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Mechanism of action by which prostaglandin E_2 mediates rabbit uterine smooth muscle contraction

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MECHANISM OF ACTION BY WHICH PROSTAGLANDIN E_2 MEDIATES RABBIT UTERINE SMOOTH MUSCLE CONTRACTION

by

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DISSERTATION

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I dedicate this dissertation to my wife, parents and family.

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Abstract

Prostaglandin E2 (PGE_2), thought to be an important effector of uterine smooth muscle contraction presents an interesting and confusing paradox. Despite being a potent stimulator of uterine contraction, PGE_2 also strikingly increases cAMP, the second messenger implicated in uterine relaxation. To study the mechanism of action for these responses, I examined the mechanism by which PGE_2 stimulated myosin light chain phosphorylation in rabbit uterine smooth muscle cells. PGE_2 at a concentration of 1 nM stimulated myosin light chain phosphorylation, a key regulatory step for uterine smooth muscle contraction, while at 100 μM myosin light chain phosphorylation was reduced. Intracellular free calcium increased at all PGE_2 concentrations without significant change in phosphoinositide hydrolysis. PGE_2-stimulated intracellular calcium increase was blocked by pretreatment with phospholipase C (PLC) inhibitor (U73122). The ability of PGE_2 to accelerate myosin light chain phosphorylation was altered in calcium free medium. At 100 μM, PGE_2 greatly stimulated adenylyl cyclase activity. Pretreatment of myocytes with a cAMP dependent protein kinase inhibitor (H8) potentiated PGE_2-stimulated myosin light chain phosphorylation, but had no significant effect on phosphoinositide hydrolysis. In rabbit uterine smooth muscle strip PGE_2 (100 μM) initially elicited contraction followed by a complete relaxation. With H8 pretreatment, PGE_2 induced a consistent contractile response. In preliminary studies, myocytes prepared from uterus of pregnant rabbits (27th day) had a much greater reduction of myosin light chain phosphorylation at high concentrations of PGE_2 than was present in myocytes from estrogen treated rabbits. In summary, PGE_2, at low concentration (1 nM), stimulates myosin light chain phosphorylation and uterine smooth muscle contraction while at high concentration (100 μM), it induces muscle relaxation. Our data support the concept that this is due to PLC-stimulated increases of intracellular calcium predominating at low PGE_2 concentration but adenylyl cyclase activation and cyclic AMP accumulation with inhibition of myosin light chain phosphorylation predominating at high PGE_2 concentration. My preliminary data also supports hormonal modulation of the PGE_2 response.

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Chapter 1 Introduction

1.1. Overview

Prostaglandin E2 (PGE2) mediates numerous physiological responses and consequently has been used extensively in medicine. An interesting and confusing feature of this response is the paradoxical response in uterine tissues. Despite being a potent stimulator of uterine contraction, PGE2 also strikingly increases cAMP, the second messenger implicated in uterine relaxation. To investigate this response paradox, I measured second messenger generation and myosin light chain phosphorylation in isolated rabbit uterine smooth muscle cells in response to PGE2.

1.1.1. Physiology of smooth muscle contraction

The contraction of smooth muscle depends on the cyclic interaction of actin-based thin filaments with cross-bridges on myosin-containing thick filaments. The distinct contractile properties of smooth muscle (compared to skeletal or cardiac muscle) are that it shortens at a considerably slower velocity and to a greater extent, and it can generate far greater force per cross-sectional area [Bagby, 1983].

1.1.1.1. Components of contraction apparatus

1.1.1.1.1. Contractile molecules

1.1.1.1.1.1. Myosin

Myosin is the principal protein of muscle contraction. The myosin filaments are approximately 15 nm in diameter in uterine smooth muscle but are more heterogeneous in diameter and longer (2.2 μm) than those of striated muscle [Ashton, 1975]. Myosin is located in myofilaments which optimizes the interaction with the other major contractile protein, the thin filament, actin. Myosin is also an enzyme that facilitates conversion of the chemical energy of ATP into mechanical force during contraction. In uterine smooth muscle, the myosin molecule is composed of two heavy chains of 200 kDa and two pairs of light chains: a 20-kDa chain and a 17-kDa chain. These two heavy chains form two globular head groups joined to a 150-nm-long tail. There are three important sites on the globular head of myosin molecule. The first is the actin combining site, where myosin interacts with actin. The second is the ATPase site, where ATP hydrolysis occurs. The third site is the 20 kDa myosin light chain (MLC), the key element of contractile regulation through reversible phosphorylation [Sommerville, 1987; Adelstein, 1980; Hartshorne, 1987; Kamm, 1985].

1.1.1.1.1.2. Thin filaments

1.1.1.1.1.2.1. Actin

Smooth muscle thin filaments are about 7 nm in diameter and circular in transverse section. Actin is the major component of thin filaments in smooth and striated muscle. Actins in uterine smooth muscle are generally organized as orderly packed bundles with a lattice-like appearance. Actin has three important properties: (1) it polymerizes to form long filaments in a reversible manner; (2) it combines with

myosin and activates its Mg^{2+}-ATPase activity; and (3) it binds to tropomyosin and other regulatory proteins, e.g., troponin in striated muscles and MLCK in smooth muscle. The 42 kDa actin molecule is present in several isoforms. In smooth muscle, there are two actin isoforms: the α and γ isoforms, which differ by only three amino acids, all at the N terminus [Vandekerchhove, 1979]. The α variant is present predominantly in vascular smooth muscles, and the γ variant in the gastrointestinal system. In uterus, there are approximately equal amounts of α and γ actins [Fatigati, 1984; Vandekerchhove, 1979].

1.1.1.1.1.2.2. Tropomyosin

Tropomyosin is also present in the thin filaments. The ratio of tropomyosin to actin in uterine smooth muscle is 1 : 6 to 1 : 7, similar to that found in skeletal muscle [Cohen, 1979]. There are differences in the amino acid composition of the tropomyosin in these two types of muscle. Smooth muscle tropomyosin exists in α and β forms. Rabbit uterine smooth muscle contains only β tropomyosin. All forms of tropomyosin increase the affinity of actin for myosin-ADP-Pi [Sobieszek, 1982]. Under most conditions, smooth muscle tropomyosins also increase actin activation, a phenomenon known as "potentiation."

1.1.1.1.1.2.3. Caldesmon

Caldesmon is the third most abundant protein component of smooth muscle thin filaments. Its molecular weight is approximately 120 to 150 kDa. Caldesmon is involved in the thin filament regulatory system controlling the interaction of actin with phosphorylated myosin [Marston,

1982; Marston, 1980]. It is well established that physiological [Ca^{2+}] can activate both the myosin filaments through myosin light chain kinase and the actin filaments through caldesmon and a calcium-binding protein [Marston, 1985; Marston, 1982].

In certain smooth muscle types, particularly vascular smooth muscles, myosin light chain phosphorylation correlates with the rise of tension of contraction after stimulation, but subsequent sustained contractions are maintained while myosin light chain phosphorylation levels decrease toward unstimulated levels. The observation of this "latch" response suggests that in these smooth muscles there are two regulatory systems for muscle contraction [Murphy, 1990]. One suggestion for the second regulatory system is modification of the thin filament system.

However, measurements relating myosin light chain phosphorylation to contractility in uterine muscle consistently indicate that myosin light chain phosphorylation correlates with contractility [Haeberle, 1985; Haeberle, 1985; Janis, 1981]. Thus these findings indicate little role for thin filament regulation in uterine smooth muscle, except as modulator of smooth muscle response to myosin light chain phosphorylation.

1.1.1.2. Regulation of contraction -- myosin light chain phosphorylation

In skeletal muscles, the predominant regulation of muscle contraction is associated with the actin filament: calcium ion binds to the troponin C subunit of the troponin-tropomyosin complex, inducing conformational changes in the protein and allowing actin-myosin

interaction and muscle contraction. In smooth muscles, however, the actin-myosin interaction is primarily regulated by the phosphorylation of the 20-kDa myosin light chain, catalyzed by myosin light chain kinase (MLCK), which is activated by calcium-calmodulin. The actin-myosin interaction can occur only if myosin light chains have been phosphorylated. Activation of actomyosin ATPase activity and muscle contraction are both dependent on myosin light chain phosphorylation (MLC phosphorylation) *in vivo* and *in vitro*. The activation of smooth muscle contraction parallels the increase in levels of P_i incorporated into myosin molecule. In smooth muscle including uterus, actomyosin ATPase activity correlated well with the phosphorylation of myosin light chains [Janis, 1981; Chacko, 1977; Lebowitz, 1979; Kerrick, 1981; Sherry, 1978]. Studies of isolated individual smooth muscle cells, Itoh *et al.* reported, activation of the calmodulin-MLCK pathway is both necessary and sufficient to trigger contraction [Itoh, 1989].

1.1.1.2.1. Calmodulin

Contractile stimuli cause a transient increase of cytoplasmic free Ca^{2+} concentration to 500 to 700 nM. This is detected by the "Ca^{2+} sensor protein" calmodulin. Calmodulin can bind 4 moles Ca^{2+}/mole with high affinity (Kd in the μM range). The calmodulin molecule, after binding with Ca^{2+}, undergoes a conformational change, which exposes a hydrophobic domain for interaction with target enzymes [Klee, 1980]. These enzymes are converted from the inactive apoenzyme to form the active holoenzyme of Ca^{2+}-calmodulin-enzyme.

1.1.1.2.2. Myosin light chain kinase (MLCK)

Myosin light chain kinase is a single polypeptide, which binds with a very high affinity to the Ca^{2+}-calmodulin complex. It is the most important calmodulin-dependent enzyme for the regulation of smooth muscle contraction. The molecular weight of this molecule is about 130 to 160 kDa. Structure-function relationships have been probed by limited proteolysis [Foyt, 1985; Walsh, 1985]. MLCK has several distinct functional domains: a catalytic site, a calmodulin-binding site, and two sites which can be phosphorylated by cyclic AMP-dependent protein kinase. The remainder of the molecule may serve to bind the kinase to the actin filaments [Walsh, 1985; Foyt, 1985]. In structure-function studies, the apoenzyme of MLCK is inactive because an inhibitory portion of the molecule is folded such that the active site is masked. After binding to Ca^{2+}-calmodulin, the molecule undergoes a conformational change, leading to the exposure of the active site. Alternatively removal of the inhibitory region of MLCK by tryptic digestion will expose the active site and activate the enzyme. The catalytic domain of MLCK is located within residues 200 to 450 and is homologous with cAMP dependent protein kinase [Guerriero, 1986].

Myosin light chain kinase contains sites for phosphorylation by protein kinase A (PKA). The 2 sites phosphorylated by cAMP dependent protein kinase are serines 491 (or 492) and 505 [Lukas, 1986; Payne, 1986]. The former is phosphorylated only in the absence of bound calmodulin, while the latter is phosphorylated in either the presence or absence of bound calmodulin. More calcium-calmodulin complexes are needed to activate MLCK after it is phosphorylated by PKA. Hence, functionally, protein kinase A inhibits MLCK in this *in vitro* setting.

The substrate for MLCK, myosin light chain, is phosphorylated at a serine residue (Ser 19) near the N terminus of this molecule [Pearson, 1984]. From *in vitro* studies of the kinetics of the myosin light chain phosphorylation, some investigators believe that the two myosin light chains on the two heads of a myosin molecule are randomly phosphorylated [Chacko, 1981; Trybus, 1985], while others feel that phosphorylation is negatively cooperative and therefore occurs by an ordered mechanism [Walsh, 1985; Persechini, 1981; Ikebe, 1982; Sellers, 1983]. Similarly, there is disagreement whether both heads of myosin need to be phosphorylated before actin activation of the myosin Mg^{2+}-ATPase can occur.

Ikebe and Hartshorne demonstrated that high concentrations of MLCK can phosphorylate myosin light chain at a second site, THR-18 [Ikebe, 1985; Ikebe, 1986]. Second site phosphorylation markedly enhanced the actin-activated Mg^{2+}-ATPase activity of myosin molecule. Haeberle and Trockman observed the formation of both the monophosphorylated and diphosphorylated forms of myosin light chain in glycerinated porcine carotid artery [Haeberle, 1986]. They suggested that the two-site phosphorylation of smooth muscle myosin might have physiological significance.

1.1.1.2.3. Myosin light chain phosphatase

Myosin light chain phosphatases have been isolated and characterized from several smooth muscles. Some of these phosphatases are ineffective to dephosphorylate intact myosin molecule *in vitro*. However, two of these enzymes, isolated from turkey gizzard can

dephosphorylate intact myosin molecules [Pato, 1985]. At present, it is unclear how the phosphatase activity is regulated.

1.1.1.2.4. Other kinases that phosphorylate myosin

Protein kinase C has been reported to phosphorylate heavy meromyosin. However, to date, it is unclear whether the protein kinase C-catalyzed phosphorylation is of importance in the contractile regulation of smooth muscle.

1.1.1.3. Contraction signal pathway

Uterine smooth muscle contractions are triggered by activation of myosin light chain kinase which phosphorylates myosin light chain. The activation of myosin light chain kinase depends on increased intracellular calcium ion. The increase of intracellular [Ca^{2+}] may come from 1,4,5-IP_3 stimulated release of intracellular calcium stores or from calcium ion entry through membrane calcium channels.

1.1.1.3.1. Inositol phosphate generation

The receptors for many agonists on uterine smooth muscle cell membrane are coupled to a guanine-nucleotide binding protein (G protein). Receptor- G protein interactions activate phospholipase C (PLC, now called by some investigators phosphoinositidase C). This PLC specifically hydrolyzes phosphatidylinositol 4,5-biphosphate.

