MINIREVIEW

Cross Talk Between Cyclic Nucleotides and Polyphosphoinositide Hydrolysis, Protein Kinases, and Contraction in Smooth Muscle[1]

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This article provides an update of a minireview published in 1996 (Abdel-Latif AA. Proc Soc Exp Biol Med 211:163–177, 1996), the purpose of which was to examine in nonvascular smooth muscle the biochemical and functional cross talk between the sympathetic nervous system, which governs the formation of cAMP and muscle relaxation, and the parasympathetic nervous system, which governs the generation of IP_3 and diacylglycerol, from the polyphosphoinositides, Ca^{2+} mobilization, and contraction. This review examines further evidence, both from nonvascular and vascular smooth muscle, for cross talk between the cyclic nucleotides, cAMP and cGMP via their respective protein kinases, and the Ca^{2+}-dependent- and Ca^{2+}-independent-signaling pathways involved in agonist-induced contraction. These include the IP_3-Ca^{2+}-CaM- myosin light chain kinase (MLCK) pathway and the Ca^{2+}-independent pathways, including protein kinase C-, MAP kinase-, and Rho-kinase. In addition, MLC phosphorylation and contraction can also be increased by a decrease in myosin phosphatase activity. A summary of the cross talk between the cyclic nucleotides and these signaling pathways was presented. In smooth muscle, there are several targets for cyclic nucleotide inhibition and consequent relaxation, including the receptor, G proteins, phospholipase C-β1–4 isoforms, IP_3 receptor, Ca^{2+} mobilization, MLCK, MAP kinase, Rho-kinase, and myosin phosphatase. While significant progress has been made in the past four years on this cross talk, the precise mechanisms underlying the biochemical basis for the cyclic nucleotide inhibition of Ca^{2+} mobilization and consequently muscle contraction remain to be established. Although it is well established that second-messenger cross talk plays an important role in smooth muscle relaxation, the many sources which exist in smooth muscle for Ca^{2+} mobilization, coupled with the multiple signaling pathways involved in agonist-induced contraction, contribute appreciably to the difficulties found by many investigators in identifying the targets for cyclic nucleotide inhibition and consequent relaxation. Better methodology and more novel interdisciplinary approaches are required for elucidating the mechanism(s) of cAMP- and cGMP-inhibition of smooth muscle contraction.

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Key words: smooth muscle; calcium-mobilizing agonists; polyphosphoinositides; cyclic nucleotides; contraction-relaxation

This article provides an update of a minireview published in 1996 (1), the purpose of which was to summarize the experimental evidence for the cross talk between cAMP and the polyphosphoinositide (PPI) signaling cascade 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. The studies described in that review were confined largely to reports on the mechanism of the inhibitory effects of cyclic nucleotides (cAMP and cGMP) on the stimulation of smooth muscle by Ca^{2+}-mobilizing agonists. Briefly, activation of G protein-coupled receptors, such as muscarinic, prostaglandin $F_{2\alpha}$ or endothelin-1, results in activation of phospholipase C-β (PLC) and the phosphodiesteric breakdown of the PPI, phosphatidyl inositol 4,5-bisphosphate (PIP_2), into inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG). Both products have intracellular second messenger functions. DAG acts by stimulating protein kinase C (PKC), whereas IP_3 causes a release of Ca^{2+} from the sarcoplasmic reticulum (SR) in smooth muscle that is

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followed by entry of Ca^{2+} across the plasma membrane. The released intracellular Ca^{2+} binds to calmodulin (CaM), and the Ca^{2+}-CaM complex activates myosin light chain kinase (MLCK). By phosphorylating the regulatory light chain of myosin (MLC_{20}), MLCK causes the smooth muscle to contract. This is the major signaling pathway for smooth muscle contraction, and it has been referred to as the "MLCK pathway." DAG, the other second messenger, activates PKC, which may lead to phosphorylation of MLC_{20} and contraction of smooth muscle. Addition of cAMP- or cGMP-elevating agents to smooth muscle pre-stimulated with Ca^{2+}-mobilizing agonists [e.g., carbachol (CCh); norepinephrine, endothelin (ET), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), etc.] results in (i) inhibition of agonist-induced contraction, i.e., relaxation, (ii) inhibition of agonist-induced IP_3 production, presumably by inhibiting PLC activity, (iii) stimulation of the conversion of IP_3 to IP_4 *via* IP_3-3-kinase, and this lowers IP_3 concentration, and (iv) inhibition of agonist-induced intracellular Ca^{2+} ($[Ca^{2+}]_i$) mobilization.

In this brief review, an update is given of more recent studies on the cross talk between the cyclic nucleotides and the IP_3-Ca^{2+} signaling cascade in smooth muscle. In addition, while the MLCK pathway is the major determinant of excitation–contraction coupling in smooth muscle (for review see Ref. 2), there is accumulating evidence for additional mechanisms, including the PKC, the mitogen-activated protein kinase (p42 and p44 MAP kinase)-, and the Rho-kinase pathways (for reviews, see Refs. 3–5). Another important mechanism of Ca^{2+} sensitization of smooth muscle contraction is through inhibition of myosin phosphatase (4). Thus, it is not unreasonable to speculate that there is also cross talk between cyclic nucleotides and the protein kinases and phosphatases. The experimental evidence for this cross talk will also be presented here.

Cross Talk Between Cyclic Nucleotides and Contraction

The primary function of smooth muscle is mechanical, to generate force. This force may be utilized to perform many functions, including maintenance and regulation of circulation, gastrointestinal motility, expulsion of the fetus, regulation of light admitted to the retina, regulation of intraocular pressure in the eye, and the behavior of the urinogenital tract. Smooth muscle contraction–relaxation also contributes significantly to many diseases, including cardiovascular disease, high blood pressure, glaucoma, asthma, etc. Agents that are able to contract smooth muscle include high K^+, α-adrenergic agonists, muscarinic agonists, endothelin, prostaglandins ($PGF_{2\alpha}$ and TXA_2) etc., and agents that are able to relax smooth muscle include low Ca^{2+}, β-adrenergic agonists, cGMP/NO, prostaglandins (EP_2), calcitonin gene-related peptide, adrenomedullin, etc. Among the drugs that are known to relax smooth muscle and regulate cellular functions are the cAMP- and cGMP-elevating agents (for review see Ref. 6). The mechanisms of these drugs are thought to lower the agonist-induced intracellular Ca^{2+} ($[Ca^{2+}]_i$) by increasing intracellular cAMP and cGMP, respectively. Smooth muscle relaxation occurs in two ways: (i) passive relaxation by removal of the contractile agent or (ii) active relaxation resulting from the activation of cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG) in the continued presence of the contractile agent. Table I shows recent examples of the relaxing effects of cAMP-elevating agents on agonist-induced contraction in smooth muscle.

