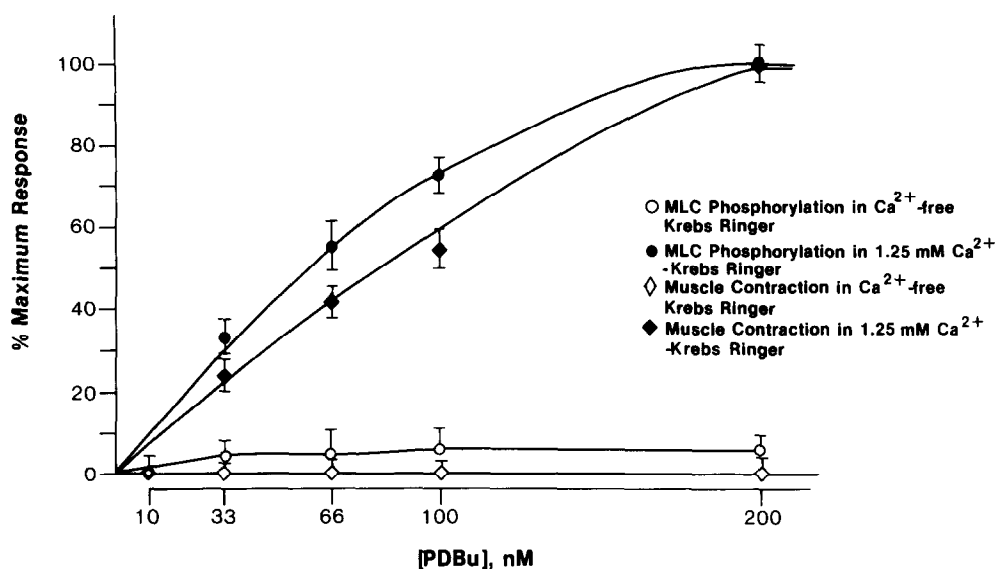


Fig.1. Dose-response effect of PDBu on MLC phosphorylation and muscle contraction in the iris sphincter in the absence and presence of extracellular Ca^{2+} . The tissues were first incubated for 90 min in 1 ml of Krebs-Ringer bicarbonate buffer containing 50 μCi of ^{32}P . Different concentrations of PDBu were added and incubation continued for 35 min. The incubations were terminated and radioactivity in tissue proteins was analyzed as described in section 2. The average ^{32}P radioactivity recovered in the MLC band in the control experiment was 7040 cpm/mg of total tissue proteins. For measurement of isometric contraction, the tissue was pre-equilibrated for 90 min before the addition of different contractions of PDBu. The data are mean $\pm$ SE of three experiments conducted in duplicate.

dependent on extracellular Ca^{2+} . Thus, in the absence of the cation PDBu failed to stimulate MLC phosphorylation or induce muscle contraction (fig.1). PDBu had no significant effect on basal IP_3 levels, however, it inhibited CCh-stimulated IP_3 accumulation in this smooth muscle (not shown).

Time course studies revealed that after an initial lag period of 10–15 min, PDBu (200 nM) increased progressively both MLC phosphorylation and contraction, and these effects were maximal at about 35 min (fig.2). Maximum effects exerted by PDBu were about 60% of that obtained with CCh (table 1). The α -isomer of PDBu, 4 α -PDBu, which has been shown to be inactive in C-kinase activation [18], was without effect on MLC phosphorylation and contraction in the iris sphincter (table 1).

Addition of the Ca^{2+} -ionophore, ionomycin (5 μM), to the iris sphincter increased both MLC phosphorylation and contraction, and when added in the presence of PDBu (200 nM) there was a 69% increase in contraction (table 1). Time-course

studies revealed that addition of the cationophore induced a slow but progressive contractile response in the iris sphincter (fig.3). Furthermore, addition of ionomycin and PDBu together augmented the