The phosphoinositides are minor components of plasma membranes and make up approximately 3 to 5% of the total

phospholipids [Abdel-Latif, 1986]. About 10 to 20% of the phosphatidylinositol is further phosphorylated at positions 4 and 5 of inositol to form phosphatidylinositol 4,5-biphosphate (PIP_2). The products formed by PIP_2 after phospholipase C is activated by agonist receptor G protein interaction are inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG). IP_3 binds to specific receptor sites causing a transient release of calcium from Ca^{2+} storage sites on sarcoplasmic reticulum. Several studies of smooth muscle demonstrate that the increase of IP_3 parallels muscle contraction and increased in myosin light chain phosphorylation [Howe, 1986]. The IP_3 is broken down sequentially to inositol biphosphate (IP_2), inositol monophosphate (IP) and free myo-inositol by specific phosphomonoesterases [Storey, 1984]. The IP phosphomonoesterases are inhibited by lithium [Hallcher, 1980] whereas the IP 5'-phosphomonoesterase is not inhibited. Thus, lithium prevents further synthesis of phosphatidylinositide and allows the accumulation of IP, IP_2 and IP_3. Phosphatidylinositol is resynthesized from myo-inositol and DAG in the endoplasmic reticulum and in the plasma membrane [Imai, 1987]. One IP_3 metabolite, 1,3,4,5-IP_4, generated by phosphorylation of 1,4,5-IP_3, seems to control Ca^{2+} flux from IP_3-insensitive calcium pool to IP_3 sensitive calcium pool [Berridge, 1989].

1.1.1.3.2. Calcium

Calcium is required for smooth muscle contraction whether the stimulus is an agonist or voltage change, and irrespective of whether the calcium originates from extracellular sources or intracellular storage. In the skeletal muscle cell, intracellular calcium stores are the primary

source responsible for contraction. However, in smooth muscle cell, calcium ion entry through plasma membrane is the major source. This is related to the high surface to volume ratio (1.1 compared to 0.1 in skeletal muscle cell) [Garfield, 1985]. The intracellular Ca^{2+} concentration in smooth muscle cell is 10^{-7} M during relaxation and about 10^{-6} M during contraction. The extracellular Ca^{2+} concentration is 10^{-3} M. Bond *et al.* reported that the intracellular calcium ion recycles and is adequate to effect a contraction in the absence of extracellular calcium [Bond, 1984]. Smooth muscle cell contraction is terminated by a reduction of intracellular Ca^{2+} concentration by active transport by a calcium pump (also called Ca, Mg-ATPase). There are two different calcium pumps, one located in the plasma membrane delivering intracellular calcium to the extracellular media and the other in the membranes of the sarcoplasmic reticulum, which sequesters calcium into this structure [Wuytack, 1984; Wibo, 1981].

1.1.1.3.3. Calcium channel

Calcium channels are on the plasma membranes of all known excitable cells [Tsien, 1987]. Despite the fact the extracellular calcium concentration is 10,000 fold higher than the intracellular concentration, calcium channels are extremely selective. There are two types of calcium channels, voltage dependent channels activated by a change in membrane potential, and ligand-gated or receptor operated channel activated by the binding of a ligand to a specific channel associated receptors [Rimele, 1984; Van Breemen, 1982]. In rat myometrium, there are four types of voltage-dependent calcium channels based on the

factors that induce their inactivated state. They are (1) voltage-dependent inactivation, (2) calcium-dependent inactivation, (3) both voltage- and calcium-dependent inactivation, or (4) neither [Hurwitz, 1986]. Any single smooth muscle may have several different types of voltage-dependent calcium channels. These include the most common, long-lasting (L)-type channels that are activated by large changes in membrane potential and are sensitive to dihydropyridine calcium antagonist [Greenburg, 1987; Hess, 1986]. In contrast, transient (T)-type calcium channels are activated by weak depolarizations and are relatively insensitive to dihydropyridine calcium antagonists [Lansman, 1986; Godfraind, 1986]. The third type of voltage dependent channel is the N type, which is activated by strong depolarization and relatively unaffected by dihydropyridines. In isolated rabbit uteri, calcium channel blocker nicardipine inhibited spontaneous contractions, as well as oxytocin activated and electrical stimulated contraction [Csapo, 1982]. Sakai *et al.* reported the presence of receptor-operated calcium channels [Sakai, 1983].

1.1.1.3.4. PKC

There are at least 20 endogenous substrates for PKC in smooth muscle [Park, 1986]. PKCs are a heterogeneous group of enzymes, frequently with more than one type in a given cell [Nishizuka, 1988]. Protein kinase C is activated by diacylglycerol (which must contain acid) with phosphatidylserine and calcium [Takai, 1979]. DAG activates PKC by increasing the affinity of this enzyme for calcium to allow full enzyme activity at normal concentration of intracellular calcium [Kikkawa, 1986].

PKC is translocated to membranes after activation in stimulated cells [May, 1985; Wolf, 1985]. Phorbol esters can activate PKC due to structure similarity to DAG [Castagna, 1982].

PKC effects smooth muscle contraction in two ways. Activation of PKC antagonizes many IP3-mediated events through phosphorylation of the receptor or phospholipase C. PKC also phosphorylates thin filaments in smooth muscle which is proposed to result in sustained contraction (latch contraction). Thus the acute contractions stimulated by agonists are antagonized by PKC. However, after prolonged exposure (20 - 60 minutes) to activated PKC, smooth muscle will contract without other stimulus. The diverse effects of PKC are due to the multitude of sites and proteins which are potential substrate for PKC phosphorylation.

1.1.1.4. Contraction pattern of smooth muscle: phasic and tonic

The contractile patterns of smooth muscle can be grouped into two categories -- phasic and tonic contractions. With phasic contraction there is a rapid rise in tension followed by relaxation. There is no "plateau phase" of steady isometric force. Phasic activity is usually associated with single action potential (e.g., cat intestine) or bursts of multiple spike (e.g., rabbit intestine). By contrast, tonic contractions reach a steady-state level of force that may be maintained for long periods of time. Sustained tonic activity is usually associated with a continuous train of action potentials [Murphy, 1980]. Tension developed in phasic smooth muscle correlates with the intracellular $[Ca^{2+}]$. In tonic smooth muscle, however, there is a continuous increase in tonic tension during prolonged depolarization, even as $[Ca^{2+}]_i$ declined [Himpens, 1988]. In tonic

contraction in tonic smooth muscle, myosin light chain phosphorylation decreases while tension is still maintained. Conversely, in phasic contraction of phasic smooth muscle, myosin light chain phosphorylation correlates well with tension and intracellular [Ca^{2+}] [Weisbrodt, 1985].

1.1.1.5. Relaxation signal pathway

Cyclic AMP and cyclic GMP are important regulators of cellular function. Most mammalian tissues including uterine smooth muscle contain both cyclic AMP and cyclic GMP and the enzymes responsible for synthesis and degradation of these two cyclic nucleotides.

1.1.1.5.1. cAMP

Cyclic AMP has been regarded as the primary tocolytic pathway in the myometrium. It's role in relaxing myometrium has been suggested by the finding that β-adrenergic receptor agonists relax uterus. Almost all known actions of cAMP in mammalian cells are mediated by protein kinase A [Krebs, 1979]. In the absence of cAMP, this enzyme exists as a tetramer of two regulatory and two catalytic subunits, R_2C_2, which is inactive. Two molecules of cAMP bind to each regulatory subunit and cause dissociation of free active catalytic subunits. The catalytic subunit of PKA phosphorylates target proteins and ultimately effects a biological response (uterine smooth muscle relaxation). Protein kinase A inhibitors such as N-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H8) or PKI inhibit PKA activity [Hidaka, 1984].

One possible mechanism by which cAMP relaxes smooth muscle is secondary to the cAMP-dependent phosphorylation of smooth muscle

myosin light chain kinase [Conti, 1981; Adelstein, 1978]. Phosphorylation of MLCK decreases the affinity of this kinase for calmodulin up to 20 fold. However, in tracheal smooth muscle, Miller *et al.* [Miller, 1983] reported that isoproterenol produced marked relaxation and reduced myosin light chain phosphorylation but did not alter the myosin light chain kinase activity ratio. Other investigators feel that the primary action of cAMP is a kinase-A mediated reduction of cytosolic free Ca^{2+} by either increasing Ca^{2+} uptake by the sarcoplasmic reticulum or by increasing Ca^{2+} efflux from the cell [Krall, 1976]. In uterus, predominance of one or the other mechanism may depend on the physiological or endocrine condition of the myometrium.

Although a good deal of evidence favors a role for cyclic AMP in uterine relaxation, there are reports that dissociate cyclic AMP elevation with relaxation of uterine smooth muscle [Polacek, 1971; Diamond, 1975; Verma, 1976]. One of the arguments against the role of cAMP as a regulator of uterine relaxation is the increase in cAMP which occurs with the potent activator of contraction, PGE_2. There are interpretations for these inconsistencies which include different compartmentalization of cAMP [Vesin, 1974], relaxation due to cAMP dependent and cAMP independent process [DoKhac, 1986] and different hormonal regulation of cAMP response [Tanfin, 1987].

1.1.1.5.2. cGMP

The suggestion for a general role for cyclic GMP as a mediator of smooth muscle relaxation comes from the finding that exogenous nitrovasodilators (nitroglycerin, sodium nitroprusside, sodium nitrite and

endogenous NO) which generate cyclic GMP relax vascular smooth muscle [Waldman, 1987; Ignarro, 1985]. A possible role for cGMP in uterine relaxation is suggested by the following observations. First, nitrovasodilators such as nitroglycerin, nitroprusside and hydroxylamine have been shown to increase cGMP levels in rat uterine preparations [Leiber, 1978; Diamond, 1975]. Phosphodiesterase inhibitors such theophylline and 1-methyl-3-isobutylxanthine, which relax many smooth muscle preparations, can increase cGMP (and cAMP) levels in rat uterus [Diamond, 1974; Leiber, 1978]. Exogenous administration of 8-bromo cGMP can relax a variety of smooth muscle preparations including rat myometrial strips [Schultz, 1979]. These observations suggest that cGMP may be involved in uterine relaxation. However, there is no direct time course or dose response relationships between the generation of cGMP and the ability to relax the uterus [Diamond, 1983]. Thus far, there is no compelling evidence that cGMP play a major role in regulation of uterine relaxation.

1.1.1.6. Integration of signal message

The signals leading to contraction or relaxation may be integrated at several levels. Messages can effect the concentrations of other second messages. An example of this is seen in rat uterine smooth muscle, where inositol phosphate generation in response to agonist stimulation can be attenuated by simultaneous cyclic AMP generation [Anwer, 1989]. Conversely, increased intracellular Ca^{2+} concentration can decrease cellular cAMP levels, through activation of phosphodiesterase [Berg, 1986]. In addition, as discussed above, intracellular $[Ca^{2+}]$ can be

decreased in uterine smooth muscle cells by cAMP. Another site of signal integration would be "downstream" where the key determinants of muscle contraction or relaxation such as myosin light chain kinase or possibly myosin light chain phosphatase are regulatory targets. Myosin light chain kinase activity can be stimulated by Ca-calmodulin complex and inhibited by cAMP-dependent protein kinase mediated phosphorylation, integrating the contractile and relaxation messages. Although the regulation of myosin light chain phosphatase activity is not yet established, it is also a likely site for message integration.

1.1.1.7. Hormonal effects upon uterine smooth muscle contraction

Hormones influence uterine contraction by regulating density of receptor concentration and gap junction protein density [Jeremy, 1986] on membranes, altering membrane permeability to different ions, and modifying synthesis of phospholipids and other components of signal transduction pathways.

I propose that the "paradoxical" effects of PGE_2 may indicate potential sites for hormonal regulation. Work in Dr. Roberts' lab in the past several years provides a model for this type of regulation. Myosin light chain kinase, the key regulatory enzyme for uterine contraction, is influenced by two regulators: calcium and cyclic AMP, which serve as messengers coupling to α- and β-adrenergic receptors. The relative dominance of α- and β-adrenergic receptors in myometrial tissues is regulated by the sex hormonal status (estrogen and progesterone concentration), at different stages of estrous cycle or during pregnancy [Marshall, 1970]. In the rabbit, α-adrenergic receptors and contractile

response predominate in the uterus from nonpregnant or estrogen-treated animals. Beta-adrenergic receptor directed responses are predominant from pregnant or progesterone treated uteri [Roberts, 1977; Roberts, 1981; Riemer, 1986; Riemer, 1987]. The same phenomena were observed in human and rat. In human, the contractile effects of catecholamine predominated in estrogen-dominated uterus, whereas inhibitory effects predominated in conditions with high progesterone concentration such as pregnancy or luteal phase of menstrual cycle [Bottari, 1985]. In addition to the receptors density, estrogen and progesterone also affect some components regulating the two pathways which couple to the α- and β-adrenergic receptors. For example, Gs protein, which couples to β-adrenergic receptor, was increased in progesterone treated animal [Riemer, 1986]. All of these hormonal effects will be integrated and present the overall influence of hormone on uterine contraction.

However, in addition to marked species differences with regard to the effect of sex hormones on adrenergic receptors, e.g., cat and rabbit [Graham, 1960; Tsai, 1964], the modification of this response by hormones may be quite different in the longitudinal and circular muscle layers of the myometrium [Kishikawa, 1981; Chow, 1981], which further complicates the picture.

1.2. Prostaglandin E_2 and uterine contraction

1.2.1. Clinical experience and its relevance to the study of signal transduction for uterine contraction

Prostaglandins (PG) are paracrine or autocrine agents, made by nearly every tissue in the body, which are intimately associated with many physiological processes. These effectors cause uterine smooth muscle contraction, especially during parturition. Prostaglandin E2 is the most potent prostaglandin in clinical use. In addition, some uterotonic agents such as oxytocin, which exerts its effect through the PIP2 hydrolysis pathway, indirectly stimulate PG production [Chan, 1977]. Diacylglycerol (DAG), one product of PIP2 hydrolysis, can be converted to arachidonic acid (AA) by the catalytic effect of DAG lipase and monoacylglycerol (MAG) lipase [Bokoch, 1984]. Phospholipase A2, which can be activated by calcium ion, releases AA from phospholipids [Okazaki, 1978]. The AA produced can be further converted into prostaglandins. Due to these potential autocrine or paracrine effects, PG plays an especially important role in smooth muscle contraction. The understanding of signal transduction of uterine contraction should enable us to better manipulate uterine contraction and should provide insights into the cause of preterm labor and possibly indicate an effective solution.