Although it is well established that in nonvascular smooth muscle, such as the iris and trachea, elevation of intracellular cAMP concentrations can lead to inhibition of agonist-stimulated PPI metabolism and contraction, there is a paucity of reports showing that cGMP is similarly involved in the mechanism of inhibition of these responses (1). However, there is general agreement now that cGMP mediates vascular smooth muscle relaxation by NO-generating vasodilators, atrial natriuretic peptide, and endothelium-derived relaxing factor (15). In bovine ciliary muscle, sodium nitroprusside stimulated cGMP formation in a time- and concentration-dependent manner and dose-dependently inhibited carbachol-induced IP_3 production and contraction (16). There is evidence from several laboratories that indicates that cGMP-dependent protein kinase may be activated by physiological increases in cAMP (cited in Ref. 6). Studies on the involvement of cGMP-dependent protein kinase in the relaxation of ovine pulmonary arteries to cGMP and cAMP revealed that a nearly four-fold higher

Table I. Recent Examples of the Relaxing Effects of cAMP-Elevating Agents on Agonist-Induced Contraction in Smooth Muscle

Smooth muscle	Species	Contracting agents	cAMP-elevating agent	References
Mesenteric artery	Rat	Phenylephrine	Isoproterenol	6, 7
Urinary bladder strips	Rat	KCl	Isoproterenol	8
Tracheal	Rabbit	Acetylcholine	Isoproterenol	9
Small mesenteric arteries	Rat	Norepinephrine	IBMX	10
Ileum and trachea	Guinea pig	Histamine, oxotremorin-M	Isoproterenol	11
Iris dilator	Rabbit	Norepinephrine	Calcitonin gene-related peptide, prostaglandin E_2	12
Iris sphincter	Cat	Carbachol	Adrenomedullin	13
Carotid artery	Bovine	Serotonin	Forskolin	14

concentration of cAMP than cGMP was required to relax arteries by 50% and to activate PKG by 50% (17). These authors concluded that relaxation of pulmonary arteries is more sensitive to cGMP than cAMP and that PKG plays an important role in both cGMP- and cAMP-mediated relaxation. Possible substrates for cyclic nucleotide-dependent kinases are the receptor, G proteins, phospholipase C-β, IP_3 receptor, the Ca^{2+}-pump (SR and plasma membrane), the protein kinases MLCK, p42/p44 MAP kinase, and Rho-kinase, and telokin, which upon phosphorylation activates MLC phosphatase (4).

Phosphorylation of the above substrates by PKA and/or PKG could cause smooth muscle relaxation through decreases in $[Ca^{2+}]_i$ and MLC phosphorylation.

Cross Talk Between Cyclic Nucleotides and IP_3 Production and Ca^{2+} Mobilization

It is well accepted that elevation of intracellular cAMP or cGMP by cyclic nucleotide-elevating agents causes relaxation of smooth muscle and that the cyclic nucleotide-dependent reduction of $[Ca^{2+}]_i$ mobilization is a critical event involved in this inhibitory pathway. Effects of carbachol on $[Ca^{2+}]_i$ mobilization and their regulation by cAMP- (18) and cGMP-elevating (19) agents in SV-40 transformed cat iris sphincter smooth muscle (SV-CISM-2) cells are shown in Figs. 1 and 2, respectively. Pretreatment of the cells with 0.5 μ*M* isoproterenol, a β-adrenergic agonist, for 5 min resulted in complete inhibition of the carbachol-stimulated increase in $[Ca^{2+}]_i$. However, the late-sustained increase in $[Ca^{2+}]_i$ was only partially decreased by the isoproterenol treatment. When these cells were incubated with different concentrations of isoproterenol, a concentration-dependent inhibition of carbachol-induced $[Ca^{2+}]_i$ mobilization was observed with an IC_{50} value of 0.17 μ*M* (Fig. 1). Similarly, pretreatment of the smooth muscle cells with the cGMP-elevating agents, atrial natriuretic peptide (ANP), C-type natriuretic peptide (CNP), sodium nitroprusside (SNP), or 8-Br-cGMP (a cell-permeable analog of cGMP), inhibited the carbachol-induced $[Ca^{2+}]_i$ mobilization by 95%, 92%, 27%, and 58%, respectively (Fig. 2). As can be seen from Fig. 2, ANP and CNP, which have been reported to exert a marked stimulatory effect on the particulate guanylate cyclase in these cells (19), had a much more pronounced inhibitory effect on CCh-induced $[Ca^{2+}]_i$ mobilization than SNP, which stimulates the soluble guanylate cyclase, or 8-Br-cGMP. The molecular mechanisms that underlie these differences are not clear. They could be due to differences in the pools and amounts of cGMP generated by the agonists, or to differences in the cGMP phosphodiesterases and cGMP-dependent protein kinases activated by these agonists, or to differences in the cGMP-dependent phosphorylation of enzymes and ion channels involved in agonist-induced $[Ca^{2+}]_i$ mobilization. These data suggest a mechanism of action for cAMP- and cGMP-elevating agents in mediating decreases in $[Ca^{2+}]_i$ mobilization through activation of cAMP- and cGMP-dependent protein kinases, respectively. Several mechanisms have been proposed to account for cAMP- and cGMP-dependent inhibition of $[Ca^{2+}]_i$ mobilization, including inhibition of IP_3 formation via inhibition of PLC or PLC-G-protein-receptor coupling, inhibition of Ca^{2+} release from the SR, stimulation of Ca^{2+} uptake and/or extrusion, and inhibition of Ca^{2+} entry. Thus, addition of forskolin to cultured canine tracheal smooth muscle pretreated with bradykinin (20) and to canine cultured aorta smooth muscle cells pretreated with 5-hydroxytryptamine (21) or with endothelin (22) caused the attenuation of agonist-induced inositol phosphates production and of $[Ca^{2+}]_i$ mobilization. In pregnant rat myometrium, β-adrenergic receptor activation attenuated the generation of inositol phosphates, and this was found to correlate well

