Effect of phorbol 12,13-dibutyrate on smooth muscle tone in rat stomach fundus

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Abstract

We investigated the effects of phorbol 12,13-dibutyrate (PDBu), a typical protein kinase C (PKC) activator, on smooth muscle tone in the rat stomach fundus. In 5-hydroxytryptamine (5-HT)-precontracted stomach fundus strips, PDBu induced dose-dependent relaxation, but 4 α -phorbol 12,13-didecanoate, a phorbol ester that does not activate PKC, did not induce relaxation. A PDBu-induced dose-dependent relaxation was also observed in strips precontracted with platelet-activating factor (PAF), carbachol, or 60 mM K⁺. In stomach fundus strips pretreated with PDBu, the contractile responses to 5-HT and PAF were completely blocked, but those induced by carbachol and endothelin-1 (ET-1) were only partially inhibited. In stomach fundus strips preincubated with carbachol in Ca²⁺-free medium, the Ca²⁺-induced contraction was decreased by preincubation with PDBu. In strips preincubated with 5-HT, PAF, or ET-1 in Ca²⁺-free medium, Ca²⁺-induced contractions were greatly inhibited by pretreatment with PDBu. These results suggest that in rat stomach fundus strips, PDBu-induced relaxation is mediated by activation of PKC. We speculate that a major factor mediating the relaxant action of PDBu in rat stomach fundus smooth muscle is represented by a reduction in Ca²⁺ influx via an inhibition of Ca²⁺ channels.

Key words: contraction, PKC, phorbol ester, relaxation, stomach

Introduction

Protein kinase C (PKC) is now known to play an important physiological role in virtually all cell types. Indeed, it appears to be involved in a wide variety of cellular responses, including proliferation, differentiation, desensitization, gene expression, membrane transport, secretion (Goodnight *et al.*, 1994; Ueda *et al.*, 2001; Hofmann, 2004), and smooth muscle contraction (Horowitz *et al.*, 1996; Walsh *et al.*, 1996; Morgan and Leinweber, 1998; Suenaga *et al.*, 2001).

However, conflicting results have been reported as to the effects of PKC on smooth muscle tone. In some, but not all smooth muscles, prolonged exposure to phorbol esters, which are

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potent stimulants of PKC (Nishizuka, 1984), induces a slowly developing contraction that takes a few minutes to start and peaks at times often in excess of one hour (Watson and Godfrey, 1988). In addition, phorbol esters potentiate the contractions induced by both high K^+ (Menkes *et al.*, 1986; Sasaguri and Watson, 1989; Xu *et al.*, 1991; Ahn *et al.*, 1997) and histamine (Holzer and Lippe, 1989). In some instances, the phorbol ester-induced response appears to be dependent on a mobilization of extracellular Ca^{2+} , while in others it appears to be Ca^{2+} -independent. In other smooth muscles, however, such activation of PKC either has no effect on tension or it induces relaxation. For example, phorbol esters reportedly inhibit the contractions induced by histamine (Baraban *et al.*, 1985; Holzer and Lippe, 1989), serotonin (5-HT) (Zhu and Sakai, 1996), carbachol (Menkes *et al.*, 1986; Sasaguri and Watson, 1989) and substance P (Holzer and Lippe, 1989), and these effects are possibly induced by activation of Na^+ - K^+ -ATPase (Sasaguri and Watson, 1990) or via a negative feed-back mechanism (Baraban *et al.*, 1985; Menkes *et al.*, 1986). Moreover, phorbol 12,13-dibutyrate (PDBu) has been found to cause a concentration-dependent decrease in basal tone in the smooth muscle of the internal anal sphincter (Chakder *et al.*, 2001).

To improve our understanding of the role played by PKC in stomach fundus smooth muscle, we examined the effects of PDBu on muscle tension in strips of rat stomach fundus.

Materials and Methods

Drugs

The following drugs were used in the present study: atropine sulfate, guanethidine, nicardipine, phorbol-12,13-dibutyrate (PDBu), 4 α -phorbol-12,13-didecanoate (4 α -PDD), ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 5-hydroxytryptamine creatinine sulfate (5-HT), carbamylcholine chloride (carbachol), and bovine serum albumin (Fraction V) (BSA) (Sigma, St. Louis, MO, U.S.A); endothelin-1 (ET-1) (Peptide Institute Inc., Osaka, Japan); and 1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine (PAF) (Bachem, Switzerland). All drugs were dissolved in saline, except PDBu and 4 α -PDD, which were dissolved in DMSO; control experiments confirmed the absence of significant effects on smooth muscle tone at the final vehicle concentration used. All concentrations are expressed as the final molar concentration of the base in the organ bath. When responses to PAF were to be examined, 0.25% BSA was added to the modified Krebs Ringer solution (KRS), because PAF was found to adhere to plastic and to glassware.