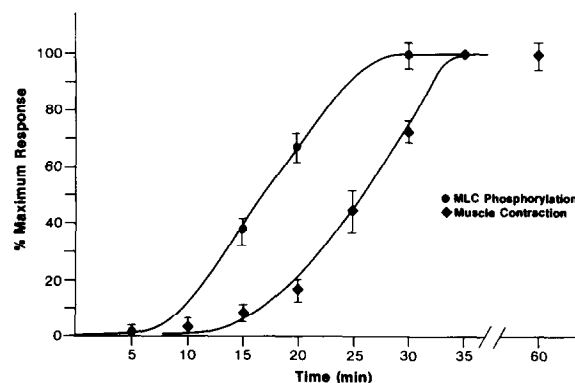


Fig.2. Time course of PDBu-induced MLC phosphorylation and contraction in the iris sphincter. Experiments were conducted as described in the legend to fig.1 and in section 2. The concentration of PDBu used was 200 nM. The data are mean $\pm$ SE of three separate experiments, each conducted in duplicate.

rapidity of contraction, and the resulting response more closely resembled that seen with CCh than with either drug used separately (fig.3). The $t_{1/2}$ values for the increase in tension by ionomycin, PDBu, ionomycin plus PDBu and CCh were 24 min, 27 min, 8 min and 11 s, respectively (fig.3).

Further support for the role of C-kinase in mediating the sustained phase of contraction in the sphincter comes from the studies on the effect of

Table 1

Effect of CCh, ionomycin and phorbol esters on muscle contraction and MLC phosphorylation in the iris sphincter smooth muscle

Addition	MLC phosphorylation (% control)	Muscle contraction (mg tension/mg wet wt)
50 μ M CCh	136 $\pm$ 1.1	14.0 $\pm$ 0.9
5 μ M ionomycin	132 $\pm$ 1.1	6.5 $\pm$ 0.9
200 nM PDBu	121 $\pm$ 0.6	8.3 $\pm$ 0.6
200 nM PMA	101 $\pm$ 1.0	3.2 $\pm$ 0.2
200 nM 4 α -PDBu	100 $\pm$ 0.6	3.7 $\pm$ 0.6
5 μ M ionomycin + 200 nM PDBu	133 $\pm$ 1.2	11.0 $\pm$ 1.3

Experiments were conducted as described in the legend to fig.1 and in section 2. Data are mean $\pm$ SE of three experiments

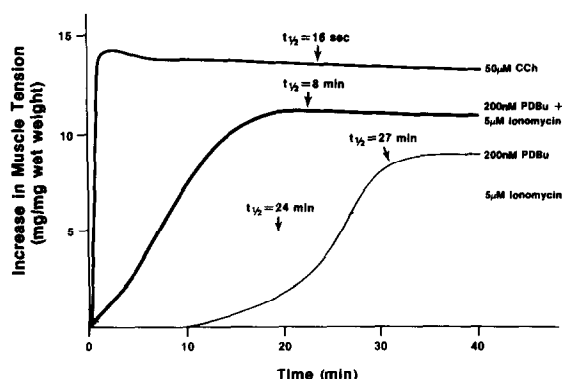


Fig.3. Time course of CCh-, ionomycin- and/or PDBu-induced contraction in the iris sphincter. Experiments were conducted as described in the legend to fig.1 and in section 2. All additions were added at time = 0. These experimental results are typical of 5 similar experiments run in triplicate.

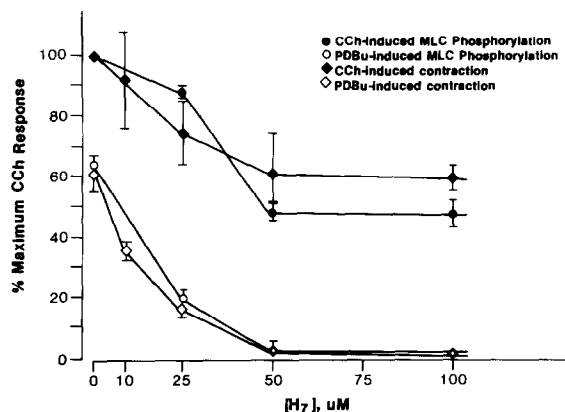


Fig.4. Dose-response effect of H-7 on CCh-induced and PDBu-induced MLC phosphorylation and contraction in the iris sphincter. Experiments were conducted as described in the legend to fig.1 and in section 2. H-7 was added 10 min prior to the addition of CCh (50 μ M) or PDBu (200 nM). The data are mean $\pm$ SE of three separate experiments, each conducted in duplicate.