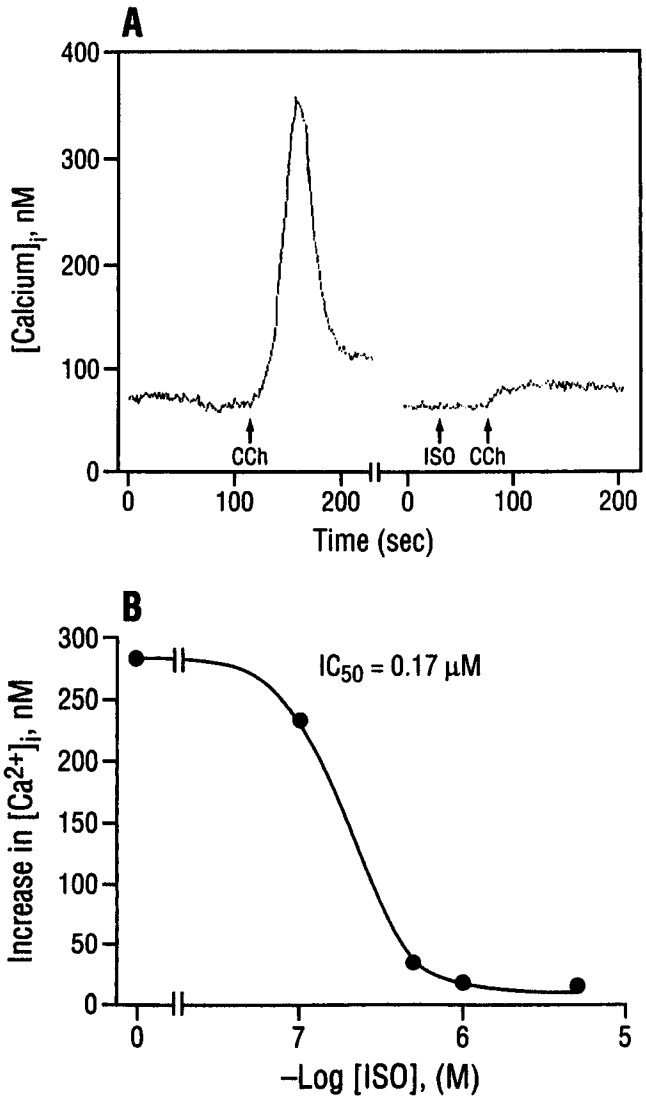

Figure 1. Effect of isoproterenol on carbachol-induced $[Ca^{2+}]_i$ mobilization in SV-CISM-2 cells. (A) Spectrofluorometer traces showing carbachol-induced changes in $[Ca^{2+}]_i$ concentrations with or without treatment of the cells with 0.5 μ*M* isoproterenol. The concentration of carbachol used was 1 μ*M*. (B) Concentration-dependent effects of isoproterenol on carbachol-induced $[Ca^{2+}]_i$. Each data point represents the peak $[Ca^{2+}]_i$ level attained with 1 μ*M* carbachol and is an average of three determinations. (Taken from Ref. 18 with permission.)

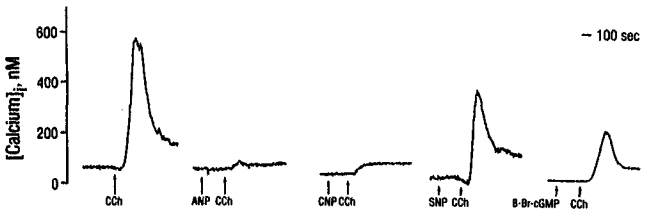

Figure 2. Typical tracings of the effects of atrial natriuretic peptide (ANP), C-type natriuretic peptide (CNP), sodium nitroprusside (SNP), and 8-Br-cGMP on carbachol-induced $[Ca^{2+}]_i$ mobilization in SV-CISM-2 cells. The confluent cells, grown as monolayers on glass cover slips, were loaded with 5 μ*M* fura-2/AM for 45 min at room temperature, washed, and then incubated in KRB buffer that contained 1.25 m*M* Ca^{2+}. The fluorescence of the Ca^{2+}-bound and -unbound fura-2 was measured by using a dual-wavelength spectrofluorometer. Tracings show the carbachol (1 μ*M*)-induced changes in $[Ca^{2+}]_i$ concentration in the absence and presence of ANP (1 μ*M*), CNP (1 μ*M*), SNP (1 m*M*), and 8-Br-cGMP (1 m*M*). Bar represents 100 sec. (Taken from Ref. 19 with permission.)

with inhibition of Ca^{2+} influx (23). In single airway smooth muscle cells, carbachol inhibited both cAMP formation and isoproterenol-stimulated decreases in $[Ca^{2+}]_i$ (24). Lee *et al.* (25), using permeabilized, arterial smooth muscle strips where membrane-associated pathways remain intact but intracellular Ca^{2+} stores are depleted, investigated mechanism(s) for the desensitization of contractile force by cGMP. They concluded that cGMP causes Ca^{2+} desensitization in this smooth muscle by activating MLC phosphatase.

Possible Mechanisms for the Cross Talk Between Cyclic Nucleotides and the IP_3-Ca^{2+} Cascade

The background, the functional antagonism between the sympathetic and parasympathetic nervous systems, and the cross talk between cyclic nucleotides and the IP_3-Ca^{2+} cascade in smooth muscle was discussed previously (1). The following is an update on the potential sites of cyclic nucleotide inhibition.