Preparation of tissues and experimental protocol

Male Wistar rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), then killed by decapitation. The stomach was excised, and stomach fundus strip preparations approximately 20 mm in length and 2 mm in width were made essentially by our previously published methods (Kamata *et al.*, 1988, 1993a, b, c). Each strip was cut out along the longitudinal muscle, and the mucous layer was completely removed to avoid reflex activity. The fundus strips were suspended in a 10 ml organ bath containing modified KRS at 37°C, aerated with 95% O_2 and 5% CO_2 . The tension loaded on each strip was 1.0 g. Contractions were

recorded isotonically using an isotonic transducer connected to an ink-writing recorder (Nihon Kohden, Tokyo, Japan). The composition of the modified KRS was as follows (in mM): NaCl 120; KCl 4.7; CaCl_2 2.0; MgCl_2 1.2; NaHCO_3 25; KH_2PO_4 1.2; and glucose 14. Each strip was placed in an organ bath containing KRS, equilibrated with 95% O_2 and 5% CO_2 , and left for 30 min at 37°C. When drugs were applied to the strip, it was preincubated with 10^{-6} M atropine and 10^{-6} M guanethidine (to avoid cholinergic and adrenergic effects being induced throughout the experiment). To allow relaxation responses to be observed, strips were precontracted with 5-HT (10^{-6} M), PAF (10^{-7} M), carbachol (10^{-6} M), or 60 mM K^+ . When a given drug-induced contraction had reached a plateau level, PDBu (10^{-10} – 10^{-6} M) or 4 α -PDD (10^{-10} – 10^{-6} M) was added in a cumulative manner. To examine the effects of PDBu on a given drug-induced contraction, strips were exposed to PDBu for 20 min, and then the test drug was added in a cumulative manner, with the PDBu remaining present thereafter. When Ca^{2+} -induced contractions in the presence or absence of agonists were to be examined, strips were first exposed to a nominally Ca^{2+} -free KRS containing 0.01 mM EGTA. Then, Ca^{2+} (10^{-6} – 3×10^{-3} M) was cumulatively applied to the depolarized fundus while it was bathed in Ca^{2+} -free KRS.

Statistical analysis

The contractile force developed by stomach fundus strips is expressed in g tension/mg tissue, the data being given as the mean $\pm$ S.E.M. When appropriate, statistical differences were assessed using Dunnett's test for multiple comparisons after a one-way analysis of variance, a probability level of $P < 0.05$ being regarded as significant.

Results

Effect of PDBu on 5-HT-induced smooth muscle tone in stomach fundus strips

As shown in Fig. 1, when 5-HT (10^{-6} M) was applied to stomach fundus strips, a contractile response was observed. This 5-HT-induced contraction was greatly inhibited by pretreatment with PDBu (10^{-6} M) (Fig. 1, upper panel). If, on the other hand, PDBu was applied when the contraction induced by 5-HT (10^{-6} M) had reached a plateau, PDBu (10^{-6} M) induced a relaxation (Fig. 1, lower panel), and the PDBu-induced relaxation was dose-dependent (Fig. 2A). In contrast, an inactive phorbol ester (4 α -PDD) did not induce relaxation (Fig. 2A).

Relaxation responses induced by PDBu in stomach fundus strips

When the contraction induced by PAF (10^{-7} M), carbachol (10^{-6} M), or high K^+ (60 mM) had reached a plateau, PDBu (10^{-10} – 10^{-6} M) was added cumulatively (Fig. 2B). At certain concentration, PDBu induced relaxation on all these drug-induced contractions as it had on that induced by 5-HT.

Effect of PDBu on various drug-induced contractions

To determine whether any agonist-induced contractile responses in the stomach fundus might be altered in strips pretreated with PDBu, we examined several agonist-induced contractions in the presence of PDBu (10^{-6} M) (Fig. 3). The 5-HT (10^{-10} – 10^{-6} M)- and PAF

Fig. 1. Effect of PDBu (10^{-6} M) on 5-HT-induced contractile response in rat stomach fundus. Upper panel shows pretreatment effect of PDBu. Lower panel shows post-treatment effect of PDBu.

Fig. 2. Concentration-response curves for relaxation responses to PDBu and 4α -PDD in rat stomach fundus precontracted (A) with 5-HT (10^{-6} M) and (B) with PAF (10^{-7} M), carbachol (10^{-6} M), or high potassium (60 mM). Each value represents mean with S.E.M. for 4–6 experiments, the SE mean being included only when it exceeds the dimension of the symbol used.