1.2.2. Biphasic effect on uterine contraction from animal study

In vitro, PGE2 contracts myometrium at low concentrations but relaxes myometrium at higher concentrations [Lundstrom, 1986]. Depending on the hormonal milieu PGE2 has different effects on uterine contractility. PGE2 stimulates uterine contraction at the time of ovulation, when the estrogen level is high, while it inhibits contraction during other phases of the menstrual cycle [Lundstrom, 1986].

1.2.3. Postulated signal transduction pathway of PGE_2 for uterine contraction

In pregnant human uterus, Carsten reported that prostaglandin E_2 inhibited ATP-dependent calcium binding, which should result in increased calcium availability for contraction [Carsten, 1973]. This finding implied that PGE_2 might mobilize calcium to effect uterine contraction. PGE_2 also increased cAMP level in cells through stimulation of adenylyl cyclase activity [Kather, 1981]. There is evidence for a biphasic PGE_2 effect on cAMP generation in human fat cells [Kather, 1979]. Studies in our lab have demonstrated that PGE_2 stimulates cAMP generation in uterine myocytes from estrogen-treated rabbits. Other investigators report that in bovine adrenal medulla, PGE_2 stimulates PLC generating DAG and IP_3, which increases $[Ca^{2+}]_i$ [Negishi, 1989]. Also, since receptor-gated ion channels play an important role in cell physiology, it is also possible that PGE_2 receptor-gated calcium channels are present in the rabbit myocytes. Currently little information is available about the exact mechanism by which PGE_2 activates this cascade in smooth muscle.

1.2.4. Significance of study of signal transduction of PGE_2 on uterine contraction

If the above mentioned pathways do mediate the PGE_2 effect on myometrial contraction, it is interesting and important to determine the interaction of these opposing pathways after activation by PGE_2 as they converge at a common effector (MLC). Do these pathways mediate signals

in parallel before they reach the common effector or are there more proximal interactions? In other systems, there is increasing evidence that the Ca^{2+} and cyclic AMP pathways interact. Although the effect of PGE_2 on myometrial contraction is well known, the underlying mechanism is not clear. Knowledge of this underlying mechanism will help to explain how myocytes integrate two pathways with opposing effects on a common response. This information should provide insight into the initiation and regulation of labor and how to better manipulate uterine contractility. It paves the way to study the hormonal modulation of the myometrial response to PGE_2 and other uterotonic agents. Also this rather unique system offers an excellent opportunity to study how transduction pathways with opposing effects convey their signals. Do they interact before they act on the common effector? The knowledge of interaction between these signal transduction pathways may help to elucidate some basic physiological and pharmacological phenomena in other systems.

1.3. The project

This project investigated the signal transduction pathways of PGE_2 in rabbit uterine smooth muscle contraction. The results of these studies resolve at least in part the apparent paradox of the coincident stimulation by PGE_2 of adenylyl cyclase and contraction.

1.3.1. Hypothesis

This study tested the hypothesis that prostaglandin E_2 effects an increase of intracellular $[Ca^{2+}]$, which leads to a uterine contraction. At high concentrations, PGE_2 also stimulates adenylyl cyclase which

generates cAMP and results in uterine relaxation. The prevalence of different pathways at different concentrations of PGE_2 determines the response to stimulation.

1.3.2. Strategy

I chose rabbits as my experimental animals. The contractile response of rabbit uteri to several agonists is well characterized. In Dr. Roberts' lab, rabbits have been used as experimental animals for several years and in addition to uterine response to agonist stimulation, the hormonal effect on these responses is well characterized.

The animals were treated with estrogen to standardize their hormonal status. I chose to study signal generation by PGE_2 stimulation in rabbit uterine myocytes grown in culture. The use of cells in culture enables us to work with a defined cell type and to be certain that second messages are all produced by this cell. In addition, cultured cells allow "dissection" of the signaling pathways with antagonists and agonists.

Myosin light chain phosphorylation is the key regulator of smooth muscle contraction. In uterine smooth muscle, the data presented above indicate that myosin light chain phosphorylation parallels the contraction force in uterine smooth muscle. It is more specific for muscle contraction than the other biochemical surrogates of contraction such as ATPase activity or actin polymerization. In addition, it is the final key step regulation of muscle contraction. Messages from different transduction pathways after stimulation will be integrated as myosin light chain phosphorylation.

I chose to study the phospholipase C - inositol phosphates generation pathway and adenylyl cyclase-cyclic AMP generation pathway involved in smooth muscle contraction. These pathways are chosen because they are well-characterized effectors of smooth muscle contraction. Also, it is known that PGE2 stimulates inositol phosphate generation and cyclic AMP generation in several systems [Negishi, 1989; Kather, 1981]. In addition, intracellular Ca^{2+}, a key effector for muscle contraction, was measured in response to PGE2 stimulation.

1.3.3. Results

Prostaglandin E2 stimulated intracellular calcium ion mobilization without detectable inositol phosphate generation, which led to uterine contraction; while at high concentration, it greatly stimulated adenylyl cyclase activity and this pathway prevailed and resulted in uterine relaxation.

Chapter 2 Materials and methods

2.1. Materials

2.1.1. Preparation and growth of rabbit uterine myocytes

2.1.1.1. Treatment of animals

Immature New Zealand rabbits (3 Kg) were used. Since uterine contractile responses are greatly affected by sex steroidal milieu, to achieve a comparably standardized hormonal status, animals received intramuscular injection of estradiol benzoate (in oil) 50 μg per kg of weight for 4 days.

2.1.1.2. Tissue preparation

On the 5th day, rabbits were sacrificed by intravenous injection of sodium pentobarbital. The uteri were removed, weighed and immersed in ice-cold Hank's balanced salt solution (Hank's BSS, University of California, San Francisco, Cell Culture Facility Center). For the preparation of isolated cells, the uterus was trimmed of fat and denuded of endometrium with a scalpel, in Hank's BSS. The resulting myometrium was minced to 3 x 3 x 1.5 mm. Each uterus weighed approximately 6 - 8 gm, and was 4 - 6 ml in volume after mince. The myometrial minces were rinsed extensively with Hank's BSS to remove blood and debris and placed in dispersion flask (Falcon) with dispersion medium.

2.1.1.3. Cell dispersal

Dispersion medium was Hank's balanced salt solution containing 0.2% collagenase (130 u/mg, CLS 2, Worthington, Biochemical Corporation) and 0.01% of deoxyribonuclease I (DNase I, from bovine pancreases, Sigma). Generally 20 ml of dispersion medium was used to disperse 5 gm of myometrial tissue. The dispersion was carried out at 37°C. Samples were aerated with 95% oxygen and 5% CO_2. After six hours the cell suspensions were spun in clinical centrifuge at 2000 rpm for 10 minutes and resuspended in 8 ml of Hank's BSS with 0.4 ml of 20% bovine serum albumin (BSA, Sigma), 2 ml of DNase I (5 mg/20 ml stock).

2.1.1.4. Cell separation

Percoll density gradients were used to separate the cells obtained from cell dispersion. The 35% Percoll solution contained 0.5% BSA solution and 0.002% DNase I. To constitute Percoll gradients, the gradient tubes were spun in Beckman centrifuge, JA-17 rotor, at 17,000 rpm for 15 minutes. Then 1 ml of the cell suspensions was added along the tube wall slowly to the top of each Percoll gradient solution. The gradient tubes were spun at 1000 g for 15 minutes (IEC centrifuge at 2000 rpm) with brake turned off. The dispersed cells were washed with Hank's BSS.

2.1.1.5. Cell culture

Cell pellets were resuspended with culture medium, Dulbecco's Minimal Essential H21, d-Valine medium (DME-H21, d-Valine medium, University of California, San Francisco, Cell Culture Facility Center), and contained 1% of penicillin-streptomycin, 100 ng/ml of insulin, 50 mg/ml of gentamycin, 500 ng/ml of fungizone and 10% of dialyzed fetal calf serum (University of California, San Francisco, Cell Culture Facility Center). The viability of the cells by trypan blue exclusion was 95%. Counts by hemocytometer indicated a recovery of 20 x 10^6 cells per gram tissue. For growth, I plated cells in either 35 mm tissue culture dishes (5 x 10^5 cells/dish) or 24 well tissue culture plates (10^5 cells/well) depending on the experiments planned. The use of l-Valine free medium (DME-H21 d-Valine medium) was to prevent the growth of fibroblasts which might still be present. Fibroblasts lack the enzyme to convert d-Valine to l-Valine, which is essential for cell growth.

To standardize the hormonal effect cultures were maintained for exactly 4 days after dispersion and then experiments were performed.

2.1.2. Myometrial contraction studies

Uterine strips 5 mm by 1 mm were prepared from uteri trimmed of fat and connective tissue. Strips were suspended in 15 ml tissue baths filled with Krebs solution (NaCl, 118.3 mM; KCl, 4.7 mM; $CaCl_2$, 2.5 mM; $MgSO_4$, 1.18 mM; KH_2PO_4, 1.17 mM; $NaHCO_3$, 25.0 mM; and glucose, 11.1 mM) aerated with 95% O_2 - 5% CO_2 (pH 7.4) at 37° C. The strips were allowed to equilibrate at 1 gm tension for 1 hour. During this time, the Kreb's solution was replaced every 30 minutes. Agonists were added

and contraction force was measured by isometric transducer (Radnoti, Monrovia, CA) and recorded by computer (Macintosh, MacLab program).

2.2. Methods

2.2.1. Myosin light chain phosphorylation

2.2.1.1. Protein preparation

Cells (1x 10^6) were incubated with and without agonist for 2 minutes and the reaction stopped by quick freezing with dry ice-cooled Freon followed by trichloroacetic acid (TCA, final concentration 10%). Cells were scraped from the dishes in TCA and proteins pelleted by centrifugation (8000 g for 5 min). Supernatant was discarded and residual TCA in the pellets extracted by 5 washes with diethyl ether containing 10 mM dithiothreitol (DTT). Pellets in Eppendorf tube were placed on the fume hood for 15 minutes to remove residual ether. The dry pellets were solubilized in 0.5 ml of solution that contained 8 M urea, 20 mM Tris base (pH 8.6), 23 mM glycine and 10 mM dithiothreitol with constant shaking for one hour. An aliquot of the solubilized protein was removed for protein quantification and the remainder stored frozen at -70°C. Before electrophoresis, the protein solutions were added to a final volume of 50 µl with an electrophoresis solution that contained 8M urea, 23 mM glycine, 20 mM Tris base, 10 mM DTT and 0.04% (W/V) bromophenol blue at pH 8.6.

2.2.1.2. Urea/glycerol polyacrylamide gel electrophoresis

The urea/glycerol gel consisted 40% of glycerol (Fisher, reagent grade), 10% acrylamide (vol/vol) (BioRad), 0.5% Bis-acrylamide (vol/vol) (BioRad), 20 mM tris(hydroxymethyl) aminomethane(Tris) base (Fisher) and 22 mM glycine (Fisher), pH 8.6 at 22°C. To polymerize, 10% ammonium persulfate and TEMED were added. After the gels were cast, the wells were cleaned with deionized and distilled H_2O (ddH_2O) and pre-run at 400 V, constant voltage, for one hour. The running buffer consisted of 20 mM Tris base, 22 mM glycine, pH 8.6 at 22°C and 1 mM thioglycolic acid (Sigma) plus 1 mM dithiothreitol. The gel running temperature was kept constant at 10°C with running cooled water by cooling pump (Hoefer) through cooling coil in the electrophoresis apparatus (BioRad, PROTEAN II). After one hour, the wells were carefully cleaned. Attention was paid to remove all the glycerol released from the gel. Immediately before loading the proteins (15 μg protein) to each well, the bottom of each well was cleaned again. After loading, the protein samples were subjected to electrophoresis at 100 V, constant voltage, at 10°C for 1.5 hours to allow all the protein molecules to enter the gel. The voltage was then increased to 400 V, constant voltage and kept at 10°C for 20 hours. After the electrophoresis, the gels were rinsed with transfer buffer (10 mM Na_2PO_4, pH 7.6).

2.2.1.3. Electrophoretic transfer of proteins to nitrocellulose sheet

Electroblotting of the polyacrylamide gels was conducted using BioRad Trans-Blot apparatus and Fisher power supply. The nitrocellulose papers were cut in size 16 x 15 cm, and immersed in transfer buffer. After assembly, the electrotransfer was conducted at 400

mA, constant current, at constant temperature 15°C, for 3 hours. After the transfer, the blot was stained with Napthal blue (or Ponseau S) and the gel was stained with stain solution (0.125% Coomassie brilliant blue R-250, Sigma; 50% methanol, 10% acetic acid) to make sure that the transfer was successful. In a successful transfer, protein bands were seen on the nitrocellulose blot and no protein was left in the gel. In the early stage of this method, incomplete transfer due to some technical problem or shortage of circuit in the transfer set was found in about 10% of transfer. Later, this problem occurred rarely.

2.2.1.4. Western blot to identify myosin light chain

After staining, those portions of nitrocellulose sheets of appropriate molecular weight and charge were excised for immunochemical processing. The blots were blocked at 22°C with 5% BLOTTO (Bovine Lacto Transfer Technique Optimizer) solution (40 ml for a blot) with change of 5% BLOTTO at 30 min twice. The BLOTTO solution was made of 5% non-fat dry milk, dissolved in ddH_2O, and included 50 mM Tris (pH 8.0), 2 mM $CaCl_2$ (Fisher), 80 mM NaCl (Fisher), 0.2% Nonidet P-40 (Sigma) and 0.02% sodium azide (Sigma). After the blocking, the blots were rinsed twice with Tris buffer solution with 0.5% Tween 20 (Fisher, TBS-Tween pH 7.5) and incubated in TBS-Tween solution for 15 min and then with exchange of solution twice at 5 minutes.

The blots were incubated in TBS-Tween solution (30 ml for a blot 16 X 5 cm in size), containing mouse anti-myosin light chain antibody (Sigma) 1:1000, at 22°C with constant shaking for 2 hours. The blots were washed as described above and incubated in TBS-Tween solution,

containing peroxidase conjugated goat anti-mouse antibody IgG, 1:2000, at 22°C for 1 hour with constant shaking. The blots were rewashed and incubated in TBS for 5 min 3 times. The blots were then incubated with substrate solution of the conjugated enzyme, using enhanced chemiluminescence reagents (ECL kit, Amersham). After incubation for one minute, the blots were removed and excess reagent drained and covered with Saran Wrap. They were exposed then to the film (Kodak) for 15 to 30 seconds.