Regulation of Phospholipase C-β by Protein Kinase A (PKA). A summary of the experimental evidence for the regulation by cAMP and cGMP, *via* their respective protein kinases, of the Gq-protein-mediated phospholipase C-β was given in an earlier review (1). Briefly, there are 10 mammalian isozymes identified to date, all of which are single polypeptides and can be divided into three types: β, γ, and δ, of which four PLC-β, two PLC-γ, and four PLC-δ proteins are known (for reviews see Refs. 26 and 27). The δ-type isozymes are smaller (M_r 85,000) than the PLC-β and PLC-γ (M_r 140,000–155,000) isoforms. All of these isoforms have been identified in both vascular and nonvascular smooth muscle (1, 27). However, these isoforms are differentially regulated (28). PLC-β, of which there are currently four isoforms described (PLC-β1 through PLC-β4), is regulated by heterotrimeric G proteins in response to agonist binding to serpentine receptors. PLC-β1, -β3, and -β4 are regulated by binding of the α-subunit of the pertussis toxin-insensitive Gq family of heterotrimeric G proteins, whereas PLC-β2 is thought to be largely regulated by G protein βγ-subunits (27).

Activation of cAMP-dependent protein kinase (PKA) attenuates the PLC signaling pathway in a wide variety of cells. Receptors coupled to Gαs subunits stimulate adenylate cyclase, leading to an increase in intracellular cAMP from ATP and the activation of PKA, PKA can inhibit the activation of PLC-β by phosphorylating it. Dodge *et al.* (29) investigated the importance of the localization of PKA to the plasma membrane for cAMP-mediated inhibition of phosphoinositide turnover in an immortalized pregnant human myometrial (PHM1-41) cell line. They found that PKA is anchored to the myometrial plasma membrane through association with a putative A kinase anchoring protein (AKAP86) which is similar to AKAP79, and that this anchoring is required for the cAMP-mediated inhibition of phosphoinositide turnover in these cells. The proposed targets for phosphorylation by PKA in the PPI cascade include the Ca^{2+}-mobilizing receptor, G proteins and PLC-β itself. Although PLC-β1 is rapidly phosphorylated in cells treated with PKA, the phosphorylation had no effect on either the basal or Gqα-stimulated activities of PLC-β1 (1, 26). However, interaction of PLC and PKA was more recently studied in COS cells transfected with cDNAs encoding PLC-β2, G protein subunits, and PKA (30). Expression of the catalytic subunit of PKA specifically inhibited Gβγ stimulation of PLC-β2 activity, without affecting Gαq-induced activation. The effect of PKA was not mimicked by PKC isozymes. Furthermore, PKA phosphorylated serine residues of PLC-β2 both *in vivo* and *in vitro*. These studies indicate that PKA can directly phosphorylate PLC-β and regulate the activation of receptor-mediated G-protein coupled phosphoinositide turnover, suggesting a molecular mechanism for the regulation of $[Ca^{2+}]_i$ by the cyclic nucleotides-dependent signaling pathways.

These findings need to be demonstrated in intact tissue, including smooth muscle, and the nature of the effects of cyclic nucleotides on phospholipase C-β isoforms (PLC-β1–4) activities remains to be elucidated. In addition, there is a need to investigate cyclic nucleotide-dependent phosphorylation of the Gq-G protein as possible cause of cyclic nucleotides inhibition of PLC and IP_3 production in stimulated smooth muscle.

Cross Talk Between Cyclic Nucleotides and IP_3-Induced Ca^{2+} Release. Another potential site for cyclic nucleotide inhibition of $[Ca^{2+}]_i$ mobilization is at the IP_3 receptor level. Phosphorylation of the IP_3 receptor by the cyclic nucleotide-dependent protein kinases represents a possible mechanism for cross talk whereby cyclic nucleotides can modulate IP_3-mediated regulation of $[Ca^{2+}]_i$ levels. The IP_3 receptor is an intracellular Ca^{2+}-release channel, of which three isoforms are identified (31, 32). High amounts of type I IP_3 receptor are seen in smooth muscle cells and in cerebellar Purkinje neurons. PKA and PKG phosphorylate the IP_3 receptor in many cell types including smooth muscle (see references cited in Ref. 33). Multiple

consensus phosphorylation sites for PKC and CaM Kinase II are present within the IP_3 receptor sequence (31). Regulation of IP_3 receptor activity is also achieved through tyrosine phosphorylation (34).

In intact rat megakaryocytes, the progenitor of platelets, agonist-induced $[Ca^{2+}]_i$ oscillations are reversibly inhibited by agents that elevate intracellular cAMP and cGMP (35). Later studies on these cells from this laboratory revealed that cGMP inhibits IP_3-induced Ca^{2+} release *via* cGMP- and cAMP-dependent protein kinases (36), and that elevation of cAMP by carbacyclin leads to the activation of PKA and thereby to the inhibition of IP_3-induced Ca^{2+} release (37). Wojcikiewicz and Luo (38) examined the ability of PKA to phosphorylate types I, II, and III IP_3 receptors in various cell lines. Type I was a good substrate for PKA, whereas types II and III were phosphorylated relatively weakly. They found that PKA enhanced IP_3-induced Ca^{2+} mobilization in a range of permeabilized cell types, irrespective of whether the type I, II, or III receptor was predominant. Schlossmann *et al.* (39), working with microsomal membranes from tracheal smooth muscle, reported that NO/cGMP/cAMP kinase I (cGKI) phosphorylated the IP_3 receptor and cGKIβ and a protein of relative molecular mass 125,000 which they identified as the IP_3 receptor-associated cGMP kinase substrate (IRAG). They concluded that in smooth muscle, phosphorylation of IRAG by cGKI is probably a major mechanism that reduces $[Ca^{2+}]_i$ and relaxes smooth muscle.

Komalavilas and Lincoln (40) examined the effects of cGMP and activation of PKG on the phosphorylation of the IP_3 receptor in intact rat aorta using the technique of back phosphorylation. They concluded that the actions of cAMP on smooth muscle relaxation may be mediated by the cGMP-dependent protein kinase-I (PKG-I), the major PKG isoform in smooth muscle cells. However, later studies by Pfeifer *et al.* (41) on PKG-I-deficient mice indicate that cAMP most likely acts through PKA. To study the biological role of cGKI and its postulated cross-activation by cAMP, these investigators inactivated the gene coding for cGKI in mice. Loss of cGKI abolished nitric oxide (NO)/cGMP-dependent relaxation of smooth muscle, resulting in severe vascular and intestinal dysfunctions. However, cGKI-deficient smooth muscle responded normally to cAMP, indicating that cAMP and cGMP signal *via* independent pathways, with cGKI being the specific mediator of the NO/cGMP effects in murine smooth muscle. More recently, Ny *et al.* (42) investigated the NO/cGMP- and vasoactive intestinal (polypeptide)/cAMP-signaling pathways in the gastric fundus of wild type and cGKI-deficient mice. They concluded that cGKI plays a central role in the NO/cGMP signaling cascade producing relaxation of mouse gastric fundus smooth muscle, and that relaxant agents acting *via* the cGMP pathway can exert their effects independently of cGKI.