the C-kinase inhibitor H-7 on PDBu- and CCh-induced MLC phosphorylation and contraction. As can be seen in fig.4 pretreatment of the tissue with H-7 for 10 min resulted in a dose-dependent attenuation of CCh-stimulated MLC phosphorylation and contraction while completely inhibiting the PDBu-induced effects. At 25 μ M concentration H-7 inhibited the CCh-induced MLC phosphorylation and contraction by 12 and 25%, respectively, and inhibited the PDBu-induced responses by 80 and 83%, respectively.

4. DISCUSSION

The data presented indicate that PDBu, in the concentration range of 10–200 nM, can elicit a slowly developing, yet progressive and sustained contractile response in the iris sphincter. This PDBu-induced contractile response is accompanied by an increase in MLC phosphorylation, while no significant effect of the phorbol ester was observed on basal IP₃ accumulation. The observed effects of PDBu are specific, since the inactive isomer, 4 α -PDBu, had no effect on MLC phosphorylation and contraction in this tissue (table 1). The differential effects of phorbol esters in the intact sphincter could be due to the possibility that the iris sphincter is permeable to PDBu but

not to PMA. This possibility is supported by more recent *in vitro* studies in which both PMA and PDBu were equally effective in stimulating C-kinase activity in a cytosolic fraction obtained from iris sphincter homogenates (P.H. Howe and A.A. Abdel-Latif, unpublished observation). PDBu induced more rapid contraction than did PMA in porcine coronary artery [12] and in skinned vascular smooth muscle [10]. Both PDBu-induced MLC phosphorylation and contraction were found to be totally dependent on the presence of extracellular Ca^{2+} , a finding similar to that previously reported for the CCh effects on the sustained phase of contraction in this tissue [5]. Thus, given the facts that the sustained phase of the contractile response, elicited by both PDBu and CCh, is dependent on extracellular Ca^{2+} , and that the phorbol esters activate C-kinase in a manner analogous to DG [18], the data presented strongly suggest the involvement of C-kinase in the tonic component of muscle contraction. The time course effects elicited by drugs which activate either the Ca^{2+} -calmodulin (ionomycin) or the DG-C-kinase (PDBu) pathways, and the synergism resulting from their combined action further support a role for C-kinase in regulating muscle contraction. Finally, our studies with the C-kinase inhibitor, H-7, show that while a complete inhibition of PDBu-induced responses is achieved, its effects on the CCh-induced responses are only attenuated (fig.4). This observation further implicates a role for C-kinase in regulating PDBu-induced muscle contraction, however, it also suggests that for CCh-induced muscle responses the Ca^{2+} -calmodulin dependent pathway is also involved.

The precise role of PDBu-induced MLC phosphorylation in the iris sphincter remains to be elucidated. C-kinase has previously been shown to phosphorylate smooth muscle MLC distinct from that phosphorylated by MLC kinase [10,19]. Other workers have shown that PDBu-induced contractions in smooth muscle are associated with low levels of MLC phosphorylation [10]. In the present work we show that maximal PDBu-induced MLC phosphorylation represents only 50–60% of that observed with CCh. A recent report demonstrates that the phorbol ester, 12-deoxyphorbol 13-isobutyrate, causes MLC phosphorylation in tracheal smooth muscle, but to a lower extent than that observed with CCh [20]. However, in the latter

study other cytoskeletal proteins thought to be involved in contraction were found to be maximally phosphorylated by the phorbol ester. Thus, further study into the identity of other biological substrates for C-kinase in the iris sphincter are needed before the physiological significance of PDBu-induced protein phosphorylation can be ascertained.

While the detailed mechanisms through which protein phosphorylation regulates smooth muscle contraction must await further research, the present studies provide additional support for the proposed role of C-kinase in mediating the sustained phase of the contractile response in the rabbit iris sphincter smooth muscle.

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