2.2.1.5. Blot reading

The films were read by densitometer (Hoefer GS370), scanning at right angle through the signal bands to generate a curve with a peak corresponding to the band. The percentage of myosin light chain phosphorylation is expressed in percentage as the sum of areas represented by monophosphorylated and diphosphorylated bands divided by the sum of the areas of both the unphosphorylated and phosphorylated bands.

2.2.2. Inositol phosphates generation

2.2.2.1. Radiolabelling and agonist stimulation

Freshly dispersed myocytes were plated in 24 well-plates at a density 5 x 10^5 cells per well and grown in DME H-21 d-Valine medium with 10% fetal calf serum for 3 days. Medium was then changed to inositol free labeling medium containing [^{3}H]-myo-inositol (3 µCi/ml) and cells were incubated 24 hours. Thirty minutes before experiments, the

labeling medium was removed and the myocytes washed with Hank's BSS and equilibrated at 37° C for 15 minutes.

Five minutes before experiments, the Hank's medium was replaced with 450 µl of Hank's medium containing 10 mM LiCl. The reaction was initiated by adding 50 µl of 10 X concentrate of agonist solutions into the reaction media at room temperature. The reactions were stopped by adding 1 ml of ice-cold methanol (at 15 minutes in some earlier experiments, usually at 2 minutes). The plates were kept on ice for 60 minutes and the methanol extracts were collected to chloroform ($CHCl_3$) resistant tube (glass or polypropylene). The wells were rinsed with 0.1 ml of water, which was added to the methanol extract.

2.2.2.2. Lipid extraction of samples

For chloroform extraction, 0.5 ml 0.1N HCl and 1 ml of chloroform were added to separate the phases (volume adjusted to allow phase separation). Samples were mixed by vortexing at least 30 seconds 3 times and centrifuged at 1000 g for 3 minutes to separate layers. The lower organic layer containing lipids was removed carefully and upper, aqueous layer, containing inositol phosphates, reextracted with 1 ml chloroform and mixed by vortexing. The tubes were spun, and the lower organic layer was discarded. Chloroform extraction was repeated 3 times.

2.2.2.3. Separation and quantitation of inositol phosphates

The method of Berridge [Berridge, 1983] was used. Each column was filled with 1 ml resin (Dowex x 1-8, formate form). The column was rinsed with 2 ml of 0.1 N HCOOH and then with 10 ml water. The

samples were loaded onto to columns. The columns were washed with 10 ml of deionized distilled water to elute [^{3}H]inositol, followed by 6 ml of 5 mM $NaBO_4$/60 mM NaCOOH to elute glycerophosphoinositol. They were then washed with 20 ml of water. The [^{3}H]inositol monophosphate was eluted by adding 6 ml of 0.2 M ammonium formate (NH_4COOH)/ 0.1 M formic acid (HCOOH). The column was washed with 20 ml of water. The [^{3}H]inositol biphosphate was eluted by adding 6 ml of 0.4 M ammonium formate (NH_4COOH)/ 0.1 M formic acid (HCOOH). After another wash with 20 ml of water, [^{3}H]inositol triphosphate was eluted by adding 7 ml of 1 M ammonium formate (NH_4COOH)/ 0.1 M formic acid (HCOOH).

Elutes were then added 10 ml of scintillant (Hydrofluor), mixed and equilibrated at room temperature for 12 hours before scintillation counting (Beckman).

The separation method by which inositol phosphates were eluted was validated by HPLC assay in our laboratory.

2.2.3. Adenylyl cyclase activity

Myocytes (10,000 cells/well) were grown in 96-well plate. Adenylyl cyclase activity was measured by permeabilizing the myocytes and stimulating with agonists in the presence of [^{32}P]αATP. The generated [^{32}P]cAMP indicated adenylyl cyclase activity.

2.2.3.1. Cell preparation and stimulation

2.2.3.1.1. Permeabilization of cells

Cells were placed in serum free media for 24 hours. Prior to cell permeabilization, the medium was changed to Hank's BSS for 15 minutes. The medium was then replaced with permeabilization solution which contained 30 μg/ml saponin and maintained at 23°C for 10 minutes. The cells were washed twice with permeabilization medium without saponin. The permeabilization solution contained 1.3% polyethylene glycol (PEG) 8000, 33 mM PIPES, 2 mM $MgCl_2$, 2 mM EGTA, 5.4 mM KCl, 0.34 mM KH_2PO_4, 137 mM NaCl, 0.34 mM Na_2HPO_4 and 5.6 mM glucose, pH 7.2.

2.2.3.1.2. Incubation

After 10 minutes the permeabilization solution was replaced with reaction mixture solution which was the same permeabilization medium but also containing 4 mM $MgCl_2$, 0.5 mM IBMX and 0.1 mM ATP, 30 x 10^3 cpm [^{3}H]AMP and 10^6 cpm [^{32}P]αATP with or without effectors. After 20 minutes, the reaction was stopped by adding 5 μl 3.3 N perchloric acid (PCA) for a final concentration of 0.33N.

2.2.3.2. Separation and recovery of cAMP

The separation was done using dowex and alumina column [Salomon, 1979]. The supernatants from samples were loaded onto dowex columns and each column washed with 0.01 N $HClO_4$. The volume was very critical. (Experiments were done to test this.) The volume of 0.01 N $HClO_4$ was adjusted such that the sample supernatant volume plus 0.01 N HClO4 volume equaled 5 ml. The dowex columns were then eluted onto alumina columns with 7 ml 0.01 N $HClO_4$. Cyclic AMP was

then eluted from alumina column into 20 ml counting vials, containing 3 ml of 0.2 M imidazole (pH 7.1). With the addition of [^{3}H]cAMP in each column for calculation of recovery rate, the recovery rate of cyclic AMP is 70.6 $\pm$ 5.2 %.

2.2.4. Intracellular Ca^{2+} measurement

Measure of intracellular Ca^{2+} was accomplished with the generous assistance of Mary Seller by examination of single cells loaded with Fura-2AM.

2.2.4.1. Loading cells with fura-2AM

Myocytes were grown for 3 days after dispersion on coverslips in serum containing medium and then serum free medium for 24 hours. Two hours before the experiment, cells were washed with Hank's BSS and incubated for one hour at room temperature in fura-2 loading medium, which contained 4 mM fura-2/AM (Molecular Probes, Eugene, OR), 0.018% Pluronic (Molecular Probe, Eugene, OR), 0.02% BSA (Sigma) and low Ca^{2+} Hank's buffer. After loading cells, they were washed with Hank's buffer and incubated for a further 30 minutes to allow the fura-2/AM to deesterify to fura-2.

2.2.4.2. Washing before agonist stimulation

The coverslip was placed in a controlled temperature (37°C) view chamber containing Hank's buffer. The microscope was adjusted for Kohler illumination. The fura-2 loading condition of the cells were

assessed by observing the fluorescence level in the cells and background which should be minimal. Based on this condition, cells (about 10 cells in a framed area) were selected and centered in the field, and cells from which fluorescence signal would be measured were framed. Fluorescence was measured with 1-s resolution in a spectrofluorometer (SPEX 2000) with excitation at 340 and 380 nm and emission at 510 nm. Washings were done until the fluorescence ratio (340 nm/380 nm) was stable.

2.2.4.3. Agonist stimulation

Prostaglandin E_2 at several concentrations or 1 μM angiotensin II were added to stimulate the cells. After stimulation, cells were washed until ratios returned to basal. At the end of each experiment, the Ca^{2+}-saturated and Ca^{2+}-free fluorescence levels of the fura-2 were determined by adding 10 μM Ionomycin in the presence of 1 mM $CaCl_2$ or adding EGTA 25 mM, respectively.

2.2.4.4. Calculation of intracellular Ca^{2+} concentration

The intracellular $[Ca^{2+}]_i$ was calculated according to the ratio method using the equation:

$$[Ca^{2+}]_i = K_d S_{f380/b380}(R - R_{min})/(R_{max} - R)$$

where K_d is 224 nM, $S_{f380/b380}$ is the ratio of the intensities of the free and bound dye forms at 380 nm, R is the fluorescence ratio (340 nm/380 nm) of the intracellular fura-2, and R_{min} and R_{max} are the minimal and maximal fluorescence ratios, respectively [Grynkiewicz, 1985].

2.2.5. Data analysis

Data are presented as the mean and standard error. Comparison of the data was done by t test for paired comparison or ANOVA. Statistical significance meant *P* value less than 0.05.

2.2.6. Assay development

2.2.6.1. Introduction

My decision to use isolated myocytes as the system in which to examine the mechanism of action of PGE_2 to contract myometrium required that I establish new assays which had not previously been used either in cultured cells or specifically in isolated myocytes. Additionally it was necessary to establish an efficient reproducible method to isolate uterine myocytes with minimal contamination by other cell types. The following section details the experiments involved in establishing these assays and arriving at the methods detailed in the above.

2.2.6.2. Myosin light chain phosphorylation

2.2.6.2.1. Approaches to reduce basal myosin light chain phosphorylation

2.2.6.2.1.1. Introduction

As I established this assay, early experiments indicated that the percentage of basal (unstimulated) MLC phosphorylation was greater than that previously reported. In my studies, the unstimulated MLC

phosphorylation data was 46.5 ± 4.1 % (n=12, in duplicates or triplicate), compared to values 4 to 9 % in published studies. Several possible explanations were considered in the following.

2.2.6.2.1.2. Possible causes of high basal myosin light chain phosphorylation

a. Basal stimulation from growth factors or other endogenous effectors in serum

b. Stimulation by mechanoreceptors (stress receptor) secondary to shear stress from mixing

c. Modification of myosin light chain which was wrongly regarded as phosphorylated myosin light chain

d. Stimulation of Ca^{2+} entry and hence MLC phosphorylation by TCA during the stop reaction

2.2.6.2.1.3. Removal from serum containing medium 24 hours before experiments to reduce basal stimulation

I initially considered the possibility that the increased basal MLC phosphorylation might be due to growth factor(s) or other stimulatory effectors in the fetal calf serum. To eliminate this possibility, samples were maintained in serum free medium (DME-H21, with BSA instead of FCS) for 24 hours prior to experiments. With this approach, the basal MLC phosphorylation was 38.6 ± 5.8 % (n=3, in triplicate) but was still higher than that in published studies.

2.2.6.2.1.4. Attempts to eliminate the effects of mechanical disturbance

The agonist stimulation was originally performed by removing Hank's medium and adding agonist stimulation solution. I felt that this might perturb the cultured cells. My initial attempt to reduce shear stress was by adding 1/9 volume of 10X concentrated agonist solution and slowly shaking the dishes (rather than replacing the medium). This didn't reduce basal MLC phosphorylation (37.8 $\pm$ 5.4 %, n=2, in triplicate, compared to 38.6 $\pm$ 5.8 % from experiments without this attempt). I next attempted to eliminate the effect of mechanical shearing by placing culture dishes on a shaker 30 minutes before experiment. With this approach, I hope to desensitize the cells to mechanical shearing before agonist stimulation and eliminate additional manual shaking of the dishes after adding agonist solution. Unfortunately, this method didn't significantly reduce the basal MLC phosphorylation either (38.5 $\pm$ 4.3 %, n=3, in triplicate).

2.2.6.2.1.5. Eliminate myosin light chain modification which might be confused with agonist-stimulated phosphorylation.

Phosphorylated MLC runs faster in urea/glycerol polyacrylamide gel than the unphosphorylated molecule because of additional negative charge (the phosphate group). If the unphosphorylated MLC molecules were modified during the process of protein preparation or during electrophoresis to a form which carried the same charge as phosphorylated MLC, they would run with the phosphorylated band, and be interpreted as phosphorylated MLC. To eliminate this possibility,

efforts were made to prevent the modification of MLC molecules. The major efforts included:

1) Include at least 1 mM Dithiothreitol (DTT) in every solution to which the protein was exposed.

2) Use freshly prepared 8 M urea solution and add ion-exchanger in 8M urea stock to prevent carbamoylation of the MLC molecules by urea degradation products.

3) Use a cooling system to keep the gel running at low temperature (10°C) to prevent protein from degrading

High temperature from the electrophoresis degraded protein molecule and forming active urea degradation products. The sum of these several approaches resulted in sharper bands and significant reduced basal MLC phosphorylations (24.7 ± 2.8 %, n=6, in triplicate, $P<0.05$, compared to 37.8 ± 5.4 %).

2.2.6.2.1.6. Test for and eliminate artifactual activation by TCA

Basal MLC phosphorylation might be related to the method used to stop the MLC phosphorylation reaction. The method I initially used was to add 10% TCA (final concentration). I was concerned that prior to destroying enzyme activity, TCA might induce an influx of Ca^{2+} and thus stimulate MLC phosphorylation. To test this possibility, I performed the following experiments.

With the standard method, after the myocytes were treated with agonists, the reaction was stopped and the cellular proteins were precipitated by adding trichloroacetic acid (TCA) and dithiothreitol to a final concentration of 10% (wt/vol) and 10 mM, respectively. This was compared with quick freezing to stop reaction.

I attempted to stop agonist stimulation by quick freezing to stop the enzyme activity prior to the addition of TCA. I tried several methods to quickly freeze the cells cultured in dishes.