The above studies add further support to the concepts (a) that the IP_3 receptor is an important site for cAMP- and cGMP inhibition of $[Ca^{2+}]_i$ mobilization and (b) that a reduction in $[Ca^{2+}]_i$ is a major underlying mechanism for cyclic nucleotide-mediated relaxation.

Cross Talk Between Cyclic Nucleotides and the Protein Kinases

Many of the same Ca^{2+}-mobilizing agonists that activate the PPI signaling cascade also activate MAP kinase and Rho-kinase. Activation of these kinases leads to phosphorylation of MLC_{20} and contraction of the smooth muscle. In the past few years several excellent reports and reviews have appeared on the involvement of Ca^{2+}-CaM-MLCK (2–4, 43), PKC (44, 45), p42/p44 MAP kinase (46–50), and Rho-kinase (51–59) in the mechanism of smooth muscle contraction. All of these kinases may directly induce contraction through MLC phosphorylation. Addition of the catalytic subunit of Rho-kinase to permeabilized vessels results in contraction (55), and Y27632, an inhibitor of Rho-kinase, inhibits contraction induced by phenylephrine or GTPγS (54). These data demonstrate involvement of Rho-kinase in smooth muscle contraction. There is also evidence indicating that PKC (52, 60) and Rho-kinase (4, 52, 56, 58) may enhance contractile force at a given submaximal concentration of free Ca^{2+} by increasing the level of MLC phosphorylation *via* inhibition of myosin phosphatase. Moreover, in swine carotid artery a Ca^{2+}-dependent isoform of PKC and CaM kinase II have been reported to be upstream activators of MAP kinase (45).

Many of these protein kinases have been implicated in smooth muscle contraction, based largely on the effects of agents that activate or inhibit them. The data shown in Table II on the effects of various protein kinase inhibitors on prostaglandin $F_{2\alpha}$- and carbachol-induced contraction in cat iris sphincter demonstrate the important role these kinases play in the mechanism of agonist-induced smooth muscle contraction. As can be seen from Table II, at 10 μ*M* concentrations, KN-93, a CaM kinase II inhibitor, and ML-7 and Wortmannin, MLC kinase inhibitors, inhibited by up to 80% both prostaglandin $F_{2\alpha}$- and carbachol-induced contraction. In contrast, at 10 μ*M* concentrations, PD98059, Apigenin, and UO126, all potent inhibitors of p42/p44 MAP kinase activity, inhibited prostaglandin $F_{2\alpha}$-induced contraction by 94%, 87%, and 80%, respectively, but had a much lesser effect on the carbachol-induced contraction. This differential effect of the inhibitors was also observed with Rock Y27632, a potent inhibitor of Rho-kinase, which at 1 μ*M* inhibited prostaglandin $F_{2\alpha}$- and carbachol-induced contraction by 95% and 26%, respectively. These data demonstrate appreciable involvement of p42/p44 MAP kinase activity and Rho-kinase activity in prostaglandin $F_{2\alpha}$- but not in carbachol-induced contraction in this smooth muscle. Furthermore, these findings indicate that Ca^{2+}/CaM-MLCK is involved in both prostaglandin $F_{2\alpha}$- and carbachol-induced contraction. These data clearly indicate that the stimulation of the iris sphincter with prostaglandin $F_{2\alpha}$ and carbachol activate two distinct pathways, namely, the MAP

Table II. Effects of Various Protein Kinase Inhibitors on Prostaglandin $F_{2\alpha}$-Induced and Carbachol-Induced Contraction in Cat Iris Sphincter Smooth Muscle[a]

Inhibitor added	Concentration (μM)	Contractile response (mg tension per mg wet weight tissue)[b]			
		Prostaglandin $F_{2\alpha}$ (5 nM)	Percent of control	Carbachol (50 nM)	Percent of control
1. Ca^{2+}/CaM kinase II inhibitor					
KN-93	1	12.1 ± 1.4	65	11.6 ± 1.0	70
KN-93	10	3.7 ± 0.2	20	3.0 ± 0.1	18
2. Ca^{2+}/CaM-MLCK inhibitors					
ML-7	1	13.0 ± 1.4	70	11.7 ± 1.0	65
ML-7	10	5.2 ± 0.4	28	5.4 ± 0.4	30
Wortmannin	1	11.2 ± 1.0	60	9.4 ± 0.6	55
Wortmannin	10	3.7 ± 0.1	20	3.1 ± 0.1	18
3. PKC inhibitor					
RO31-8220	1	15.8 ± 0.9	80	18.9 ± 1.2	100
RO31-8220	10	7.4 ± 1.0	40	13.6 ± 1.0	72
4. p42/p44 MAP kinase inhibitors					
PD 98059	1	14.0 ± 1.2	75	17.2 ± 1.2	100
PD 98059	10	1.1 ± 0.1	6	14.1 ± 1.0	82
Apigenin	1	14.5 ± 1.0	78	18.1 ± 1.0	100
Apigenin	10	2.4 ± 0.2	13	15.7 ± 1.0	87
U0126	1	16.5 ± 1.2	89	18.0 ± 1.2	100
U0126	10	3.6 ± 0.2	20	15.5 ± 1.3	86
5. Rho-kinase inhibitor					
Rock Y27632	0.1	10	60	19	100
Rock Y27632	1.0	1.0	5	14	74
6. Protein tyrosine kinase inhibitor					
Genistein	1	9.0 ± 1.0	48	18.0 ± 1.2	97
Genistein	10	2.8 ± 0.12	15	14.79 ± 1.2	85

