MINIREVIEW

Cross Talk between Cyclic AMP and the Polyphosphoinositide Signaling Cascade in Iris Sphincter and Other Nonvascular Smooth Muscle (43959B)

ATA A. ABDEL-LATIF[1]

Department of Biochemistry and Molecular Biology, Medical College of Georgia, Augusta, Georgia 30912

Abstract. **Nonvascular smooth muscle, such as the iris sphincter, receives double reciprocal innervation: stimulation of the parasympathetic nervous system (cholinergic muscarinic), which functions through the polyphosphoinositide (PPI) signaling pathway, contracts it, while activation of the sympathetic nervous system (β-adrenergic), which functions through the cAMP system, relaxes it. Interactions between the two second messenger systems are important in regulation of smooth muscle tone and represent an important focal point for pharmacological manipulation. Here, I have summarized the experimental evidence in support of the hypothesis that the cross talk between cAMP and the PPI cascade could constitute a biochemical correlate for this functional antagonism. Recent studies suggest that cAMP inhibition is on Ca^{2+} mobilization rather than myosin light chain phosphorylation. Thus, cAMP-elevating agents, which inhibit agonist-induced PPI hydrolysis, are effective relaxants. Furthermore, inositol 1,4,5-trisphosphate (IP_3) appears to be involved in both Ca^{2+} release from the sarcoplasmic reticulum and in Ca^{2+} influx through the plasma membrane, and since a reduction in intracellular Ca^{2+} ($[Ca^{2+}]_i$) is the underlying mechanism for cAMP-mediated relaxation, an important target for cAMP inhibition would be either to inhibit IP_3 production or to stimulate IP_3 inactivation. In the iris sphincter and other nonvascular smooth muscle there is reasonable experimental evidence that shows that cAMP inhibits phospholipase C activation and stimulates IP_3 3-kinase activity, both of which can result in: (i) reduction in IP_3 concentrations and (ii) reduction in IP_3-dependent Ca^{2+} mobilization, which may lead to muscle relaxation. In addition to IP_3-induced Ca^{2+} mobilization, changes in $[Ca^{2+}]_i$ are the result of the interplay of many processes which may also serve as potential sites for cAMP inhibition. A great deal of progress has been made on the cross talk between cAMP and the PPI signaling cascade in the past decade, and there will be more made on the regulation of the second messenger systems and their involvement in smooth muscle tone in the coming years. Clearly, an understanding of the physiological and pathophysiological regulation of smooth muscle tone is central to the development of novel therapeutic agents for the treatment of diseases such as asthma and glaucoma, where cAMP-elevating drugs are currently employed.** [P.S.E.B.M. 1996, Vol 211]

[1] To whom requests for reprints should be addressed at Department of Bio chemistry and Molecular Biology, Medical College of Georgia, Augusta, GA 30912.

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A great deal of effort has been made in the past few years to understand the biochemical basis for the functional antagonism between agonist-induced smooth muscle contraction and β-adrenoreceptor–mediated relaxation. It is well established now that in smooth muscle agents that elevate intra-

cellular cAMP concentrations, such as β-adrenergic agonists, cAMP phosphodiesterase inhibitors, and E-type prostaglandins (PGs), inhibit agonist-stimulated hydrolysis of the polyphosphoinositide (PPI), phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG), Ca^{2+} mobilization and contraction. A large body of evidence has recently accumulated, in both vascular and nonvascular smooth muscle, which indicates that cross talk between the cAMP and the PPI signaling cascade plays an important role in the functional antagonism between the sympathetic and parasympathetic nervous systems. In this brief review, a summary is given of more recent progress on the experimental evidence for the cross talk between cAMP and the PPI signaling system in nonvascular smooth muscle, with major emphasis on the iris sphincter and the trachea, both of which are innervated by cholinergic and β-adrenergic nerve terminals, and both of which have been thoroughly investigated in the past few years.

Background

Ca^{2+} plays an essential role in the mechanism of smooth muscle contraction and relaxation. In agonist-stimulated smooth muscle, Ca^{2+} regulates contraction by regulating myosin light chain kinase (MLCK), which, in turn, controls the actin-myosin interaction (1). Briefly, Ca^{2+} binds to calmodulin (CaM), the Ca-CaM complex then activates MLCK to phosphorylate serine at position 19 on the 20-kDa regulatory light chain (MLC_{20}), thereby changing the myosin from an inactive to an active form (2, 3). A single residue (Ser^{19}) is the only amino acid on the MLC_{20} that is phosphorylated upon physiological stimulation (4). Phosphorylation of MLC_{20} at position 19 allows the Mg^{2+}-dependent ATPase of myosin to be activated by actin and the muscle to contract (2, 3, 5). It is now generally accepted that the resultant phosphorylation of the MLC_{20} leads to the interaction of actin with myosin, triggering a contractile response. Relaxation of an agonist-contracted smooth muscle can be achieved: (i) by removal of the contractile agonist or (ii) by activation of either the adenylyl cyclase (AC)/cAMP or guanylyl cyclase/cGMP second messenger systems. While there has been a considerable amount of research conducted on the mechanisms underlying agonist-induced smooth muscle contraction, there has been less work devoted to our understanding of muscle relaxation (6). Several drugs, neurotransmitters, and hormones that are known to induce relaxation seem to involve increases in the intracellular concentrations of the second messengers cAMP or cGMP (for recent reviews see Refs. 7 through 12). Thus, pharmacological agents such as isoproterenol (ISO), which activates β-adrenergic receptors, or forskolin, which directly stimulates AC, elevate intracellular cAMP and induce relaxation. While the functional antagonism of agonist-induced contraction by cAMP elevating agonists is well documented and, in spite of the fact that several mechanisms have been proposed for a role for cAMP in mediating smooth muscle relaxation (7–12), the precise mechanism by which this second messenger induces relaxation has yet to be determined. This is true because cAMP mediates the actions of many stimuli through activation of protein kinase A (PKA).

Two hypotheses have been proposed to explain the mechanism through which cAMP relaxes smooth muscle (see Refs. 10 and 13 for more detail): Firstly, activation of PKA could alter the Ca sensitivity of MLC_{20} phosphorylation by phosphorylation of MLCK. In support of this hypothesis, De Lanerolle *et al.* (14) reported that MLCK is phosphorylated by PKA and that phosphorylation of MLCK may decrease the affinity of MLCK for the Ca-CaM complex. This could result in inhibition of MLC_{20} phosphorylation and subsequently may lead to smooth muscle relaxation. However, more recent work has demonstrated that PKA phosphorylation of MLCK is not a physiologic mechanism nor likely to be responsible for cAMP-induced relaxation (15). This hypothesis remains controversial at the present time and there is a need to carry out more experiments in intact smooth muscle, especially to demonstrate that there is a decrease in MLCK activity following an increase in cAMP levels (10). Very recently, Van Riper *et al.* (16) working on MLCK phosphorylation in swine carotid artery contraction and relaxation concluded that, in this tissue, MLCK phosphorylation appears to be regulated exclusively by Ca^{2+} and plays little role in stimulus-dependent differences in Ca^{2+} sensitivity of MLC phosphorylation or in mediating forskolin-induced relaxation. Extensive studies in our own laboratory on the effects of pretreatment of bovine iris sphincter smooth muscle or immortalized cat iris sphincter smooth muscle (SV-CISM-2) cells with isoproterenol (ISO) on MLCK activity revealed no changes in the activity of this enzyme (Wang X-L, Akhtar RA, Ding K-H, Abdel-Latif AA, unpublished observations).