2.2.6.2.1.6.1. Quickly freeze cells with dry ice-cooled ethanol.

The agonist stimulation was stopped by quickly removing the reaction solution and immersing the culture dishes into the cooled 100% ethanol (-60°C) with 1 mM dithiothreitol. After incubation for 15 minutes, the dishes were quickly moved to dry ice-cooled ethanol bath, which containing 10% TCA and 1 mM dithiothreitol and incubated for 30 minutes. Then they were moved to dry ice-cooled 10% TCA bath with 1 mM dithiothreitol and incubated for another 30 minutes. Finally, they were removed and incubated in another dry ice-cooled ethanol bath, which containing 10% TCA and 1 mM dithiothreitol for 20 minutes before they were added ice-cold 10% TCA with 1 mM DTT and put on ice ready for scraping and protein preparation. By doing so, it significantly reduces the basal level (control) of phosphorylated myosin light chain to 18.3 ± 3.2% (n=3, in triplicate), compared to 26.5 ± 3.1 % (n= 3, in triplicate) in control TCA group ($P < 0.05$). The disadvantage of this method is that the dead cells stuck to the dishes firmly and it was hard to scrape the dead

cells off the dishes. This, and materials lost into wash solutions, resulted in a low yield of protein (0.1-0.2 mg/ml vs. 0.5-1.5 mg/ml).

2.2.6.2.1.6.2. Quickly freeze cells with dry ice-cooled Freon

The procedure used Freon as a freezing medium but otherwise was exactly the same as the above ethanol method. The advantage of this method was that it was easy to completely scrape cells off the dishes and the protein yield was high. The disadvantage was cost and environmental concern. The phosphorylated myosin light chain in the control group was 17.9 ± 3.7 % (n=3, in triplicate), significantly lower than 26.5 ± 3.1 % (n= 3, in triplicate) in control group ($P < 0.05$). Hence Freon freezing was the chosen method to stop reaction in MLC phosphorylation assay experiments.

2.2.6.2.2. Details found to be important with urea/glycerol polyacrylamide gel electrophoresis to improve band sharpness

One of the major problems with urea/glycerol polyacrylamide gel is obtaining sharp bands to allow quantitation by scanning. Through trial and error and consulting both at the University of California and externally the following approaches were developed empirically. This was greatly aided by the generous assistance of Dr. Haeberle at the University of Vermont who allowed me to work in his laboratory to gain hands on experience with these assays and their pitfalls. It is obvious that few of these strategies is included in any published description of the methods.

a. The TCA in the protein sample must be completely removed. If TCA was not completely removed, the protein bands smeared. Thus it was necessary to wash extensively to remove TCA completely.

b. Prior to loading the gels, glycerol which had leaked into the protein loading notch during prerunning the gel must be removed. This greasy material which was barely visible could be removed with a pointed pipette immediately prior to protein loading. This approach prevented the dumbbell shape and also improved the sharpness of the bands.

c. The gel was run at 400 V, constant voltage and generated a great deal of heat, leading to protein and urea degradation and resulting MLC modification. Cooling at low temperature (e.g., 10°C) improved sharpness of the bands as well as preventing MLC modification (see above).

d. It also helped to run the gel at lower voltage (100 V) in the beginning, and turn the voltage up to 400 V after 1.5 hours, when the dye in all lanes showed the sample had run completely into the gel. With this approach bands were much sharper.

2.2.6.2.3. Considerations in the choice of technique to visualize antibody myosin light chain complex

To visualize the complex of MLC with anti-MLC antibody, a second antibody is used which is directed against the anti-MLC antibody and carries a signal. The second antibody can be radioactively labeled, a detection method that is sensitive and precise. The disadvantage is the

use of radioisotope and a relative long time to get a developed film and the result.

The second antibody can also be linked to an enzyme, e.g., peroxidase or alkaline phosphatase, which is visible when incubated with its substrate (e.g., 4-chloro-1-naphthol for peroxidase). The advantage is convenience and results are readily visible immediately after incubation with substrate solution. However, there were three disadvantages. The method is relatively less sensitive than the radiolabelled method. Color on the blot sheet will fade with time. The blot couldn't be reused for repeated washing and hybridization if the first attempt is unsatisfactory.

The chemiluminescent reaction of cyclic diacyhydrazides can also be used with horseradish peroxidase-biotinylated antibody. In this reaction, the peroxidase catalyzes the oxidation of luminol in the presence of hydrogen peroxide, exciting luminol which decays to the ground state via a light emitting pathway. This method is very sensitive and provides results rapidly. We compared this assay system to the radioisotope assay. Two proteins in quadruplicate were run in the same gel. After incubation with first antibody and washing, one group in duplicate was incubated with radiolabelled second antibody, the other with alkaline peroxidase-biotinylated anti-mouse antibody. The percentages of myosin light chain phosphorylation were 28.6 $\pm$ 1.2 % (n=2, in duplicate) and 45.7 $\pm$ 1.4 % (n=2, in duplicate) respectively in radiolabeled group. In the group using enhanced chemiluminescence detection method, they were 29.1 $\pm$ 1.3 % (n=2, in duplicate) and 46.5 $\pm$ 1.1 % (n=2, in duplicate). Since the enhanced chemiluminescence detection method was as accurate and sensitive as the radioisotope

labeling method, yet more rapid and economical, it was used for my assays.

2.2.6.2.4. Validation and optimization of assay

2.2.6.2.4.1. Validation of MLC phosphorylation assay

The validation of the identity of the MLC bands was done by electrophoresis of my samples in parallel with samples from Dr. Haeberle. He provided samples to us which had been analyzed by phosphopeptide mapping and phosphoamino acid analysis to verify the phosphorylation of the MLC protein [Haeberle, 1985 & 1986]. The locations of the MLC bands in my samples were exactly the same as theirs. Another support of the identity of the bands is the phosphorylation or dephosphorylation of myosin light chain induced by the stimulation by ionomycin or forskolin.

2.2.6.2.4.2. Time to stop agonist stimulation

To determine the time of peak phosphorylation of myosin light chain, the reaction in response to 10 nM PGE_2 was stopped at different times (Figure 1). The data from 3 experiments in triplicate indicated that the percentage of peak MLC-PO_4 with 10 mM PGE_2 was at 2 min (45.8 $\pm$ 4.4 %) and fell thereafter to a minimum value of 20.8 $\pm$ 2.8 % at 15 min. Based on these findings, agonist stimulation was stopped at 2 minutes in all MLC-PO_4 experiments.

2.2.6.2.4.3. Protein amount to be used in electrophoresis (protein linearity)

To determine if the extent of myosin light chain phosphorylation was affected by the amount of protein used in the western blot, proteins with high percentage of phosphorylation (protein A, stimulated by 1 μM bradykinin) and low percentage of phosphorylation (protein B, unstimulated) were compared adding protein amounts from 7.5 μg to 20 μg (Figure 2). No significant difference of percentage phosphorylation was found for either protein using different amount of protein in the range tested (P>0.05). We chose 15 μg as the protein amount used in all urea/glycerol PAGEs.

2.2.6.3. Adenylyl cyclase activity

2.2.6.3.1. Introduction

There are 3 general methods to measure cyclic AMP in biological systems. Radioimmunoassay or protein kinase binding assay can be used to measure cAMP generation in tissue or cells. Alternatively, the adenine pools of cells can be radiolabeled with [^{3}H] adenine and radioactive cAMP measured. A third approach is to measure radioactive cAMP generation from [^{32}P]αATP by adenylyl cyclase using cell membrane preparations. With the first and second methods, cyclic AMP generation is determined by enzyme (adenylyl cyclase) but also endogenous substrate concentration. With both of these techniques, the cyclic AMP measured is total cAMP in cells including the content and agonist stimulated and newly generated portions. For the second method, the signal is also determined by how well the cells are labeled. If the stimulation of cAMP generation is small, it may not be detectable by these two methods. The third method measures the stimulated adenylyl

cyclase activity with substrate present in excess. Because cell membranes are not permeable to ATP, the conventional way to make radiolabelled [^{32}P]αATP available to adenylyl cyclase is with membrane preparations. Although this is a sensitive assay, there are some disadvantages. Firstly, membrane preparations will frequently result in loss of material, a major problem with limited amounts of tissue or cells. Secondly, some membranes form inverted vesicle with the receptors within the vesicle not accessible to ligands. Probably at least in part due to this, the stimulation of cAMP generation by an agonist as a multiple of basal cAMP formation is much lower in membrane preparations than intact cells. Several reports indicated that those problems could be overcome by using permeabilized cells which allowed control of substrate and ionic concentration but maintained intracellular architecture and relationship.

2.2.6.3.2. Cell permeabilization

2.2.6.3.2.1. Cell permeabilization reagents

Experiments were performed to determine the optimal method of permeabilizing myocytes. Digitonin and saponin were tested. Different concentrations of digitonin (0.005% to 0.01%) and saponin (15 - 120 μg) were used to permeabilize myocytes for 15 minutes. The myocytes were washed with permeabilization medium (without digitonin or saponin) twice and 1% trypan blue added. Microscopic assessment indicated that only a few dead cells (abnormally enlarged cell size and nucleus) were stained. Increasing incubation time to 30 minutes didn't increase trypan blue inclusion. I thought that it might be possible that the cells were

permeabilized but that the holes on the membranes were too small for trypan blue molecule.

2.2.6.3.2.2. Cell permeabilization proved by stimulated adenylyl cyclase activity

To determine more sensitively whether myocytes were permeabilized by digitonin or saponin, they were incubated in permeabilization medium (control group) or permeabilization solution with 0.005% digitonin or 30 μg/ml saponin for 5 minutes. The medium was changed and cells stimulated with 100 μM GTP, 100 μM GTPγS or 10 μM forskolin. The adenylyl cyclase activity of digitonin permeabilized myocytes stimulated by 100 μM GTP was 16.2 ± 1.3 femtomoles/min/well. Using this as a standard, the fold stimulation by 100 μM GTPγS and 10 μM forskolin were 1.93 fold and 18.1 fold (Figure 3). Those for saponin permeabilized cells were 2.49, 3.66 and 39.2 fold by 100 μM GTP, 100 μM GTPγS and 10 μM forskolin respectively and no evidence of stimulation was present in the non-permeabilized cells (0.26, 0.41 and 0.43 fold). The impermeability of cell membrane to [^{32}P]αATP made the radiolabelled substrate unavailable to adenylyl cyclase in nonpermeabilized cells. Thus, despite the results with trypan blue it was evident that the cells were permeabilized by these detergents.

2.2.6.3.3. Dose response and time of cell permeabilization

Myocytes were permeabilized with different concentrations of digitonin (0.001% to 0.01%) and saponin (15 μg/ml to 60 μg/ml) for 5 minutes. They were then exposed to 100 μM GTP, 100 μM isoproterenol

plus 100 μM GTP, 100 μM GTPγS or 10 μM forskolin. The reaction was stopped at 20 minutes. The data revealed that permeabilization with 0.005% digitonin or 30 μg/ml saponin was optimal for response to most effectors (Figures 4).

To test the optimal time of permeabilization, myocytes were permeabilized with 0.005% digitonin or 30 μg/ml saponin for 5, 10 and 15 minutes and exposed to 100 μM GTP, 100 μM isoproterenol plus 100 μM GTP, 100 μM GTPγS or 10 μM forskolin. Two experiments were done in triplicate. For most effectors, permeabilization for 10 minutes either using digitonin or saponin resulted in optimal adenylyl cyclase activity (Figure 5).

2.2.6.3.4. Optimization of reaction mixture

2.2.6.3.4.1. Selection of reaction medium

In adenylyl cyclase assays done in membrane preparations, a reaction mixture was usually used which contained 50 mM HEPES (pH 8.0), $MgCl_2$ 4 mM, EGTA 0.2 mM, 2-mercapto-ethanol (β-MeOH) 2 mM, BSA 0.1 mg/ml, creatine phosphate 10 mM, creatine phosphokinase 10 u/ml, ATP 0.4 mM, cAMP 1 mM. In this permeabilized cell adenylyl cyclase assay, permeabilization was used as a reaction medium. I compared the two media. The cells were treated exactly the same except that agonist stimulation was done in permeabilization medium in control group and in the standard reaction mixture in the experiment group. Results revealed that permeabilized cells in permeabilization medium responded better that those in reaction mixture (Table 1). My explanation is that the permeabilization medium is needed for the cells to remain permeabilized.

2.2.6.3.4.2. Response in permeabilization medium with ATP regeneration system

I was concerned that ATP availability might limit response because of rapid hydrolysis, I tested the importance of an ATP generating solution generally used in adenylyl cyclase assays. Permeabilized cells were incubated in reaction mixture prepared from permeabilization buffer with and without an ATP regeneration system (creatine phosphate 10 mM, creatine phosphokinase 10 u/ml and ATP 0.4 mM). The fold stimulation was shown in Table 2. The results demonstrated that in permeabilized cells there is no need to add ATP regeneration system in permeabilization medium for adenylyl cyclase activity study. Hence it was not included in subsequent experiments.

2.2.6.4. Cell population in culture

It is important to mention that the cells used in all the experiments in this study were prepared following exactly the same methods and were grown in the same culture condition and for exactly the same duration.

Immunocytochemistry with anti-actin antibody (smooth muscle-specific, Sigma) was used to identify uterine myocytes after 3 or 7 days in culture. After 3 days, the cytoplasm of the cells was well extended. They were stained with smooth muscle specific anti-actin antibody. The smooth muscle cells were characterized by the presence of bundles of myofilaments in the cytoplasm, which were stained by actin antibody and were easily identified under microscope (Figure 6). The percentage of

myocytes in the total cell population was calculated by the number of cells with actin stained myofilaments divided by the total cell count in ten high power fields. After 3 days in culture, 90% of the cells were myocytes by this assessment. Even after 7 days in culture, the myocytes were well extended and the myofilaments were clearly visible (Figure 6).