(10^{-10} – 10^{-6} M)-induced dose-dependent contractile responses were completely blocked by PDBu pretreatment (Fig. 3, A, B). The carbachol (10^{-9} – 10^{-5} M)-induced contraction showed a significant right-ward shift in the presence of PDBu (10^{-6} M), but it was unaffected by the presence of 4α -PDD (10^{-6} M) (Fig. 3C). The ET-1 (10^{-10} – 10^{-7} M)-induced contraction was significantly reduced in the presence of PDBu (10^{-6} M) (Fig. 3D). Isotonic high K^{+} (10–60 mM) induced a concentration-dependent rise in tension, and this tended (non-significantly) to be

Fig. 3. Concentration-response curves for contractile responses of rat stomach fundus to (A) 5-HT, (B) PAF, (C) carbachol, (D) ET-1, and (E) potassium in the absence (closed symbol) and presence (open symbol) of PDBu (10^{-6} M) or 4 α -PDD (10^{-6} M). Each value represents mean with S.E.M. for 6 or 7 experiments, the SE mean being included only when it exceeds the dimension of the symbol used. *, $P < 0.05$, **, $P < 0.01$ vs. control.

decreased in the presence of PDBu (10^{-6} M) (Fig. 3E).

Effects of PDBu on Ca^{2+} -induced contraction

As shown in Fig. 4A, Ca^{2+} ($CaCl_2$) induced a concentration-dependent contraction in stomach fundus strips bathed in an initially Ca^{2+} -free solution, and this contraction was completely suppressed by treatment with 10^{-6} M PDBu. Preincubation with 5-HT did not significantly alter these Ca^{2+} -induced contractile responses in the absence of PDBu (Fig. 4A). Ca^{2+} -induced contractile response in the presence of 5-HT was also completely blocked by treatment with PDBu (Fig. 4A). As reported previously (Kamata *et al.*, 1993a), PAF (10^{-7} M) augmented the contraction induced by Ca^{2+} in Ca^{2+} -free solution, and this augmentation was completely blocked by 10^{-6} M PDBu (Fig. 4B). A similar result was observed when ET-1 was given as pretreatment

Fig. 4. Concentration-response curves for contractile responses of rat stomach fundus to CaCl_2 in the absence (closed symbol) and presence (open symbol) of PDBu (10^{-6} M). Tissue were preincubated in Ca^{2+} -free medium with (A) 5-HT (10^{-6} M), (B) PAF (10^{-7} M), (C) carbachol (10^{-6} M), or (D) ET-1 (10^{-7} M). Each value represents mean with S.E.M. for 6 or 8 experiments, the SE mean being included only when it exceeds the dimension of the symbol used. *, $P < 0.05$, **, $P < 0.01$ vs. control. #, $P < 0.05$, ##, $P < 0.01$ vs. 5-HT-preincubated control (A) or nicardipine-preincubated control (C).

instead of PAF (Fig. 4D). Carbachol (10^{-6} M) augmented the contraction induced by Ca^{2+} in Ca^{2+} -free solution, and this augmentation was partly antagonized by 10^{-7} M nicardipine, a selective L-type Ca^{2+} channel inhibitor, and 10^{-6} M PDBu (Fig. 4C). A combination of nicardipine and PDBu (same concentrations as above) almost completely suppressed the contraction induced by Ca^{2+} in Ca^{2+} -free solution.

Discussion

The main observations made in the present study were that in rat stomach fundus longitudinal smooth muscle, the PKC activator PDBu induced a relaxation response, and also attenuated the contractile responses induced by various agonists and by Ca^{2+} .

The effect of PKC on smooth muscle tone is controversial, with results depending on the species, the type of smooth muscle, and the membrane receptor agonist studied (See Introduction). In the present study, when rat stomach fundus strips were precontracted with any of a number of agonists (5-HT, PAF, carbachol, and isotonic high K^+ solution), PDBu-induced a dose-dependent relaxation (Figs. 1, 2). The maximal PDBu-induced relaxation was

weaker in strips precontracted with carbachol than in those precontracted with either 5-HT or PAF (Fig. 2). These results suggest that the magnitude of the PDBu-induced relaxant effect in this tissue depends on the nature of the drug used to produce the precontraction. To extend our investigation of the effect of PDBu on smooth muscle tone, we therefore examined the contractions induced by various agonists in the presence of PDBu.