Secondly, a reduction in intracellular Ca^{2+} ($[Ca^{2+}]_i$) is the underlying mechanism for cAMP-mediated relaxation (17). A reduction in intracellular Ca^{2+} will inactivate MLCK kinase and this will lead to relaxation. This could occur as a result of a decrease in Ca^{2+} release from the sarcoplasmic reticulum (SR), a decrease in Ca^{2+} influx, or an increase in Ca^{2+} outflux or sequestration into internal stores. This hypothesis is supported by more recent findings on the inhibitory effects of cAMP-elevating agents on agonist-induced increases in IP_3. It is well established now that agonist-stimulated PIP_2 hydrolysis, with the concomitant production of the two second messengers IP_3, which mo-

bilizes Ca^{2+} from the SR, and DAG, which activates protein kinase C (PKC), is essential for initiation and maintenance of the contractile response in smooth muscle (for reviews see Refs. 5, 11, and 18 through 23). The rationale and the experimental evidence for the hypothesis that cAMP inhibition via PKA of the PPI signaling pathway may constitute the biochemical correlate for the functional antagonism between agonist-induced contraction and β-adrenoreceptor–mediated relaxation will be summarized in the following sections.

Functional Antagonism between the Sympathetic and Parasympathetic Nervous Systems in Smooth-Muscle

An important mechanism by which the peripheral nervous system regulates smooth muscle tone is through a reciprocal interaction between the parasympathetic (cholinergic) nervous system, which liberates acetylcholine (ACh), and sympathetic (adrenergic) nervous system, which liberates norepinephrine (NE) (Fig. 1). Adrenergic nerves can regulate, through changes in intracellular cAMP levels, the muscarinic stimulation of smooth muscle contraction, and in many instances smooth muscle relaxation (inhibition). In addition to muscarinic cholinergic and β-adrenergic receptors, the iris sphincter contains receptors for substance P, endothelin and prostaglandins (PGs) which also function through the cAMP and PPI signaling cascade (25). Representative recordings of mechanical responses of bovine iris sphincter to CCh and inhibition by the cAMP elevating agents, NE, ISO, and 3-isobutyl-1-methylxanthine (IBMX), a cAMP phosphodiesterase inhibitor, are shown in Figure 2. L-N^G-nitroarginine methyl ester (L-NAME), a commonly used competitive inhibitor of nitric oxide synthase (26), did not inhibit relaxation of sphincter muscle induced by NE or ISO. This suggests that the observed relaxant effects of the cAMP-elevating agents in this muscle (Fig. 2) are mediated through cAMP and not through cGMP. In contrast, in rat aorta rings L-NAME inhibited endothelium-dependent relaxation induced by ACh (26). As in guinea pig taenia (27), the relaxant response to NE in the bovine sphincter (Fig. 2) could be mediated through β_3-adrenoreceptors.

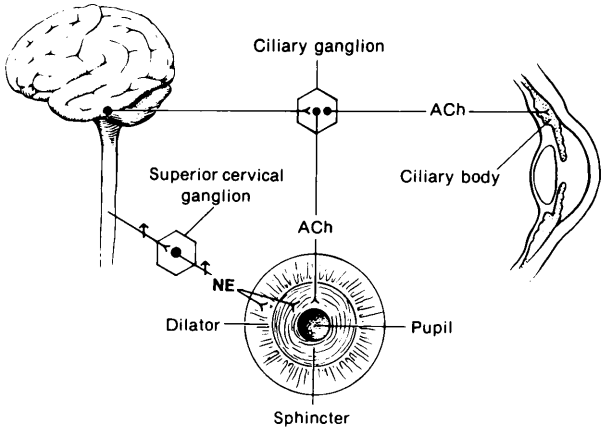

Figure 1. Autonomic innervation of the iris smooth muscles and site (arrows) of surgical sympathetic denervation. The two iris muscles receive double reciprocal innervation: cholinergic activation contracts the sphincter and relaxes the dilator to give miosis, while activation of the adrenergic nervous system contracts the dilator and relaxes the sphincter to give mydriasis. The sphincter muscle is innervated predominantly by parasympathetic nerves; it is also innervated by sympathetic nerves from the superior cervical ganglion. Activation of β-adrenergic receptors in the sphincter results in relaxation of the muscle. (From Ref. 24, with permission.)

Cross Talk between cAMP and the PPI Cascade in Nonvascular Smooth Muscle: A Scheme

A scheme showing potential sites of cross talk between cAMP and the PPI cascade in iris sphincter and other nonvascular smooth muscle is given in Figure 3. Briefly, activation of m_3 muscarinic receptors (m_3-mAChRs) by ACh increases Ca^{2+} mobilization through the generation of IP_3 from PIP_2 hydrolysis by phospholipase C (PLC), inhibits cAMP formation, via m_2-mAChRs, and results in contraction of the muscle. This concurrent activation of a stimulatory pathway and blunting of an inhibitory pathway could enhance the contractile efficiency of ACh on the smooth muscle. On the other hand, activation of β-adrenergic receptors by NE or ISO, and activation of AC by ISO or forskolin or inhibition of cAMP phosphodiesterase by IBMX, and activation of EP_2 receptors by PGE_2 increases cAMP accumulation and inhibits IP_3 production and Ca^{2+} mobilization, which result in relaxation of the muscle. Activation of PKC by DAG inhibits agonist-induced IP_3 production and stimulates AC and phospholipases A_2 and D (PLA_2, PLD). The increases in arachidonic acid release, and its subsequent conversion into PGs, due to activation of PLA_2 by PKC and/or activation by Ca^{2+}-mobilizing agonists contributes to some of the cellular effects of phorbol esters and the various agonists (Fig. 3).

In the past decade we have demonstrated that the effects of neurotransmitters, hormones, and PGs on the generation of cAMP and IP_3, and on the contraction in the smooth muscles of the iris of the eye are both species and cell-type specific (25). In general, smooth muscle cells obtained from different tissues vary considerably in their responsiveness to different stimuli and since these variations are important in the control of $[Ca^{2+}]_i$, it must be emphasized here that results obtained from one cell type may not be universally applicable. Ca^{2+} needed for smooth muscle contraction may come from the outside of the cell through ion channels on the plasma membrane or from the intracellular storage sites through IP_3-induced Ca^{2+} release located on the SR (Fig. 3). The source of trigger