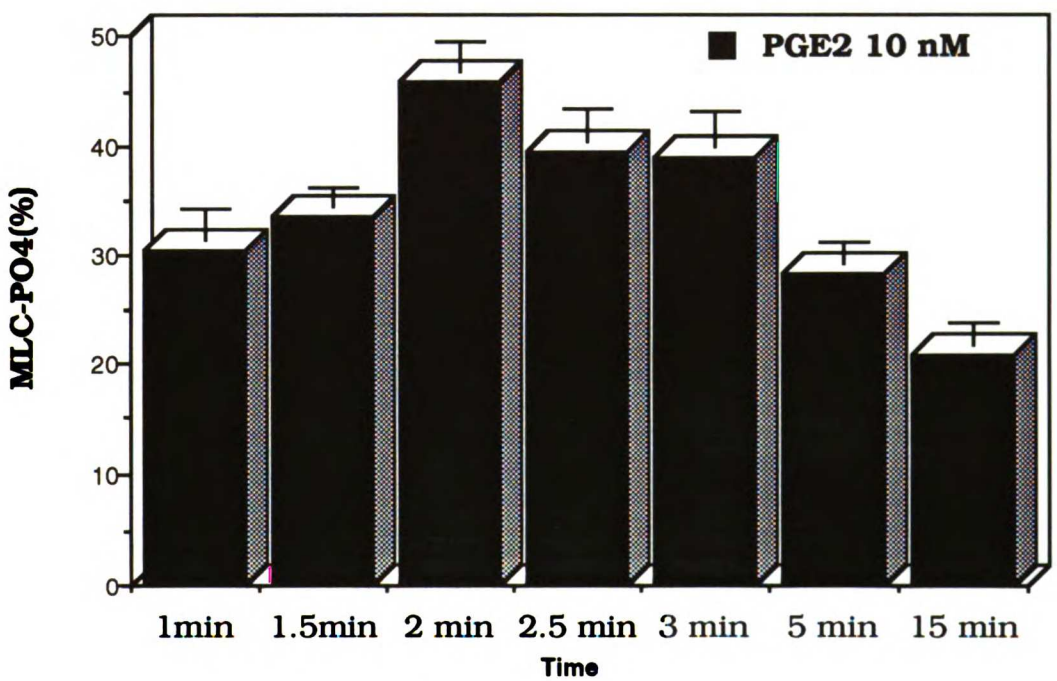


FIGURE 1: Time course of myosin light chain phosphorylation. Cells were treated with 10 nM prostaglandin E2 and reactions were stopped at those designated time points. Data are from 3 experiments conducted in triplicate.

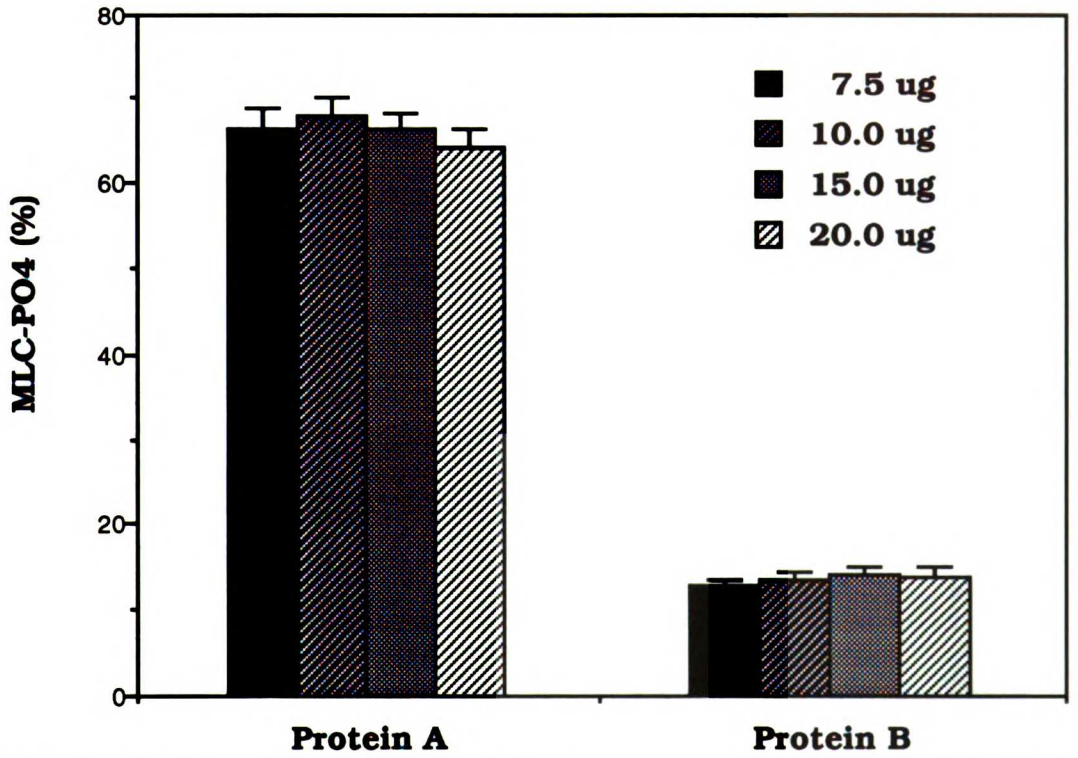


FIGURE 2: Effect of protein quantity on percentage of myosin light chain phosphorylation. Protein A was from myocytes treated with 1 μM bradykinin and protein B from unstimulated myocytes. Electrophoresis of both proteins loaded in different amounts (7.5, 10, 15, 20 μg) and western blots were performed to determine percentage myosin light chain phosphorylation in each sample as described in text. No significant difference was found within each group ($P > 0.05$). Data are from two experiments in triplicate.

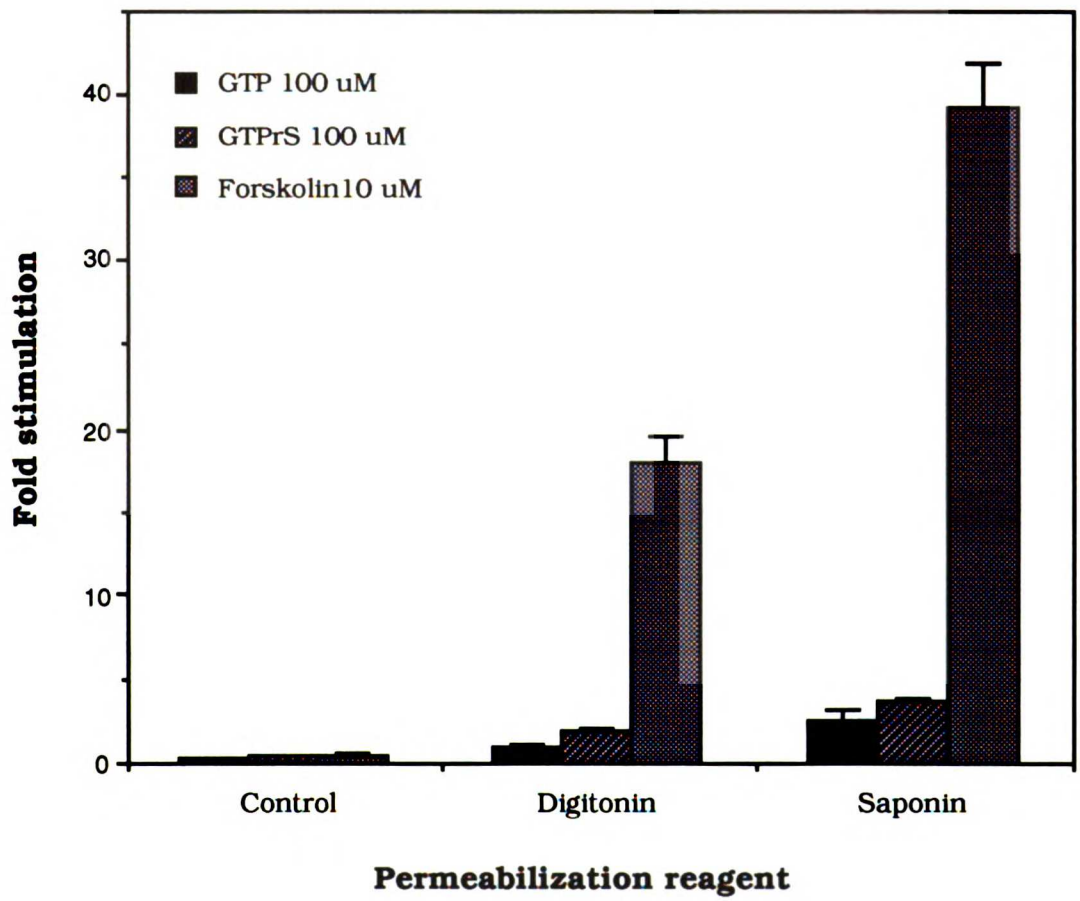


FIGURE 3: Myocyte permeabilization by digitonin or saponin. Myocytes were permeabilized with 0.005% digitonin or 30 μg/ml saponin for 5 minutes and stimulated by 100 μM GTP, 100 μM GTPγS or 10 μM forskolin for 20 minutes. Stimulation was presented as fold stimulation of 100 μM GTP (16.2 ± 1.3 femtomolar/min/well). Data are from 3 experiments were conducted in triplicate.

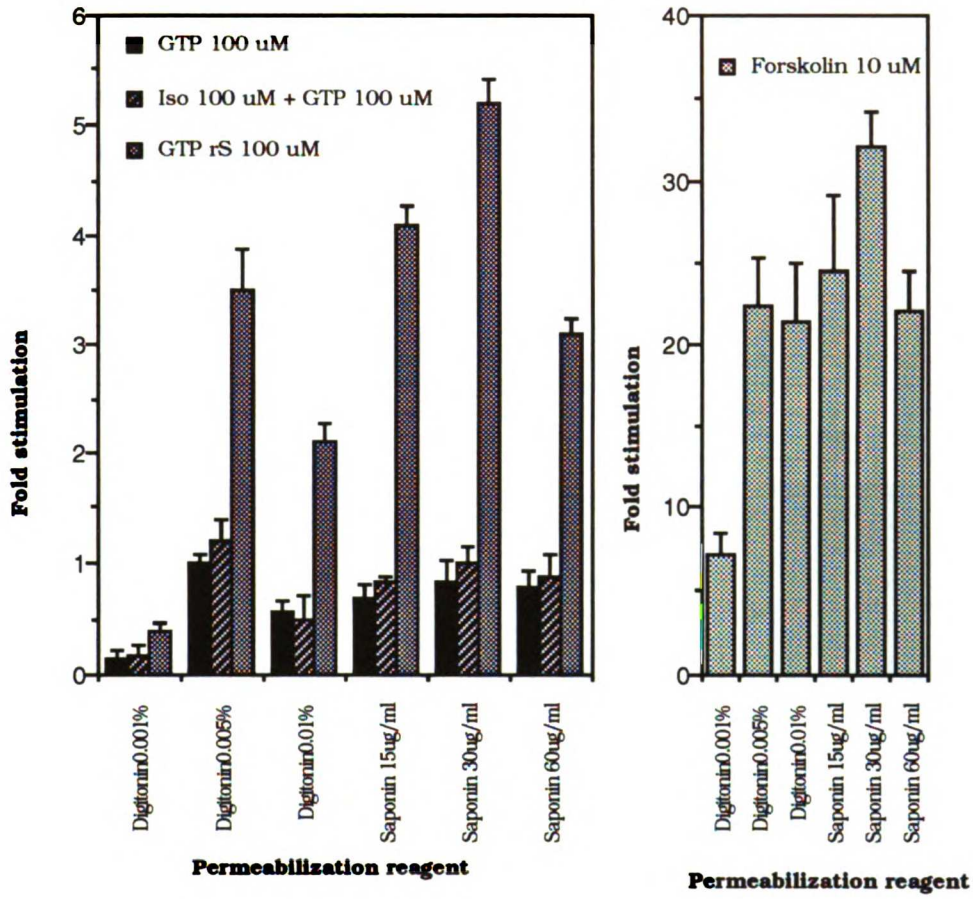


FIGURE 4: Dose response of permeabilization reagent to activate adenylyl cyclase. Myocytes were permeabilized with 3 concentrations of digitonin (0.001, 0.005 or 0.01%) or saponin (15 µg/ml, 30 µg/ml or 60 µg/ml) for 5 minutes and then exposed to 100 µM GTP, 100 µM isoproterenol plus 100 µM GTP, 100 µM GTPγS (left panel) or 10 µM forskolin (right panel). The reaction was stopped at 20 minutes. Stimulation is presented as fold of adenylyl cyclase activity by 100 µM GTP in digitonin permeabilized cells. Left panel, data of forskolin stimulation are not shown to demonstrate isoproterenol effect. There was no detectable stimulation with any effector in the absence of permeabilization. Data are from 2 experiments done in triplicate. (Iso = isoproterenol)

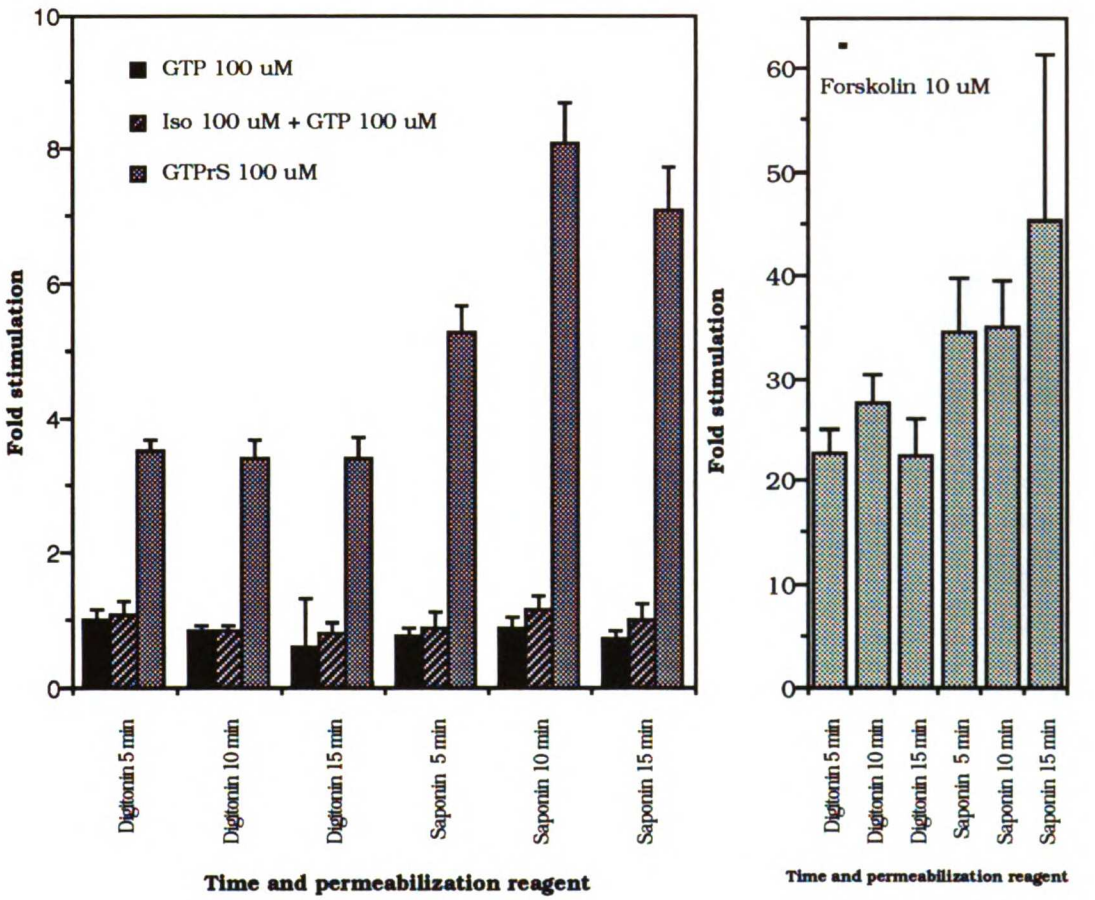


FIGURE 5: Time course of permeabilization and adenylyl cyclase activity. Myocytes are permeabilized by 0.005% digitonin or 30 μg/ml saponin for 5, 10 or 15 minutes and then exposed to 100 μM GTP, 100 μM isoproterenol plus 100 μM GTP, 100 μM GTPγS (left panel) or 10 μM forskolin for 20 minutes (right panel). Stimulation was presented as fold of adenylyl cyclase activity by 100 μM GTP in 5-minute digitonin permeabilized cell. Reaction was stopped at 20 minutes. There was no detectable stimulation with any effector in the absence of permeabilization agent. Data are from 2 experiments in triplicate. (Iso = isoproterenol)