[a] Part of this data was taken from Refs. 50 and 61.
[b] Sphincter muscles were contracted by 5 nM prostaglandin $F_{2\alpha}$ (18.6 mg tension per mg wet weight tissue) or by 50 nM carbachol (17.4 mg tension per mg wet weight tissue) for 3 min followed by addition of the inhibitors as indicated for 15 min. The data are the mean ± SEM of 3–5 different experiments.

kinase pathway and the Ca^{2+} mobilization pathway. In cat iris sphincter, protein tyrosine phosphorylation is involved in the mechanism of prostaglandin $F_{2\alpha}$- but not in that of carbachol-induced contraction, IP_3 accumulation, and Ca^{2+} mobilization (61). It is interesting to note that the protein tyrosine kinase inhibitor, genistein, inhibited both prostaglandin $F_{2\alpha}$-induced contraction and IP_3 production and Ca^{2+} mobilization in this tissue (61). In contrast, the inhibitors of MAP kinase and Rho-kinase blocked prostaglandin $F_{2\alpha}$-induced contraction but not that of phosphoinositide hydrolysis. This could suggest that MAP kinase and Rho-kinase exert their contractile effects *via* direct phosphorylation of MLC_{20} and not through the IP_3-Ca^{2+}-CaM-MLCK pathway. The differential effects of the protein kinase inhibitors on prostaglandin $F_{2\alpha}$- and carbachol-induced contraction could be due to the fact that the prostaglandin promotes changes in smooth muscle contractility and it is a smooth muscle mitogen, whereas the muscarinic agonist promotes only smooth muscle contractility. These findings suggest an important role for the protein kinases in muscle contraction.

In smooth muscle, as well as in other tissues, there is extensive cross talk between cyclic nucleotides and other signaling cascades, including those that involve CaM-dependent protein kinases and MAP kinases. Thus, in the 1980s Adelstein and colleagues (62, 63) suggested that PKA phosphorylates purified MLCK resulting in a 10-fold decrease in the sensitivity of MLCK to activation by Ca^{2+}-CaM. It was hypothesized that cAMP promotes relaxation of smooth muscle by inhibiting MLC_{20} phosphorylation *via* phosphorylation of MLCK by PKA. This biochemical mechanism by which cAMP would lead to desensitization of the contractile response to $[Ca^{2+}]_i$ remains controversial (6). There is evidence that suggests a cross talk between cyclic nucleotides and the CaM kinase cascade (64–66); however, there is little information about the inhibitory effects of cyclic nucleotide-elevating agents on Ca^{2+}/CaM-dependent protein kinase II which has been shown to phosphorylate MLCK in smooth muscle (2).

In addition to activation of the PPI signaling pathway, activation of G protein-coupled receptors also results in stimulation of the MAP- and Rho-kinases pathways (58). Despite considerable investigation, the precise mechanisms involved in coupling G protein activation to stimulation of the MAP- and Rho-kinases cascades remain unclear. However, it is well established that G-protein coupled receptors use multiple pathways that ultimately converge upon the activation of these kinases (67). Recent discoveries indicate

considerable cross talk between cAMP, via PKA, and the MAP kinase signal transduction pathway (for review see Ref. 68). There are receptors that couple to pertussis toxin-sensitive Gi proteins and others that activate Gq, in addition to Gi proteins. Activation of the MAP kinase pathway can be mediated by Gi protein-linked receptors (69). G_i-coupled receptors stimulate MAP kinase via $G\beta\gamma$ subunits, and many $G\alpha q$-coupled receptors stimulate MAP kinase activity by increasing PPI turnover leading to the activation of PKC, and resultant activation of Raf. Raf phosphorylates and activates MAP kinase, which phosphorylates and activates the MAP kinase (the ERK MAP kinase). PKC and cAMP, via PKA, have been reported to activate and inhibit Raf, respectively (70). PKA can phosphorylate Raf kinase, resulting in reduction in its catalytic activity. cAMP-elevating agents have been reported to inhibit activation of ERK and Raf-1 in fibroblasts and vascular smooth muscle cells (68, 71). However, phosphorylation of Raf-1 by PKA catalytic subunits *in vitro* does not inhibit the ability of Raf to activate MAP kinase kinase (MAPKK) (72). One likely mechanism of PKA inhibition of the MAP kinase cascade seems to be a reduced ability of GTP-loaded Ras to interact with Raf (68).

In contrast to the inhibitory effects of cAMP-elevating agents on the MAP kinase cascade, cGMP-elevating agents have been reported to stimulate the MAP kinase pathway in vascular smooth muscle cells (73), isolated cardiomyocytes (74), and rat pinealocytes (75). In contrast, Pandy *et al.* (76) examined the effect of atrial natriuretic peptide (ANP) and its guanylyl cyclase/natriuretic peptide receptor-A (NPRA) on MAPK/ERK2 activity in mesangial cells. They found that the ANP/NPRA system negatively regulates MAPK/ERK2 activity and proliferation in a PKG-dependent manner.