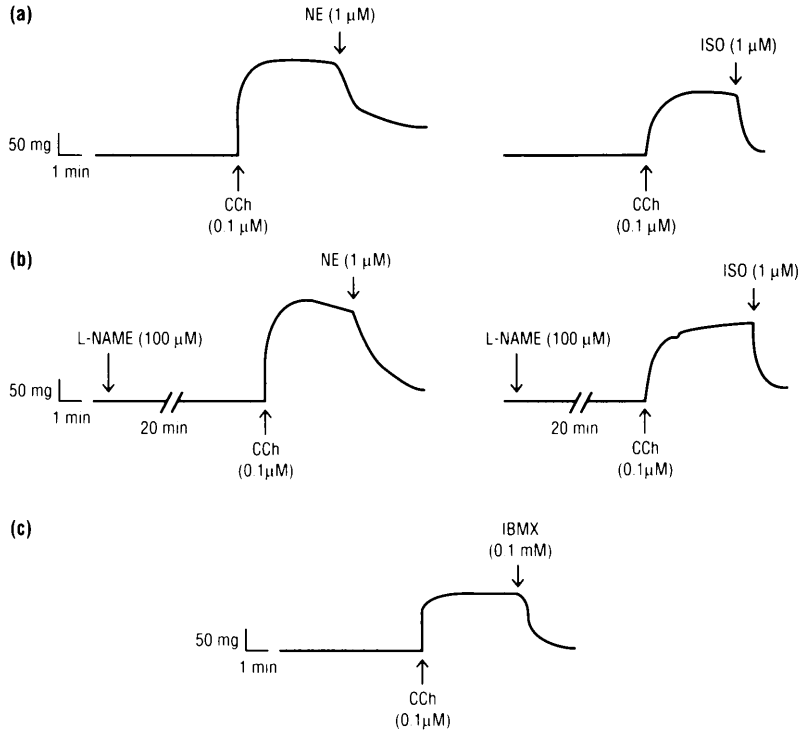

Figure 2. Representative recordings of mechanical responses of bovine iris sphincter to (a and b) CCh and the cAMP-elevating agents NE and ISO, in the absence (a) and presence (b) of L-NAME, and to (c) CCh and IBMX.

Ca^{2+} is dependent on the stimulus as well as on the type of smooth muscle. The return to basal $[Ca^{2+}]_i$ is due to Na^+-Ca^{2+} exchange located at the plasma membrane and to the Ca^{2+} pumps located at the plasma membrane and the SR (Fig. 3). IP_3 is one of the messengers involved in agonist-induced Ca^{2+} release from the SR, which results in the initial transient phase of contraction (5, 20). The contribution of external Ca^{2+} follows and is mostly responsible for the sustained phase of muscle contraction. The cellular mechanisms by which IP_3 stimulates Ca^{2+} influx remain poorly understood. In some cells IP_3-induced Ca^{2+} release, which depletes intracellular Ca^{2+} stores, is accompanied by activation of the capacitative Ca^{2+}-influx pathway (28). The steps that link the depletion of intracellular Ca^{2+} stores to Ca^{2+} influx remain unresolved. Thus, if we accept the hypothesis that a reduction in $[Ca^{2+}]_i$ is the underlying mechanism for cAMP-mediated relaxation, then it is not unreasonable to assume that an important target of cAMP actions, presumably through activation of PKA, would be either to inhibit IP_3 production or to stimulate IP_3 metabolism (Fig. 3). There is little information about the effects of cAMP inhibition at the plasma membrane receptor or at the G protein levels. Phosphorylation of the porcine atrial mAChR by PKA did not affect its interaction with CCh or the CCh-induced stimulation of the GTPase activity of G_i in the reconstituted system (29). Furthermore, phosphorylation of mAChR, purified from porcine cerebrum, by PKC did not affect the interaction between the receptor and G proteins (30). Therefore, the emphasis here will be on the regulation by PKA of PLC and the enzymes which metabolize IP_3, namely IP_3 5-phosphatase and IP_3 3-kinase (Fig. 3).

Cyclic AMP and PPI Turnover in Smooth Muscle

Since our early discovery of agonist-stimulated phosphodiesteratic breakdown of PIP_2 into IP_3 and DAG in the iris muscle and the suggestion that this was involved in the contractile response of this smooth muscle more than 18 years ago (18, 21, 31, 32), a large body of evidence has accumulated showing that IP_3-induced Ca^{2+} release is involved in the phasic component of smooth muscle contraction (for reviews see Refs. 5, 11, 18, 20, 23, and 33 through 35) and that DAG may be involved in the tonic phase of the contractile response (for reviews see Refs. 19, 36, and 37). A cross talk between receptors that accelerate the hydrolysis of membrane phosphoinositides following agonist stimulation and those that stimulate AC activity and produce cAMP was first reported in lymphocytes (38). These authors observed that in the lymphocyte system, cells are activated by phytohemagglutinin that induce phosphatidylinositol (PI) turnover, and this PI turnover and cellular activation are profoundly blocked by dibutyryl cAMP as well as by PGE_1, which markedly increases cAMP. Since then considerable amount of experimental evidence for cAMP-mediated inhibition of agonist-stimulated phosphoinositide turnover has been obtained from nonsmooth muscle (for reviews see Refs. 39 and 40) as well as from smooth muscle, including the iris sphincter (22) and tracheal (23) smooth muscles.

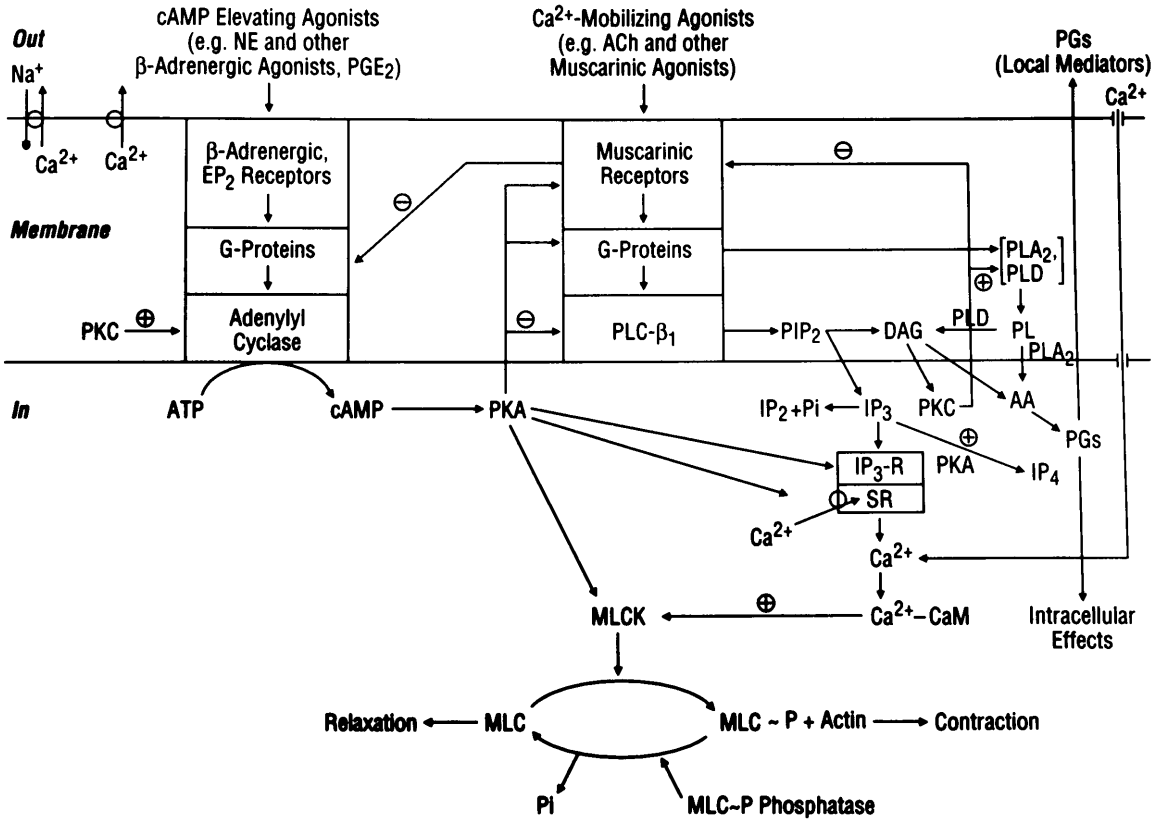

Figure 3. Scheme showing a biochemical correlate for the functional antagonism between receptors mediating contraction and relaxation in iris sphincter isolated from different species. Potential sites of cross talk between cAMP and the PPI signaling cascade are indicated by arrows. Abbreviations are the same as given in the text; (+) and (−), stimulation and inhibition, respectively.