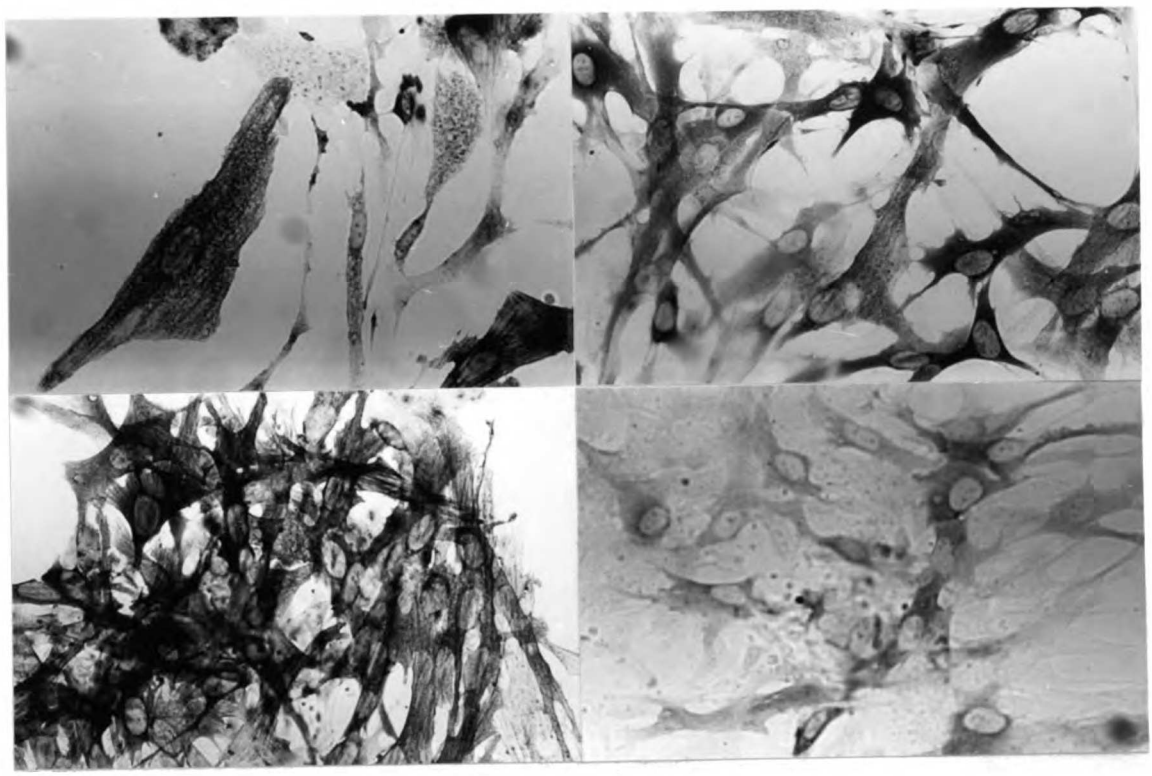


FIGURE 6: Characterization of myocytes in culture. Myocytes were grown on slides in DME-H21 d-Valine medium for 7 days. Immunocytochemistry with anti-actin antibody (smooth muscle - specific, Sigma) was used to identify smooth muscle cells after 3 days and 7 days in culture. Upper left panel: Cells were plated and grown for 7 days. The smooth muscle cells were characterized by bundles of myofilaments in the cytoplasm stained by actin antibody. Upper right panel: Cells were plated and grown on slides for 3 days. Lower left panel: Cells were plated and grown for 3 days on slides under conditions used for the experiments. The percentage of myocytes in the total cell population was calculated by the number of cells with actin stained myofilaments divided by the total cell count in ten high power fields. After 3 days in culture, 90% of the cells are myocytes. Lower right panel shows the negative control.

Table 1

	Permeabilization	Membrane
GTP 100 μM	20.0 ± 3.2	10.2 ± 2.5
Iso + GTP 100 μM	30.1 ± 5.1	10.4 ± 2.1
GTPγS 100 μM	29.2 ± 2.9	12.3 ± 3.5
Forskolin 10 μM	1210.1 ± 115.1	50.4 ± 7.1

Table 1: Different reaction mixtures effect upon adenylyl cyclase activity (femtomole/min/well). Cells were permeabilized with 30 μg/ml saponin for 10 minutes. Stimulation was in permeabilization medium or standard membrane preparation reaction mixture (see Text). There was greater adenylyl cyclase stimulation in permeabilization medium than in membrane preparation reaction mixture ($P < 0.05$, one way ANOVA). Data are from two experiments in triplicate. (Iso = isoproterenol 100 μM)

Table 2

	Without ATP regeneration	ATP regeneration
GTPγS 100 μM	2.1 ± 0.3	1.2 ± 0.2
Forskolin 10 μM	18.9 ± 2.3	10.2 ± 2.1
Prostaglandin E_2	15.2 ± 2.5	11.6 ± 2.6

Table 2: Adenylyl cyclase activity (as fold stimulation of 100 μM GTP) with or without ATP regeneration system. Cells were permeabilized with 30 μg/ml saponin for 10 minutes. Stimulation was in permeabilization medium with or without ATP regeneration system (creatine phosphate 10 mM, creatine phosphokinase 10 u/ml and ATP 0.4 mM). Greater adenylyl cyclase stimulation was present in medium without ATP regeneration system than with ATP regeneration system ($P < 0.05$, one way ANOVA). Data are from two experiments in triplicate.

Chapter 3 Results

3.1. Myosin light chain phosphorylation

3.1.1. Biphasic prostaglandin E_2 dose response

To investigate the PGE_2 effect upon uterine contraction through myosin light chain phosphorylation, cultured myocytes were exposed to different concentrations of PGE_2 and the percentage of myosin light chain phosphorylation was measured.

Myosin light chain phosphorylation with different concentration of PGE_2 is shown in Figure 7. The percentage of MLC-PO_4 increased from 23.2% to 42.1% at concentrations of PGE_2 from 10^{-11} M to 10^{-7} M. At higher concentrations phosphorylation decreased to a minimum of 24.8% at 10^{-4} M PGE_2 resulting in a biphasic dose response. When expressed as fold stimulation, the percentage of myosin light chain phosphorylation was 2.14 fold of control at 10^{-7} M and 1.20 fold at 10^{-4} M of PGE_2. In the same experiment, the response to 1 μM angiotensin II was 2.16 fold of control. In other experiments, the percentage of myosin light chain phosphorylation in response to 1 μM ionomycin was 65.2 ± 7.8 %, about 3.43 fold of control.

3.1.2. The role of extracellular calcium in Prostaglandin E_2 stimulated MLC phosphorylation

Some agonists stimulate smooth muscle contraction by opening calcium channels in cell membranes. In the absence of extracellular Ca^{2+}, these agonists lost their stimulatory effect. To test the dependence

of PGE_2 response on extracellular Ca^{2+}, the reaction media were changed to Ca^{2+}-free Hank's media 30 minutes before stimulation.

In Ca^{2+}-free Hank's medium, the percentage MLC-PO_4 in response to PGE_2 was less than in cells stimulated in standard media at all PGE_2 concentrations (Figure 8, $P < 0.05$, one way ANOVA). The percentage myosin light chain phosphorylation in experimental group (Ca^{2+}-free medium) was about 60% of that in control group. However, since stimulation in the absence of agonist was also reduced, fold stimulation by PGE_2 was unchanged ($P > 0.05$, two way ANOVA). The biphasic response to PGE_2 persisted in Ca^{2+}-free Hank's medium.

This experiment indicated that response of myocytes to PGE_2 stimulation doesn't require the presence of extracellular calcium ion. This suggests that the primary source of Ca^{2+} increase in response to PGE_2 stimulation was not due to calcium entry from extracellular pool but was from within the cells. It has been established that the response to some agonists which generate IP_3 and mobilize intracellular calcium will be augmented by a secondary response--extracellular calcium entry. Our finding of a generalized reduction in MLC phosphorylation in the absence of extracellular calcium indicates that an amplification of the response by calcium entry is also present in the uterine myocyte.

3.1.3. The effect of pretreatment with phospholipase C inhibitor (U73122) on myosin light chain phosphorylation in response to PGE_2 stimulation.

The best characterized pathway to effect smooth muscle contraction is the receptor-G protein activated phospholipase C activity, which hydrolyzes phosphatidylinositol 4,5-biphosphate (PIP_2) to generate inositol 1,4,5-triphosphate (IP_3) which mobilizes calcium ion and thus stimulates myosin light chain kinase. To investigate if PGE_2 activated PLC to effect uterine smooth muscle contraction, I tested whether inhibition of PLC activity abolished PGE_2 stimulated MLC phosphorylation. Myocytes were pretreated with 1 μM, 10 μM or 50 μM U73122 [Bleasdale, 1989; Wu, 1992] 10 minutes before exposed to 10 nM PGE_2. The percentage MLC phosphorylation were 38.1 ± 2.5 in control group (pretreated with 1% DMSO, the vehicle in which U73122 was solubilized), 22.5 ± 5.3 in group treated with 1 μM U73122 ($P < 0.05$, compared to control), 16.8 ± 4.9 in those treated with 10 μM U73122 ($P < 0.01$, compared to control), and 12.9 ± 4.3 in those pretreated with 50 μM U73122 ($P < 0.01$, compared to control). Analyzed by one way ANOVA, there is significant reduction of MLC phosphorylation in U73122 treated group ($P< 0.05$). This data suggests that PGE_2 effects uterine smooth muscle contraction through PLC.

I also tested PLC inhibition at different concentration of PGE_2 stimulation. Cells were pretreated with 50 μM U73122 or 1% DMSO (control group) for 10 minutes, and then exposed to different concentrations of PGE_2 or 1 μM angiotensin II (AII). The result, shown in Figure 9, demonstrated pretreatment of U73122 inhibited myosin light chain phosphorylation at all concentrations of PGE_2 tested ($P<0.05$).

3.1.4. The effect of cAMP on PGE_2-stimulated myosin light chain phosphorylation

As will be discussed later, PGE_2 stimulated adenylyl cyclase activity to generate cyclic AMP, regarded as the second messenger to inactivate myosin light chain kinase, reduce myosin light chain phosphorylation and induce muscle relaxation. To test if PGE_2-stimulated adenylyl cyclase activity affected myosin light chain phosphorylation, I first tested if cAMP generation in myocytes influenced MLC phosphorylation.

3.1.4.1. Forskolin inhibited bradykinin-stimulated myosin light chain phosphorylation

To address this question, I examined the effect of bradykinin, a potent stimulant of MLC phosphorylation and forskolin, an activator of adenylyl cyclase, on MLC phosphorylation. In the control group, myocytes were treated with 1 μM bradykinin. The experimental groups included myocytes treated with 1 μM bradykinin and several concentrations of forskolin from 1 μM to 100 μM. The percentage MLC phosphorylation in control group was 62.5 ± 8.3 %. Those for the experimental group were 11.6 ± 2.2 %, 6.3 ± 2.1 % and 6.1 ± 2.5 % for 1 μM, 10 μM and 100 μM of forskolin (Figure 10). Compared to the control (bradykinin treatment), forskolin treatment significantly inhibited myosin light chain phosphorylation ($P < 0.001$). This demonstrated that adenylyl cyclase activity generated by 10 μM forskolin abolished the myosin light chain kinase activity which would be activated by 1 μM bradykinin in the absence of forskolin.

3.1.4.2. Kinase-A inhibitor (H8) reversed the forskolin effect on myosin light chain phosphorylation.

To test that the inhibitory effect of forskolin on MLC phosphorylation was mediated by cAMP, a kinase-A inhibitor (H8) was used to treat the cells prior to exposure to forskolin. Myocytes in the experimental group were pretreated with several concentrations of H8, from 0.1 μM to 10 μM and followed by 1 μM bradykinin or 1 μM bradykinin plus 10 μM forskolin. The control groups included myocytes treated with Hank's BSS (as basal), 1 μM bradykinin, 1 μM bradykinin plus 10 μM forskolin, or plus 10 μM H8. The result is shown in Figure 11. There was no statistical significant difference between basal and H8 treatment ($P > 0.05$). Pretreatment with H8 significantly increased percentage MLC phosphorylation after exposure to forskolin and bradykinin ($P < 0.005$, H8 0.1 μM; $P < 0.001$, H8 1 μM to 10 μM). H8 at 1 μM concentration reversed the inhibition of forskolin on MLC phosphorylation induced by bradykinin.