Another potential target for cyclic nucleotide inhibition of agonist-induced smooth muscle contraction is the Rho/Rho-kinase pathway. There is evidence that, like MLCK, Rho-kinase can regulate smooth muscle contraction by directly phosphorylating MLC (51, 55). Since Rho-kinase is regulated by Rho A, a small GTPase, but not by $[Ca^{2+}]_i$, MLC should be phosphorylated without changing $[Ca^{2+}]_i$ when Rho A is activated by receptor stimulation, this may also be a mechanism for Ca^{2+} sensitization (77). Muscarinic agonists act both to increase $[Ca^{2+}]_i$ and to enhance the effectiveness of Ca^{2+} for inducing contraction, and this phenomenon has been referred to as Ca^{2+} sensitization (78). Y-27632, a selective inhibitor of Rho-kinase, selectively inhibits smooth muscle contraction by inhibiting Ca^{2+} sensitization (54). Although the mechanism underlying Ca^{2+} sensitization has not yet been fully elucidated, it appears that inhibition of MLC phosphatase due to a small GTPase Rho-associated coiled coil-forming protein kinase (ROCK I) and its isoform, ROCK II, is partly involved (54, 79, 80). Koyama *et al.* (81) investigated the phosphorylation of CPI-17, an inhibitory phosphoprotein of smooth muscle myosin phosphatase, by Rho-kinase. They found that phosphorylation by Rho-kinase dramatically increased the inhibitory effect of CPI-17 on myosin phosphatase activity. Inhibition of MLC phosphatase increases the level of MLC phosphorylation and helps to develop and/or maintain tension. Therefore, increases in the activity of MLC phosphatase by blockade of Rho/ROCK-mediated signaling pathway would attenuate the contractile responses via inhibition of the Ca^{2+} sensitizing mechanism. Ca^{2+} sensitization by the Rho/Rho-kinase pathway contributes to the tonic phase of agonist-induced contraction in smooth muscle (82). Recently, Nakahara *et al.* (83) examined how Y-27632 affects the relaxant responses to β_2-adrenoceptor agonists in bovine tracheal smooth muscle preparations precontracted with methacholine. Y-27632 (0.3–30 μM) caused a concentration-dependent attenuation of precontraction with methacholine (0.3–3 μM). Pretreatment with Y-27632 (1 μM) significantly ($P < 0.05$) augmented salbutamol (0.3–100 nM) and terbutaline (0.3 nM—1 μM)-induced relaxations. These results suggest that the ROCK inhibitor could become a new type of bronchodilator and its combination with β_2-adrenoceptor agonists may become a novel strategy for the long-term treatment of asthma. These results demonstrate cross talk between the cAMP- and Rho/Rho-kinase pathways. In rabbit ileum smooth muscle, cyclic nucleotide (cAMP or cGMP)-activated kinases, possibly through the activity of phosphorylated telokin, accelerate dephosphorylation of MLC_{20} leading to muscle relaxation (84). There also is accumulating evidence for the regulation of Rho-dependent pathways through cAMP, and, thus, conceivably through $G\alpha s$ (58). The mechanism(s) underlying the inhibitory effect of cAMP on Rho is not fully understood, but cAMP or PKA may act at several sites. Sauzeau *et al.* (85), working with cultured vascular myocytes, demonstrated cGMP effects mediated by cGMP-dependent protein kinase that inhibit Rho A-dependent Ca^{2+} sensitization of contraction of blood vessels and actin cytoskeleton organization. Sanders *et al.* (86) investigated the possibility that p21-activated kinases (PAKs) phosphorylate and activate MLCK activity. They found that PAK1, which is activated by either Rac or Cdc42, decreases MLCK activity and consequently blocks the phosphorylation of MLC. This results in decreased myosin activity, a reduction in contractility, and the disassembly of stress fibers. It remains to be determined whether these effects of Rac and Cdc42 are due to activation of PAK1. Recently Goeckeler *et al.* (87), working with saponin-permeabilized endothelial monolayers, reported that phosphorylation of MLCK by p21-activated kinase PAK_2, a member of the Rho family of GTPase-dependent kinases, can inhibit its activity and limit the development of isometric tension.

The above findings demonstrate cross talk between cyclic nucleotides and the protein kinases. However, the amount that each of these interactions may contribute to the relaxing effects of cAMP- and cGMP-elevating agents remains to be determined.

Summary and Conclusions

It is clear from the above discussion that cross talk between the second messengers plays an important role in the contraction and relaxation of smooth muscle. A scheme summarizing the possible cross talk points of interaction between cyclic nucleotides (cNTs) and the IP_3-Ca^{2+}-CaM-MLCK-, PKC-, MAP kinase-, Rho-kinase-, and myosin phosphatase pathways is given in Fig. 3. Two separate signal transduction pathways exist in smooth muscle. One leads to a contractile response and another to a proliferative response. For example, prostaglandin $F_{2\alpha}$ and endothelin-1 stimulate both responses, whereas carbachol, an M3 muscarinic agonist, stimulates mainly the contractile response. This could explain the differential effects of protein kinase inhibitors on carbachol- and prostaglandin $F_{2\alpha}$-induced contraction reported in iris sphincter smooth muscle (Table II) (50, 61). The question arises as to what extent these proposed pathways for contraction may be operating in smooth muscle *in vivo*. The major signaling pathway for smooth muscle contraction is the IP_3-Ca^{2+}-CaM-MLCK pathway

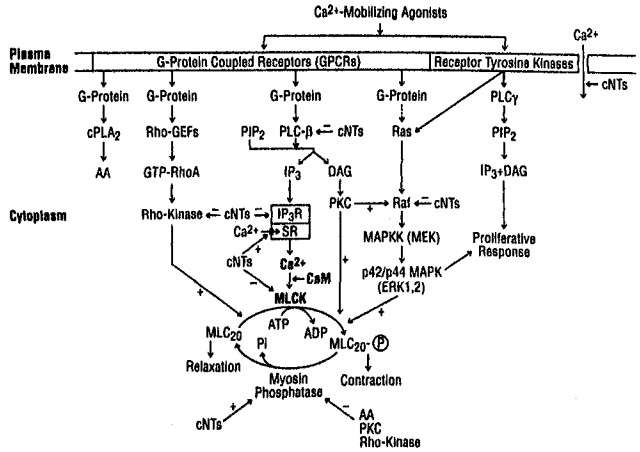

Figure 3. Scheme showing possible cross talk points of interaction between cyclic nucleotides (cNTs (cAMP and cGMP)) and the IP_3-Ca^{2+}-, Ca^{2+}-CaM-MLCK-, MAP kinase-, Rho-kinase-, PKC-, and myosin phosphatase pathways in smooth muscle. Upon activation of smooth muscle by activation of the GPCRs through Ca^{2+}-mobilizing agonists the $[Ca^{2+}]_i$ increases due to an influx of Ca^{2+} through plasmalemmal Ca^{2+} channels or IP_3-mediated release from the SR. Ca^{2+} binds to CaM and activates MLCK to phosphorylate MLC_{20} resulting in contraction. Myosin is dephosphorylated by myosin phosphatase. Activation of $G\alpha_i$-coupled receptors inhibits adenylate cyclases, decreasing cAMP and inactivating PKA (not shown in the scheme). Activation of GPCRs also leads to stimulation of $cPLA_2$ and the release of AA for prostaglandin (PG) biosynthesis. Activation of receptor tyrosine kinases leads to the stimulation of PLCγ and Ras and subsequently to proliferative response. In addition to the MLCK pathway, which comprises the major pathway in muscle contraction, MLC_{20} can also be directly phosphorylated by Rho-kinase, PKC, and p42/p44 MAPK. Inhibition of myosin phosphatase by AA, PKC, or Rho-kinase increases MLC_{20} phosphorylation and contraction. Several targets for cNTs regulation have been reported, including the receptor, G proteins, PLCβ, IP_3 receptor, the Ca^{2+}-pump in the SR, MLCK, Rho-kinase, MAP kinase, myosin phosphatase, and the plasmalemmal Ca^{2+} channel. Cross talk between the cNTs and these targets could constitute the biochemical mechanisms underlying relaxation in smooth muscle. Abbreviations are the same as given in the text; (+) and (−), stimulation and inhibition, respectively.