In smooth muscle, although opposing effects of Ca^{2+}-mobilizing agonists and agents that increase the intracellular concentrations of cAMP at the functional level are well established (see above), a cross talk between the cAMP and the phosphoinositide systems in nonvascular smooth muscle began to emerge in 1988 when Madison and Brown (41), working with canine tracheal smooth muscle, reported that ISO, forskolin, and dibutyryl cAMP significantly decreased inositol phosphates accumulation in response to histamine but not to methacholine stimulation of phosphoinositide turnover. In the same year, Hall and Hill (42) independently reported that in bovine tracheal smooth muscle both the nonselective β-agonist ISO (IC_{50} = 0.08 μM) and the β_2-selective agonist salbutamol (IC_{50} = 0.29 μM) produced a dose-related inhibition of the inositol phosphate response to 0.1 mM histamine. Furthermore, when NE (0.1 mM) was added simultaneously with histamine it significantly reduced the inositol phosphate response to high (>0.1 mM) concentrations of histamine. However, NE had no inhibitory effect on the CCh-induced inositol phosphate response. This is in contrast to the bovine sphincter where NE inhibited CCh-induced contraction (Fig. 2). Later studies on the effects of cAMP elevating agents, such as IBMX, in bovine tracheal smooth muscle confirmed these findings (43, 44). These observations correlate well with the functional studies which had shown that the ability of β-adrenoreceptor stimulation to cause relaxation was dependent upon the concentration and nature of the contracting agonist (23, 45–47). In this connection, recent studies in our laboratory revealed a lack of relaxant effect of ISO, but not forskolin, in bovine ciliary smooth muscle precontracted with CCh (Yousufzai SYK, Abdel-Latif AA, unpublished observations), this is in contrast to its inhibitory effects in the bovine sphincter (see below). However, the β-adrenergic agonist relaxed cat ciliary muscle precontracted with CCh (48). In addition, PGE_2, which increases intracellular cAMP concentrations, relaxed bovine (49) and cat (48) ciliary muscles precontracted with CCh. ISO relaxes bovine iris sphincter smooth muscle precontracted with CCh (50). These findings demonstrate that the relaxant effects of ISO are both species and cell-type specific. It has been suggested that the large M_3-mAChR reserve in tracheal smooth muscle might account for the resistance of IP_3 production, stimulated by full mAChR agonists, to inhibition by cAMP elevating agents (23, 51). However, Offer *et al.* (44) reported that in bovine tracheal smooth muscle, while inositol phospholipid hydrolysis stimulated by full mAChR agonists is completely resistant to inhibition by β-adrenoreceptor stimulation, some partial muscarinic agonist-stimulated responses are susceptible to

such inhibition. It is possible that this could be due to the fact that partial muscarinic agonists, such as pilocarpine, exert weak effects on PLC stimulation and AC inhibition, whereas the full mAChR agonists CCh and oxotremorine exert strong effects on both of these biochemical responses. In contrast to the bovine and canine tracheal smooth muscles and the bovine ciliary smooth muscle, where ISO had no inhibitory effects on muscarinic stimulation of IP_3 production and contraction, in the bovine iris sphincter which contains mainly the m_3 muscarinic receptor subtype (52) ISO, forskolin, and IBMX completely blocked these muscarinic responses (50, 53). A time course experiment on the effects of CCh and ISO on cAMP accumulation, IP_3 production, and relaxation in the bovine iris sphincter is shown in Figure 4. In these experiments, we have investigated the temporal relationships between ISO-induced cAMP formation, inhibition of IP_3 accumulation and muscle relaxation. Upon addition of ISO (0.5 μM) to muscle precontracted with CCh (1 μM), the level of intracellular cAMP was increased by 40% within 15 sec, and the maximal effect was observed at about 5 min. In contrast to its stimulatory effect on cAMP formation, ISO inhibited IP_3 accumulation in a time-dependent manner. Inhibition of IP_3 accumulation by ISO was evident after 30 sec of incubation and reached maximal (30% inhibition) at about 5 min. In general, the time course profile for ISO-induced muscle relaxation parallels that of cAMP formation. About 50% of muscle relaxation by the agonist is achieved within 15 sec, and it is complete within 2 min. These data clearly demonstrate a temporal relationship between the ISO-induced stimulation of cAMP, inhibition of IP_3 and relaxation of the bovine iris sphincter. Inhibitory effects of cAMP-elevating agents on agonist-induced phosphoinositide hydrolysis has also been reported in rat aorta (54, 55), DDT_1-MF_2 smooth muscle cell line (56, 57), isolated gastric muscle cells (58), and cat iris sphincter smooth muscle cell

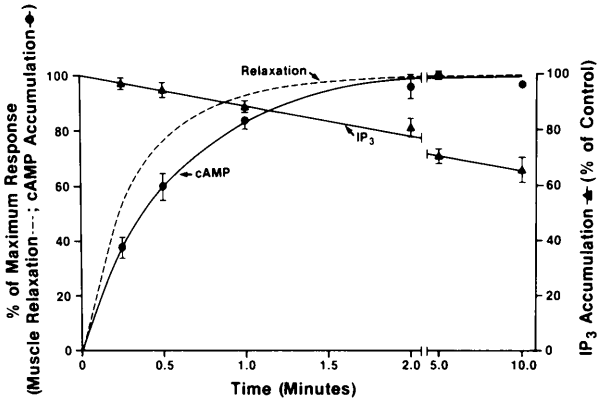

Figure 4. Time course of the effects of ISO (0.5 μM) on cAMP formation, IP_3 accumulation and muscle relaxation in bovine iris sphincter precontracted with CCh (1 μM). (From Ref. 50, with permission.)

Table I. Effects of ISO on CCh-Induced Inositol Phosphates Production and MLC_{20} Phosphorylation in SV-CISM-2 Cells

Additions	^3H-inositol phosphates (% of control)	[^{32}P] radioactivity in MLC_{20} (% of control)
None	100	100
CCh (25 μM)	142	170
ISO (5 μM)	104	108
CCh (25 μM) + ISO (5 μM)	107	93

Note. Incubation of the cells with ^3H-inositol or $^{32}P_i$ and analysis of ^3H-inositol phosphates or ^{32}P-MLC_{20} were the same as described previously (59). The average ^3H radioactivity recovered in inositol phosphates in the control experiment was 37109 dmp/mg of total tissue proteins. The average ^{32}P radioactivity recovered in the MLC_{20} band in the control experiment was 8992 cpm/mg of total tissue proteins.

line (59). Data from our own laboratory on the effects of ISO on CCh-induced IP_3 production and MLC_{20} phosphorylation in SV-CISM-2 cells are given in Table I. The β-adrenergic agonist blocked the muscarinic stimulation of both IP_3 production and MLC_{20} phosphorylation, suggesting that a decrease in Ca^{2+} release from the SR by IP_3 could result in a decrease in MLC_{20} phosphorylation, which may lead to relaxation.