Smooth muscle contractile responses are the final result of an influx of extracellular calcium and/or a mobilization of intracellular calcium. Several types of Ca^{2+} -channels, such as voltage-operated Ca^{2+} -channels (VOCCs) and receptor-operated Ca^{2+} -channels (ROCCs), are activated when agonists act on a range of G-protein-coupled receptors, whereas store-operated calcium channels (SOCCs) are activated by depletion of the calcium stores within the sarcoplasmic reticulum (Meisheri *et al.*, 1981; Karaki and Weiss, 1984; McFadzean and Gibson, 2002). Reportedly, 5-HT and PAF have no significant effects on phosphatidylinositol (PI) turnover in the rat stomach fundus (Secrest *et al.*, 1989; Kamata *et al.*, 1993b). Indeed, these reagents primarily utilize extracellular calcium to contract this tissue (Secrest *et al.*, 1989; Kamata *et al.*, 1993a). On the other hand, in a variety of smooth muscles carbachol and ET-1 increase PI turnover (Yang *et al.*, 1994; Prestwich and Bolton, 1995) and activate Ca^{2+} -channels (Kawanabe *et al.*, 2002; So and Kim, 2003). In the present study, the 5-HT- and PAF-induced contractile responses were completely blocked in the presence of PDBu (Fig. 1, Fig. 3A, 3B). This suppressive effect of PDBu on the 5-HT-induced contraction in rat stomach fundus is consistent with a previous finding (Zhu and Sakai, 1996). On the other hand, the carbachol- and ET-1-induced contractions were only partially inhibited by PDBu (Fig. 3C, 3D). High KCl is known to cause membrane depolarization and hence to stimulate Ca^{2+} entry through VOCC (Khalil and van Breeman, 1995). In our study, the isotonic K^+ -induced contraction tended to be decreased in the presence of PDBu (Fig. 3E). Moreover, Ca^{2+} is able to cause contraction of rat stomach when added to an initially Ca^{2+} -free bathing solution (Kamata *et al.*, 1993a). Since such a Ca^{2+} -induced contraction in Ca^{2+} -free solution is completely abolished by treatment with the calcium antagonist nifedipine (Kamata *et al.*, 1993a), it is likely that VOCC activity in the fundus is increased in Ca^{2+} -free solution. In the present study, PAF, but not 5-HT, augmented the Ca^{2+} -induced contraction observed in Ca^{2+} -free solution (Fig. 4A, 4B), and the contractions induced by these two reagents were completely abolished by PDBu. Furthermore, the carbachol-induced augmentation of the Ca^{2+} -induced contraction was significantly weaker in the presence of PDBu, and the residual contraction was completely abolished by additional treatment with nifedipine (Fig. 4C). Further, ET-1 augmented the Ca^{2+} -induced contraction seen in Ca^{2+} -free solution (Fig. 4D), and the ET-1-induced augmentation of this contraction was completely abolished by PDBu. These results suggest (a) that PAF, carbachol, and ET-1 depolarize the stomach fundus and hence open VOCC, thereby causing contraction via an influx of extracellular Ca^{2+} through the open VOCC, and (b) that PDBu inhibits this pathway. Taken together, the above findings lead us to conclude that the suppressive effects of PDBu on stomach fundus smooth muscle tone are due to reduced Ca^{2+} -channel activity, rather than to reduced PI turnover. Although we did not investigate the effects of PDBu on Ca^{2+} movement or electrophysiological Ca^{2+} current, this speculation is supported by the finding that addition of PDBu to intact canine gastric smooth muscles reduced Ca^{2+} transients and contractions (Ozaki *et al.*, 1993).

The precise relationship between PKC activation and the PDBu-induced relaxation of rat stomach fundus smooth muscle remains unknown. Data from several reports suggest that the inhibition of agonist-induced contraction by phorbol esters may occur via a direct inhibition of inositol phosphates or via an activation of Na⁺-K⁺-ATPase, resulting in a membrane hyperpolarization and inhibition of VOCC (Holzer and Lippe, 1989; Sasaguri and Watson, 1990; Mitsui and Karaki, 1993). However, a direct inhibition of Ca²⁺ channels (VOCC, ROCC) by phorbol esters has also been reported (Prestwich and Bolton, 1995; Sena *et al.*, 1995; Korchak *et al.*, 2001). The results obtained in the present study implicate a reduced Ca²⁺ influx via an inhibition of Ca²⁺ channels as a potential underlying mechanism for negative regulation by PKC and the consequent induction of relaxation, at least in the smooth muscle of the rat stomach fundus. Further investigations will be necessary, however, to confirm or deny this idea.

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