(Fig. 3). There are interactions between the signal transduction pathways that subserve the G-protein coupled receptors and the receptor tyrosine kinases. Jin *et al.* (88), working with vascular smooth muscle, demonstrated interrelationship between the tyrosine kinase pathway and the MLCK pathway. There is also cross talk among the signal transduction pathways. Thus, in addition to the direct phosphorylation of MLC_{20}, MAP kinase may phosphorylate and activate MLCK (89). PKC can directly phosphorylate MLC_{20} and also phosphorylate and activate the MAP kinase pathway. In skinned preparations of arterial smooth muscle, PKC enhanced the contractile response at a given submaximal concentration of free Ca^{2+} by increasing the level of MLC phosphorylation via inhibition of myosin phosphatase (55). Similarly, Rho-kinase can directly phosphorylate MLC_{20} and also phosphorylate, and inactivate, myosin phosphatase. Activation of G-protein coupled receptors also results in the stimulation of cytosolic phospholipase A_2 ($cPLA_2$) and the release of arachidonic acid (AA) for prostaglandin (PG) synthesis (90, 91). In iris sphincter smooth muscle, endothelin-1-induced activation of $cPLA_2$ and AA release is mediated through PKC-α and/or p38 MAP kinase, but not through p42/p44 MAP kinase (90, 91). AA, a Ca^{2+}-sensitizing agent, can activate Rho-kinase (80).

An important pathway for Ca^{2+} desensitization in smooth muscle is mediated *via* cyclic nucleotide-dependent protein kinases (PKA and PKG). It is clear from the findings outlined in this review that there are several cross talk points of interaction between cyclic nucleotides and the IP_3-Ca^{2+}-CaM-MLCK-, MAP kinase-, Rho-kinase-, PKC-, and myosin phosphatase pathways. These interactions could underlie the mechanism of cyclic nucleotide inhibition, i.e., relaxation, of agonist-induced contraction in smooth muscle. Phosphorylation of these protein kinases by the cyclic nucleotide-dependent protein kinases provides additional mechanisms by which GPCRs can regulate the various signaling pathways (Fig. 3). There is general agreement that cAMP- and cGMP-elevating agents reduce agonist-induced $[Ca^{2+}]_i$ mobilization and relax smooth muscle (Figs. 1 and 2, Table I). However, the net consequence of cAMP-dependent phosphorylation may quite be complex, because this process may either increase the Ca^{2+} concentration of the SR by simulating the Ca^{2+}-pump or inhibits the agonist-stimulated hydrolysis of polyphosphoinositides (PPI) (Fig. 3) (92). Thus, cAMP-dependent phosphorylation could produce both enhancement of Ca^{2+} uptake and inhibition of Ca^{2+} release from the SR (Fig. 3). In spite of the intensive effort made by many investigators in the past few years, the precise mechanisms underlying cyclic nucleotide inhibition of stimulated PPI hydrolysis and Ca^{2+} mobilization remain to be clarified. The finding by Liu and Simon (30) that in COS cells, transfected with cDNAs encoding PLC-β2, G protein subunits, and PKA, the catalytic subunit of PKA specifically inhibited Gβγ stimulation of PLC-β2 activity, without affecting Gαq-induced activation, is interesting, however, the nature of the effects of cyclic nucleotides on

the activities of phospholipase C-β isoforms (PLC-β1–4) remains to be demonstrated in smooth muscle and other tissues. In addition to studies on PLC phosphorylation there is a need to investigate cyclic nucleotide-dependent phosphorylation of the Gq-G protein as possible cause of cyclic nucleotides inhibition of PLC and IP_3 production in stimulated smooth muscle. In the last four years more information was reported on the effects of cyclic nucleotides on the IP_3 receptor, and on the activities of MAP kinase and Rho-kinase. A potential mechanism for smooth muscle relaxation could be the stimulation of myosin phosphatase by cyclic nucleotide-elevating agents (Fig. 3). Thus, 8-Br-cGMP, at constant Ca^{2+}, accelerated the dephosphorylation of MLC_{20} and relaxation of permeabilized rabbit ileum smooth muscle (93). These authors concluded that cGMP-dependent protein kinase, activated by 8-Br-cGMP, increases myosin phosphatase activity. There is a need to establish whether myosin phosphatase is regulated by the second messenger pathways.

The complexity of the multiple pathways involved in agonist-induced contraction (Fig. 3), as evidenced by the effects of the protein kinase inhibitors on muscle contraction (Table II), could explain the slow progress that has been made in the past four years on the cross talk between cyclic nucleotides and the PPI signaling cascade and protein kinases and contraction in smooth muscle. It is evident that smooth muscle contraction is not regulated only by the Ca^{2+}-CaM-MLCK pathway but also by modulation of Ca^{2+} sensitivity. Thus, future work will have to (i) establish the role of PKC, MAP kinase, Rho-kinase, and myosin phosphatase in smooth muscle contraction and (ii) determine the biochemical mechanisms underlying cyclic nucleotide (cAMP and cGMP) inhibition of agonist-induced $[Ca^{2+}]_i$ mobilization and contraction. There is a need to develop better methodology and employ more novel interdisciplinary approaches in investigating these interactions in smooth muscle. The combination of novel physiological, biochemical, and pharmacological techniques and molecular biology including transgenic animals will increase our understanding of the cross talk between cyclic nucleotides and the signal transduction pathways involved in smooth muscle contraction. This will help in designing more effective drugs for the treatment of high blood pressure, cardiovascular diseases, renal inefficiency, glaucoma, and asthma.

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