Cyclic AMP-elevating agents have also been shown to inhibit fluroaluminate-induced inositol phosphate production and contraction in both bovine tracheal smooth muscle (60) and bovine iris sphincter smooth muscle (53). Aluminum fluoride (ALF_4^-), which mimics the γ-phosphate group of GTP when GDP is bound to the α subunits, is believed to stimulate PLC activity by activating $G_{\alpha q}$ directly via the GTP binding site (61), thus bypassing the agonist-receptor binding step in the activation of the enzyme. These findings suggest that inhibition of agonist-stimulated inositol phosphates production by cAMP in these muscles occur at the postreceptor level.

It can be concluded from the above that in nonvascular smooth muscle elevation of intracellular cAMP concentrations can lead, depending on the species and cell type, to inhibition of agonist-stimulated PPI hydrolysis.

Cyclic GMP and PPI Turnover

There are few studies that have investigated the ability of cGMP-elevating agents to modulate PPI turnover in smooth muscle. Langlands *et al.* (62) reported that treatment of guinea pig tracheal rings with zaprinast (M & B 22948), a cyclic GMP phosphodiesterase inhibitor, abolished methacholine- and histamine-stimulated IP_3 production without exerting any effect on the contractile response. The authors concluded that in guinea pig tracheal smooth muscle, IP_3 did not

appear to be responsible for the contractions induced by these agonists. Later, Hall *et al.* (43) reported no effect on zaprinast upon either CCh-induced inositol phosphate formation or contraction in bovine tracheal smooth muscle, and this was confirmed by Chilvers *et al.* (63). In bovine iris sphincter zaprinast had no effect on CCh-induced PIP_2 hydrolysis, IP_3 production, or contraction (64). This is supported by the data given in Figure 2, from which it was found that pretreatment of the tissue with L-NAME, a nitric oxide synthetase inhibitor, had no effect on CCh-induced contraction and subsequent relaxation by NE or ISO. More recently, Challiss and Boyle (23) observed that cGMP elevation failed to inhibit histamine-stimulated PPI hydrolysis, suggesting that differences exist between cAMP- and cGMP-mediated cross talk mechanisms.

In vascular smooth muscle, cGMP-elevating agents inhibited α-adrenoreceptor–stimulated PPI turnover and contraction in rat aorta (65), and inositol phosphate formation in membrane fractions isolated from rat aorta and cultured bovine aortic smooth muscle cells (66).

These data suggest that cGMP may not be involved in the mechanism of agonist-induced inhibition of phosphoinositide turnover and relaxation in nonvascular smooth muscle.

Regulation of Phospholipase C by Protein Kinase A

The studies on the inhibitory effects of cAMP-elevating agents on PPI hydrolysis and Ca^{2+} mobilization in nonvascular smooth muscle suggest that PLC could be a target for PKA regulation. Inhibition of receptor-mediated PIP_2 hydrolysis and IP_3 production could occur by phosphorylating, via PKA, the receptor, the G protein, or PLC (Fig. 3). Activation of PLC and AC by hormone or neurotransmitter receptors is mediated via stimulatory G proteins, namely G_q and G_s, respectively. However, activation of certain receptors, such as m_2-mAChR, can directly inhibit cAMP synthesis via an inhibitory GTP-binding protein, G_i (Fig. 3). In contrast, there is little information about involvement of an analogous inhibitory GTP-binding protein in the negative regulation of PLC. More recently, Watkins *et al.* (67) reported that in stem cells (F_9 terato-carcinoma) or rat osteosarcoma 1712.8 cells in which $G_{i\alpha 2}$ expression is abolished by antisense RNA displays markedly elevated basal IP_3 accumulation and a potentiated PLC response to stimulatory hormones. Expression of the Q205L mutant of $G_{i\alpha 2}$, which is constitutively active, was found to block persistently hormonally stimulated PLC activity, implicating $G_{i\alpha 2}$ as an inhibitory regulator of PLC signalling. These authors suggested that $G_{i\alpha 2}$ could serve as an ideal locus for cross talk, integrating the PLC and AC signaling pathways. The ubiquitously expressed $G_{i\alpha 2}$ has been implicated in activation of cytoplasmic phospholipase A_2 in CHO cells (68, 69), activation of MAP kinase in fibroblasts (70) and inhibition of AC in various cell types (71). There is little evidence at this time for a direct interaction between $G_{i\alpha 2}$ and the phospholipases. Therefore, the identity of the G protein that is involved in the inhibitory regulation of PLC remains unknown.

While PKA has been reported to phosphorylate PLC purified from a wide variety of tissues, this phosphorylation does not appear to have an effect on the enzyme activity as measured *in vitro*. There are multiple PLC enzymes in mammalian tissues as deduced from direct protein isolation and molecular cloning studies. There are three families of mammalian PLC: PLC-β, PLC-γ, and PLC-δ. This classification is made on the basis of amino acid sequence conservation. At present, there is a total of 10 known PLC isozymes: four PLC-βs, two PLC-γs, and four PLC-δs (72). PLC-γ activity is regulated through receptor tyrosine kinases, PLC-β subtypes are regulated by G-protein subunits, and the mechanism of regulation of PLC-δ is not known at the present time. PLC-β is localized in the plasma membrane, and several lines of evidence have demonstrated both *in vitro* and *in vivo* that the GTP-bound α subunits of the G_q family activates this enzyme (73, 74). In addition, the subunits βγ of the heterotrimeric G protein activate β_2 isoform of PLC (75). Activation of PLC by G_q and by βγ subunits have been reported to be insensitive and sensitive to pertussis toxin treatment, respectively. Recently, purified M_1-mAChRs and $G_q/11$ were reconstituted in lipid vesicles, and addition of purified PLC-β_1 stimulated the receptor-promoted steady-state GTPase activity of $G_q/11$ up to 20-fold (73); the stimulation depended upon receptor-mediated GTP-GDP exchange. In contrast, PLC-γ_1 is stimulated by the tyrosine kinase-linked receptors, which interact directly with this form of the enzyme (76).

In smooth muscle, PLC was purified from guinea pig uterus (77) and from the bovine iris sphincter (78). In the bovine iris sphincter PLC-δ_1 and PLC-γ_1 were identified in the supernatant fraction and PLC-β_1 in the membrane fraction. Incubation of the purified enzymes with the catalytic subunit of PKA and [γ^{32}p]ATP resulted in increased phosphorylation of the soluble isozymes, but it had no inhibitory effect on their enzyme activities. PLC-β1 in iris membranes did not act as a substrate for PKA. To throw more light on the mechanism of muscarinic stimulation of PLC in this tissue, we have investigated the properties of this enzyme and its regulation by GTP analogs and protein phosphorylation in bovine iris sphincter membranes (79). In this study: (i) the presence of PLC-β_1 and a GTP-binding protein, $G_{q\alpha}$, was detected in the microsomal (membrane) fraction by anti-PLC β_1 and

anti-$G_{q\alpha}$ antibodies, respectively, and (ii) CCh-stimulated PIP_2 hydrolysis was found to be significantly reduced in membrane fractions prepared from iris sphincter pretreated with ISO. It was concluded that in bovine iris sphincter: (i) muscarinic stimulation of PLC-β_1, and subsequently PIP_2 hydrolysis, is mediated through the G-protein, $G_{q\alpha}$; and (ii) that phosphorylation of $G_{q\alpha}$ and/or muscarinic receptor by PKA could be a mechanism through which ISO inhibits CCh-stimulated hydrolysis of PIP_2 and contraction in this tissue. Wen *et al.* (80) working with rat myometrial plasma membranes reported that PKA inhibits PLC activity and alters phosphorylation in this tissue. They demonstrated that one or more GTP-binding proteins mediate activation of membrane-bound PLC in this tissue and showed that activation of endogenous and exogenous PKA inhibits the PLC activity. They concluded that phosphorylation of protein components is key to the regulation of PLC by uterine relaxants that elevate cAMP.

In conclusion, at this time little direct experimental evidence exists for the ability of PKA to phosphorylate and thereby inhibit the activities of either G_q or isozymes of PLC purified from smooth muscle or non-smooth muscle tissues. However, in nonvascular smooth muscle there is evidence that (i) phosphorylation of one or more membrane-associated proteins in rat myometrial plasma membranes by PKA may regulate myometrial PLC activity and this could play a role in the inhibition of oxytocin-induced increase in $[Ca^{2+}]_i$ and inositol phosphate formation by ISO and relaxin (80), and (ii) phosphorylation of mAChR, $G_{q\alpha}$, and/or PLC, presumably by PKA, in membranes isolated from bovine iris sphincter pretreated with ISO, results in impaired coupling between the two proteins and subsequently in attenuation of CCh-induced inositol phosphate production (79). Therefore, while elevation of intracellular cAMP concentrations can result in inhibition of agonist-stimulated PPI hydrolysis the precise site of cAMP inhibition (receptor, G-protein, or PLC) remains to be elucidated.

Regulation of IP_3 5-Phosphatase and IP_3-Kinase by Protein Kinase A

Another potential site for PKA regulation of the PPI signaling pathway is at the level of IP_3 inactivation (Fig. 3). Release of Ca^{2+} by IP_3 is terminated through the concerted actions of a series of phosphatases and kinases which convert IP_3 into a number of inositol phosphates. In most cells, inactivation of IP_3 occurs either through the action of IP_3 5-phosphatase, which dephosphorylates IP_3 at the 5-position to yield IP_2, or is phosphorylated by IP_3-kinase to yield IP_4. Thus, possible sites for cAMP actions could be either the stimulation of IP_3 hydrolysis by IP_3 5-phosphatase, or IP_3 phosphorylation by IP_3 3-kinase. This will terminate IP_3-induced Ca^{2+} mobilization and consequently inhibit muscle contraction. Recently, we have characterized IP_3 metabolism in the bovine iris sphincter and investigated the effects of protein phosphorylation by PKA and PKC on the activities of IP_3 5-phosphatase and IP_3 3-kinase both *in vitro* and in the intact muscle (81, 82). The iris sphincter contains IP_3 5-phosphatase and IP_1 monophosphatase which can rapidly degrade IP_3 to free myo-inositol, thus terminating IP_3-induced Ca^{2+} mobilization and muscle contraction. However, these phosphatases do not appear to be regulated by protein phosphorylation mediated by PKA or PKC (81). The studies on IP_3 3-kinase in the bovine sphincter revealed that the enzyme is a 50-kDa protein when analyzed by SDS/PAGE and is strongly stimulated by Ca-CaM. PKA was found to phosphorylate and stimulate IP_3 3-kinase activity both directly, when used with the purified enzyme, and indirectly, when the tissue was treated with ISO. PKC also phosphorylated the purified enzyme but this led to a decrease in its activity. However, treatment of the iris sphincter with PDBu resulted in increased activity of IP_3 3-kinase in the soluble fraction. The stimulatory effects of ISO and PDBu on the enzyme activity in the intact muscle was preserved on SDS/PAGE and renaturation. An autoradiogram of a representative experiment following phosphorylation of IP_3 3-kinase is given in Figure 5. It shows the effect of protein phosphorylation on the enzyme activity. As can be seen from this figure, when the purified enzyme was incubated with [γ-^{32}P]ATP and PKA or PKC, and the reaction products analyzed by SDS/PAGE, a 3- to 4-fold increase in ^{32}P-incorporation occurred, compared with the control, in the protein band (50 kDa) corresponding to IP_3 3-kinase. When IP_3 3-kinase was assayed following phosphorylation with PKA, a significant increase (62%) in enzyme activity was observed. In contrast, phosphorylation of the enzyme with PKC resulted in a significant decrease (25%) in its activity. These data support a role for IP_3 3-kinase in limiting the availability of IP_3 for Ca^{2+} release from the SR and suggest that the kinase could be an important site for cAMP inhibition (Fig. 3). In addition, a decrease in IP_3 in the cell could inactivate the capacitative Ca^{2+}-influx pathway.

That IP_3 3-kinase is a key regulatory enzyme in IP_3 metabolism and the fact that its activity can be modulated by PKA suggest that this enzyme could serve as a target for the cross talk between cAMP and the PPI signaling cascade.

Regulation of IP_3-Induced Ca^{2+} Release by Protein Kinase A

Another potential site for a cross talk between cAMP and the PPI signaling cascade is at the level of the IP_3 receptor (IP_3R) (Fig. 3). In the past decade, an enormous amount of progress has been made in our

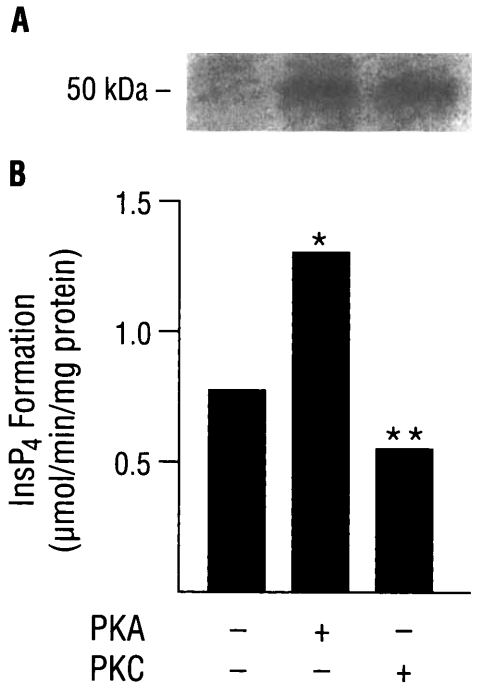

Figure 5. Phosphorylation of purified IP_3 3-kinase by PKA and PKC (A) Autoradiogram of ^{32}P-labeled IP_3 3-kinase (50-kDa bnad) following incubation of the enzyme with [γ-^{32}P]ATP and PKA or PKC and SDS/gel electrophoresis. The moles of phosphate incorporated into each mole of IP_3 3-kinase were: control, 0.6; PKA, 2.1; PKC, 1.7. (B) Effect of protein phosphorylation on IP_3 3-kinase activity. The purified enzyme was phosphorylated with PKA or PKC and then assayed. *Significantly higher than the control, $P < 0.05$; **significantly lower than the control, $P < 0.05$. (From Ref. 82, with permission.)

understanding of the mechanism of IP_3-induced Ca^{2+} release in various tissues, including smooth muscle (for recent reviews, see Refs. 83 through 86). Briefly, IP_3 binds to the IP_3R on the SR, and this activates a Ca^{2+} channel to release Ca^{2+} from the intracellular stores into the cytoplasm of the cell. The purified IP_3-R protein contains both the recognition site for IP_3 and associated Ca^{2+} channel, since reconstituted lipid vesicles containing the IP_3-R protein mediate IP_3R-induced Ca^{2+} flux (87). Recent evidence indicates that the IP_3R exists as a tetrameric complex to form a functional IP_3-gated Ca^{2+} channel. This is analogous to the plasma membrane receptor-operated Ca^{2+} channels, except that the IP_3R is localized in the intracellular membranes of the cell. The IP_3R contains an IP_3-binding domain, a regulatory (coupling) domain, and a Ca^{2+} channel domain, and its function can be modulated by cytosolic Ca^{2+}, CaM, ATP, and protein phosphorylation (84).

In smooth muscle the IP_3R was purified from rat vas deferens (88) and bovine aorta (89). Furthermore, direct binding of IP_3 was demonstrated in microsomal preparations isolated from bovine and rabbit tracheal smooth muscle (90, 91), bovine iris sphincter smooth muscle (92), and canine colonic circular smooth muscle (93). The purified IP_3R from aortic smooth muscle forms an IP_3-gated and heparin-sensitive Ca^{2+} channel in planar bilayers with functional properties characteristic of IP_3 triggered Ca^{2+} release (94). Smooth muscle and brain IP_3 receptors have been shown to be structurally and functionally similar (95). Nixon *et al.* (96), using immunogold labeling techniques, demonstrated that IP_3 receptors are present and associated with the SR in a phasic smooth muscle (guinea pig vas deferens) and a tonic muscle (guinea pig aorta) both at the cell periphery, the presumed site of IP_3 production, and deep within the cell several microns distance from the plasma membrane. These results suggest that IP_3-induced Ca^{2+} release can occur locally from both peripheral and central SR.

Studies on the effects of phosphorylation on the functional properties of the IP_3R have yielded conflicting results. The IP_3R can serve as a substrate for PKA, PKC, and Ca-CaM–dependent protein kinase II (97, 98). The IP_3R is also phosphorylated by cGMP-dependent protein kinase (99). The regulatory domain, which probably functions as the transducing domain in the coupling of IP_3 binding to Ca^{2+} channel opening, is the site of phosphorylation. With the exception of PKA phosphorylation, there is little information about the effects of PKC, CaM-dependent protein kinase II or cGMP-dependent protein kinase phosphorylation on IP_3R function. Thus, phosphorylation of the IP_3R from rat brain cerebellum using the catalytic subunit of PKA caused a 10-fold (100) or 2-fold (101) shift to the right in the concentration dependence of IP_3-induced Ca^{2+} release without affecting the IP_3 binding to the receptor. An inhibitory action of cAMP on IP_3-induced Ca^{2+} release was reported in saponin-permeabilized platelets (102) and platelet membranes (103), but it had no effect on Ca^{2+} uptake into platelet membrane vesicles or on subsequent release by IP_3 (104). In contrast, cAMP enhanced IP_3-induced Ca^{2+} mobilization of intracellular Ca^{2+} in cultured aortic smooth muscle cells (105) and in premeabilized hepatocytes (106).

While cAMP, via PKA, does seem to regulate IP_3-induced Ca^{2+} release in certain tissues, there is no experimental evidence at this time to show that this can also occur in nonvascular smooth muscle. Phosphorylation of the IP_3R could provide a target for second messenger cross talk (Fig. 3), however, there is a need to demonstrate functional consequences of IP_3R phosphorylation in the various tissues. This is especially critical because it is not yet established whether or not the alterations observed in IP_3-induced Ca^{2+} release due to PKA phosphorylation are a direct consequence of phosphorylation at the IP_3R.

Cross Talk between cAMP and Protein Kinase C

The cAMP and PPI signal transduction pathways regulate each other's activity at several steps (Fig. 3).

Agonist-induced PIP_2 hydrolysis produces simultaneously IP_3, which releases Ca^{2+} from the SR, and DAG which activates PKC. PKC is also activated by DAG derived from PC via the actions of PLD and phosphatidic acid phosphohydrolase, by arachidonic acid derived from phospholipids via the action of PLA_2 (Fig. 3), and by phorbol esters which mimic DAG. There are at least 12 distinct isoforms of PKC which have been identified by molecular cloning (107). In the iris sphincter smooth muscle, we have demonstrated the presence of α, β, γ, ϵ, δ and ζ, the remaining six isoforms were not investigated (126).

PKC has been suggested to play an important role in smooth muscle homeostasis and regulation of contractility (19, 36, 108). Phorbol esters induce slowly developing, sustained contractions in many smooth muscles, and there is reasonable experimental evidence that shows that PKC may be involved in the tonic phase of agonist-induced contraction. The mechanism and role of phorbol ester–induced contraction is under active investigation in many laboratories. Phorbol 12,13-dibutyrate (PDBu) induced contractile responses in rabbit (109, 110) but not in bovine sphincter (Fig. 6) (111). In airway smooth muscle studies, phorbol 12-myristate 13-acetate (PMA) alone failed to induce contraction in calf tracheal smooth muscle, although the phorbol ester induced a contractile response in the presence of Ca^{2+} ionophore (112). PDBu induced contraction in bovine bronchial rings (113) and human airway smooth muscle (114). PDBu, through activation of PKC, inhibits CCh stimulation of inositol phosphate formation and contraction in many types of smooth muscle (Fig. 6) (10, 18, 19, 36, 108, 109, 115, 116). Here, PKC appears to be involved indirectly in the regulation of contraction, mainly through feedback inhibition of agonist-induced PIP_2 hydrolysis (Fig. 3), the mechanisms underlying these effects are still speculative. Pretreatment of the bovine iris sphincter with staurosporine, a potent PKC inhibitor, had no effect on CCh-induced contraction (Fig. 6). Furthermore, densensitization of rabbit vas deferens with PMA for 90 min had no effect on CCh-induced contraction in this smooth muscle (117). These data do not support a role for PKC in CCh-induced contractions in these muscles.

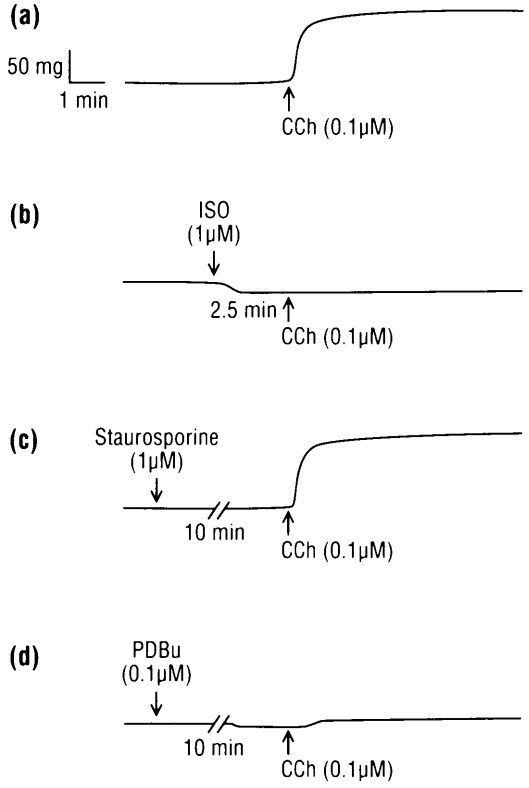

Figure 6. Representative recordings of the effects of (b) ISO, (c) staurosporine, and (d) PDBu on CCh-induced contraction in bovine iris sphincter. The muscles were preequilibrated in buffer for 90 min, then pretreated with ISO (1 μM), staurosporine (1 μM), or PDBu (0.1 μM) for 10 min followed by contraction with CCh (0.1 μM). (a) Control.

In the past decade several studies have shown that phorbol esters and other activators of PKC can also alter intracellular cAMP levels in various tissues and cultured cells (for review see Ref. 118). In many tissues, activation of PKC can induce significant changes, either stimulatory or inhibitory, in cAMP formation, elicited either by receptor activation or by forskolin, a direct activator of AC. Phorbol ester–activated PKC induces the phosphorylation of AC (see Ref. 119 and the references therein). However, it is not known how PKC modulates the catalytic activity of AC; this has been controversial. PKC phosphorylates and inactivates G_i, and this may relieve the inhibitory effect of G_i on AC. There are more than six types of AC: Types I and III are Ca-CaM stimulable, Types II, IV, and V are insensitive to Ca-CaM, and Type VI is Ca-CaM inhibitable (120). Phorbol esters increased cAMP formation by Type II AC expressed in human embryonic kidney 293 cell line (121, 122) and Types I and III expressed in the kidney 293 cells (123).

In bovine iris sphincter, PDBu stimulated cAMP production in a dose-dependent manner, over a concentration range of 0.01 to 10 μM ($EC_{50} = 8.8 \times 10^{-8}$ M) (Fig. 7). Pyne *et al.* (124) and Moughal *et al.* (125) have recently reported PKC-dependent cAMP formation in airway smooth muscle. In contrast, phorbol esters had no effect on cAMP levels in intestinal smooth muscle (115). Thus, several effects on PKC activation could contribute to the inhibition of agonist-stimulation of inositol phosphate production and contraction, including the increase in intracellular levels of cAMP, and phosphorylation of the mAChR and/or $G_{\alpha q}$ resulting in inhibition of receptor G-protein coupling.

While a type-specific stimulation of AC by PKC has not yet been reported in nonvascular smooth muscle, both the PKC and IP_3-Ca^{2+} arms of the PPI-

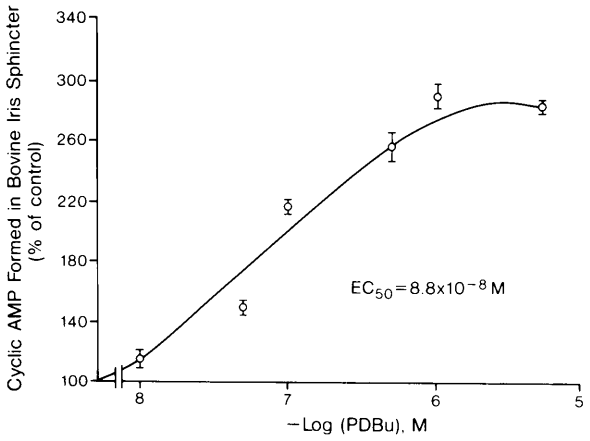

Figure 7. Dose-response effects of PDBu on cAMP formation in bovine iris sphincter. The tissue was exposed to different concentrations of PDBu for 15 min as indicated. The basal value for cAMP formation in bovine iris sphincter was (pmol/mg protein): 26.2 ± 0.5. (From Ref. 111, with permission.)

signaling cascade exert many effects on cAMP accumulation. The PKC-stimulated cAMP response can limit the activation of PLC due to muscarinic stimulation (Fig. 3). Regulation of AC by PKC may play important roles in cross talk between the PPI and the cAMP signaling pathways, and furthermore these mutual interactions provide both a negative feedback mechanism to autoregulate mAChR activity and a mechanism to integrate the various intracellular signals generated at the cell surface receptors.

Conclusion

It is clear from the findings outlined above that in nonvascular smooth muscle, cAMP-elevating agents are effective relaxants and that cross talk between cAMP and the PPI signaling cascade plays an important role in the regulation of contraction-relaxation responses. Furthermore, recent evidence indicates that in this smooth muscle the cross talk between these two signaling pathways may underlie the functional antagonism between contraction and β-adrenoreceptor relaxation, and that the site of cAMP inhibition is on Ca^{2+} mobilization rather than MLC phosphorylation. A scheme for cAMP inhibition of agonist-induced PPI hydrolysis and contraction was proposed (Fig. 3). There is compelling evidence now that indicates that IP_3 is involved in both the release of Ca^{2+} from the SR and in Ca^{2+} influx through the plasma membrane. Therefore, if we accept the hypothesis that a reduction in $[Ca^{2+}]_i$ is the underlying mechanism for cAMP-mediated relaxation, then it is not unreasonable to assume that an important target of cAMP actions, presumably through activation of PKA, would be either to inhibit IP_3 production or to stimulate IP_3 inactivation (Fig. 3). A reasonable amount of experimental evidence was presented here in support of: (i) cAMP inhibition of PLC activation, both in intact muscle and in smooth muscle membranes, and (ii) cAMP stimulation of IP_3 3-Kinase. Both of these cAMP actions can result in a reduction in IP_3 concentrations. This, in turn, will result in a reduction in IP_3-dependent Ca^{2+} mobilization and subsequently will lead to relaxation. While evidence obtained from muscle pretreated with ISO points to G protein activation as the site of cAMP inhibition, there is a need to determine whether the G protein coupling and/or PLC activation is altered by the PKA phosphorylation. This is especially significant since PKA phosphorylation had no effect on the activities of the purified PLC isozymes. In addition to IP_3-induced Ca^{2+} mobilization, changes in $[Ca^{2+}]_i$ are the result of the interplay of many processes (Fig. 3) which could also serve as potential sites for cAMP inhibition. More information about the effects of PKA phosphorylation on these processes in nonvascular smooth muscle is needed. The role, if any, of PKC activation of adenylyl cyclase in cAMP inhibition of the PPI signaling cascade needs to be explored. In addition to activation of adenylyl cyclase, PKC also activates phospholipases A_2 and D, and inhibits agonist-stimulated PLC activity (Fig. 3). This is an example of how activation of one signaling pathway can either activate or inhibit the other pathway. Activation of PLA_2 increases the release of arachidonic acid and subsequently the eicosanoids, and this could contribute to the cellular effects of phorbol esters and Ca^{2+}-mobilizing agonists.

In spite of the considerable amount of effort made by many on cross talk between cAMP and the PPI signaling pathways in both muscle and nonmuscle tissues, we still know little about the actual sites for cAMP inhibition. This is in spite of the significant progress that was made recently in our understanding of: (i) how Ca^{2+}-mobilizing receptors are coupled to PLC isozymes, (ii) the nature of the G proteins that couple these receptors to the effector, (iii) the physiological functions of IP_3, and (iv) the regulation of the enzymes involved in PPI metabolism and its derived second messengers. In the coming years there will be more advances made on the regulation of these second messenger systems and their modulation of smooth muscle contraction-relaxation. Cyclic cAMP-IP_3-Ca^{2+} interactions are important in regulation of smooth muscle tone and represent an important focal point for pharmacological manipulation. Studies on interactions between cAMP and PPI signaling cascade in smooth muscle cells transfected with β-adrenoreceptors and m_3-mAChRs could throw more light on the cross talk between the two second messenger systems. Clearly an understanding of physiological and pathophysiological regulation of smooth muscle tone is central to the development of novel therapeutic agents for the treat-

ment of diseases such as asthma and glaucoma where cAMP-elevating drugs are used routinely.

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