The interaction of H8, forskolin and bradykinin indicates that H8 reverses the inhibitory effect of forskolin on myosin light kinase activity. H8 alone did not appear to increase MLC phosphorylation (basal was slightly reduced but not statistically significant $P > 0.05$).

3.1.4.3. H8 blocked the inhibitory phase of the biphasic response of PGE_2

Based on the above data and by the same analogy, I tested if the biphasic effect of PGE_2 on MLC phosphorylation was due to the

stimulation of adenylyl cyclase activity by PGE_2 at high concentrations. Myocytes were pretreated with or without 1 μM H8 for 10 minutes, and then exposed to different concentrations of PGE_2 or 1 μM AII. The result was shown in Figure 12. No significant difference was found with H8 pretreatment in control or angiotensin II treated group ($P > 0.05$). Pretreatment with H8 significantly increased PGE_2 stimulated myosin light chain phosphorylation at PGE_2 concentrations 10^{-9} to 10^{-4} M (**$P < 0.05$, PGE_2 10^{-9} to 10^{-6} M; ***$P < 0.01$, PGE_2 10^{-5} to 10^{-4} M). By contrast, in the control group, the biphasic effect of PGE_2 persisted (in triplicate, n=3). This experiment demonstrated that the inhibition of MLC phosphorylation at high concentrations of PGE_2 was due to its stimulation of adenylyl cyclase activity.

3.1.5. Summary of PGE_2 effects on MLC phosphorylation

In summary, PGE_2 demonstrated a biphasic stimulation of myosin light chain phosphorylation in rabbit uterine myocytes. The peak stimulation was at 10 nM (2.12 fold of basal stimulation), and at higher concentrations the stimulation decreased (1.21 fold at 100 μM). In the absence of extracellular Ca^{2+}, the biphasic pattern of stimulation persisted although the percentage myosin light chain phosphorylation was less. Pretreatment with a kinase-A inhibitor reversed the inhibitory phase of PGE_2 treatment (from 1 μM to 100 μM) and attained a plateau on MLC phosphorylation at concentration of 10 μM. Stimulation by PGE_2 of myosin light chain phosphorylation was blocked by pretreatment with phospholipase C inhibitor U73122. This suggested a role for PLC in PGE_2 stimulated uterine contraction.

3.2. Inositol phosphates generation

Since PGE_2 stimulation of MLC phosphorylation was blocked by PLC inhibition, I tested if PGE_2 activated PLC which hydrolyzes PIP_2 to generate inositol phosphates.

3.2.1. Pretreated myocytes with LiCl

Lithium chloride is known to inhibit inositol phosphatase and is widely used in inositol phosphate generation assay. To test if a better stimulatory effect on inositol phosphate generation could be shown in the absence of lithium chloride, it was omitted in some angiotensin II treatment experiments. The result indicated that the fold stimulation to 1 μM angiotensin II treatment was decreased compared to LiCl pretreatment group (1.5 fold versus 2.3 fold, $P < 0.05$). In subsequent experiments, myocytes were pretreated with LiCl.

3.2.2. Time course of inositol phosphate generation

3.2.2.1. Time course of IP generation in response to 10 nM prostaglandin E_2

Myocytes were radiolabelled, pretreated with LiCl and treated with 10 nM PGE_2 for 0.5 min, 1 min, 2 min, 5 min or 15 min respectively. The control group was Hank's BSS or AII 1 μM treatment for 15 min. Statistical analysis was done by one-sided T test. No stimulation of IP generation was found in PGE_2 treatment group at any time points tested

(*$P > 0.05$). Significant IP generation was found in angiotensin treatment group (IP_3 generation 2.56 fold) (**$P < 0.0$ 1) (Figure 13).

3.2.3. Dose response of prostaglandin E_2 on inositol phosphates generation.

3.2.3.1. Dose response of PGE_2 for 15 minute stimulation

Since I saw no peak time of prostaglandin E_2 stimulation on IP generation in the time course experiment, I chose 15 minutes as time point to stop reaction. This is a time commonly used in inositol phosphate generation experiments. Myocytes were radiolabelled, pretreated with LiCl and exposed to PGE_2 at concentrations from 10^{-11} M to 10^{-4} M, AII 10^{-6} M or bradykinin 10^{-6} M. The dose response of generation of inositol monophosphate (IP), inositol 4,5-biphosphate (IP_2) and inositol 1,4,5-triphosphate (IP_3) is shown in Figure 14. No significant generation of IP, IP_2, IP_3 was found after stimulation with PGE_2 for 15 minutes while AII and bradykinin attained a 2.13 and 3.64 fold stimulation on IP_3 generation respectively. The result from PGE_2 concentration response at 15 minutes raised the possibility that although the accumulated IPs increased with time, the fold stimulation in agonist treated group might not necessarily increase.

3.2.3.2. Time course of IP generation in response to 1 μM angiotensin II

I also chose 15 minutes to stop reaction since in the presence of LiCl to inhibit IP breakdown, inositol phosphates accumulated to a

plateau in 30 minutes in most cell types. To look at the relationship between fold stimulation and reaction time, I examined the time course of IP generation by angiotensin II, a known stimulant of inositol phosphates generation. I examined the fold stimulation (compared to control at the same time point) in response to 1 μM angiotensin II in the presence of LiCl at 1, 1.5, 2, 3, 5, 10 or 15 minutes. The peak stimulation was at 2 minutes after angiotensin II stimulation (Figure 15). Hence for subsequent inositol phosphate generation experiments, reactions were stopped at 2 minutes.

3.2.3.3. Dose response of PGE_2 for 2 minute stimulation

Experiments were conducted as previous if except reactions were stopped at 2 minutes. No significant IP generation from PGE_2 stimulation was noted at two minutes ($P>0.05$).

3.2.3.4. Temperature factor

I also considered the possibility that myocytes might not respond to PGE_2 at room temperature. To test this possibility, 2 experiments were conducted at 37°C with the reactions stopped at 2 minutes. Still no perceivable IP generation was found in response to PGE_2 stimulation.

3.2.3.5. Inositol phosphates generation corrected by protein amount in each sample.

I considered the possibility that the stimulation of IPs generation by PGE_2 might be small and more careful characterization of the

stimulation was necessary to demonstrate the effect. Although the myocytes in each well grew from 5 x 10^5 dispersed cells, difference might be present after growth in medium for 3 days. This might affect the calculated fold stimulation on IPs generation. Proteins from each well were measured. The mean of protein amount from each well was 0.25 $\pm$ 0.04 mg. Still no significant fold stimulation by PGE_2 was found after correction by protein amount.

3.2.4. Dose response of PGE_2 to alter inositol phosphate generation after pretreatment with kinase-A inhibitor (Hs)

In rat myometrial cells, Anwer *et al.* reported that forskolin inhibited IP generation by uterine contractants [Anwer, 1989]. By the same pathway crosstalk analogy, cyclic AMP generated by PGE_2 stimulated adenylyl cyclase activity, might inhibit the generation of inositol phosphates. To test this hypothesis, myocytes were radiolabelled for 24 hours, pretreated with 1 μM Hs (the same concentration used in MLC phosphorylation experiment) 10 minutes before agonist stimulation, and LiCl 5 minutes before agonist stimulation. The reactions were stopped at 2 minutes. As shown in figure 16, the result did not demonstrate that pretreatment with Hs at 1 μ M affected inositol phosphates generation in response to PGE_2 treatment.

3.3. Adenylyl cyclase activity

3.3.1. Dose response of prostaglandin E_2 on Adenylyl cyclase activity

Due to the previously mentioned advantages of permeabilized cell system, adenylyl cyclase activity by PGE_2 stimulation was examined in permeabilized cells. Myocytes were permeabilized with 30 μg/ml saponin for 10 minutes and the cells incubated in reaction mixture prepared from permeabilization media with effectors including 100 μM GTPγS, 10 μM forskolin or several concentrations of PGE_2. The results presented as fold stimulation of 100 μM GTP stimulated adenylyl cyclase activity indicate marked (15 fold) adenylyl cyclase stimulation by PGE_2 (Figure 17). In previous experiments using a conventional cyclase assay with membranes prepared from myocytes, PGE_2 stimulation was 1.3 times GTP stimulation compared to 6 fold with the permeabilized cells. To further validate this assay, myocytes were radiolabelled with radioactive [^{3}H]adenine and then stimulated with PGE_2, the result is shown in Figure 18. Compared to the adenylyl cyclase activity in permeabilized cells, the fold stimulation in [^{3}H]adenine radiolabelled group was significantly lower ($P < 0.05$). Generally speaking, the fold stimulation of adenylyl cyclase activity done in permeabilized cells is twice that done with radiolabelling of the adenine pool.

At concentrations higher than those at which MLC-PO_4 was detectable, PGE_2 significantly stimulated adenylyl cyclase. At 100 μM, PGE_2 stimulation of adenylyl cyclase activity was equivalent to that by 10 mM forskolin (15 vs. 17 fold). The potent stimulation of adenylyl cyclase activity at high concentrations may explain its inhibitory effect on myosin light chain phosphorylation and resulting muscle relaxation.

3.4. Intracellular [Ca^{2+}] measurement

3.4.1. The effect of prostaglandin E2 on intracellular [Ca^{2+}]

Since PGE2 stimulated myosin light chain kinase activity with a biphasic response, I examined the dose response of PGE2 to increase intracellular [Ca^{2+}]. Cells loaded with fura-2 were treated with different concentrations of PGE2 or 1 μM angiotensin. The order of stimulation was from low concentration to high concentration of PGE2 with 3 minute interval between the return to basal state and next stimulation. Basal $[Ca^{2+}]_i$ was 105 ± 9 nM (n= 3), consistent with previous studies of uterine smooth muscle cells [Mackenzie, 1990]. PGE2 elicited a rapid rise of $[Ca^{2+}]_i$ in a dose-dependent manner. An increase of $[Ca^{2+}]_i$ could be detected at PGE2 concentration of 1 nM. Intracellular [Ca^{2+}] increased gradually with addition of PGE2 from 1 nM to 10 μM and increased sharply from 10 μM to 100 μM. The mean values of the peak $[Ca^{2+}]_i$ in response to different concentrations of PGE2 are shown in Figure 19. The intracellular calcium concentration returned to basal level in 20 second to 1 minute after each peak. $[Ca^{2+}]_i$ increased at all concentrations of PGE2. Thus the biphasic MLC phosphorylation response is secondary to effects of PGE2 distal to calcium.

3.4.2. Effect of phospholipase C inhibitor U73122 on PGE2-stimulated increased $[Ca^{2+}]_i$

U73122, a putative PLC inhibitor, blocks agonist-induced increased intracellular Ca^{2+} concentration in neutrophils and platelets (Bleasdale, 1990). Myocytes were prepared for intracellular [Ca^{2+}] measurement and responded by increasing $[Ca^{2+}]_i$ to several

concentrations of prostaglandin E2 or 1 μM angiotensin II. As shown in Figure 20, incubation of 10 μM U73122 for 10 minutes blocked the $[Ca^{2+}]_i$ increase of myocytes by 100 μM PGE2 or 1 μM AII.

The possibility that this inhibition of $[Ca^{2+}]_i$ response to PGE2 stimulation was due to homologous desensitization rather than the effect of U73122 was excluded by the responsiveness of myocytes to the sequentially increased concentrations of PGE2 in dose response experiments.

3.5. Hormonal effect of PGE2-mediated myosin light chain phosphorylation

3.5.1. Biphasic effect of prostaglandin E2 on uterine contraction in pregnant rabbit uterus

On occasions, I had access to pregnant rabbit uterine tissue left over from some colleague's experiments (excess tissue). I examined the PGE2 stimulated response in this tissue as a preliminary approach to examine hormonal modulation of PGE2 response. Myocytes from 27 day pregnant (term 31 days) rabbit were cultured and treated exactly the same as those from estrogen treated uteri. The basal MLC phosphorylation (20.2 ± 0.8 %) was not significantly different from that in myocytes from estrogen treated uterus (19.2 ± 0.9 %). In response to 10 nM PGE2, the percentage myosin light chain phosphorylation in myocytes from pregnant uterus was significantly less than that in those from estrogen treated nonpregnant uterus ($P < 0.05$). So was the response to 1 μM angiotensin II ($P < 0.01$). The most striking change was

the low percentage of MLC phosphorylation in response to high concentrations of PGE_2 from 1 μM to 100 μM (3.0 to 7.2%, $P< 0.005$, compared to those in myocytes from estrogen treated non-pregnant uterus) (Figure 21). Nonetheless the biphasic response persisted with suppression of MLC phosphorylation at high concentrations of PGE_2.

The data of MLC phosphorylation from the estrogen treated and pregnant rabbits demonstrated that the biphasic response to PGE_2 persisted in rabbits of different hormonal status. The difference in their response to agonists especially at high concentration of PGE_2 clearly indicates that the PGE_2 effect on uterine contraction is subject to hormonal modulation.

3.6. Myometrial contraction study

3.6.1. Dose response of PGE_2-stimulated myometrial contraction

To test the physiological relevance of the blunting of MLC phosphorylation by PGE_2 stimulation of cyclic AMP generation, I examined H8 effects on PGE_2 stimulated myometrial contraction. Uterine strips contracted in response to PGE_2 concentrations from 1 nM to 100 nM. However at higher concentration (1 μM to 100 μM), inhibition of spontaneous muscle contraction was observed. The results of uterine strip contraction are consistent with those of myosin light chain phosphorylation of myocytes in response to PGE_2 stimulation.

3.6.2. Response in the presence of adenylyl cyclase activity inhibition

After pretreatment with the protein kinase A inhibitor, H8, PGE_2 stimulated contractions at concentrations from 1 μM to 100 μM. Additionally, the inhibitory effect of PGE_2 on uterine strip contraction could be reversed by the addition of 1 μM H8.

The contraction data from H8 treatment is also consistent with the hypothesis that the adenylyl cyclase activity stimulated by high concentrations of PGE_2 blocked its stimulatory effect and this can be reversed with protein kinase A inhibitor H8.

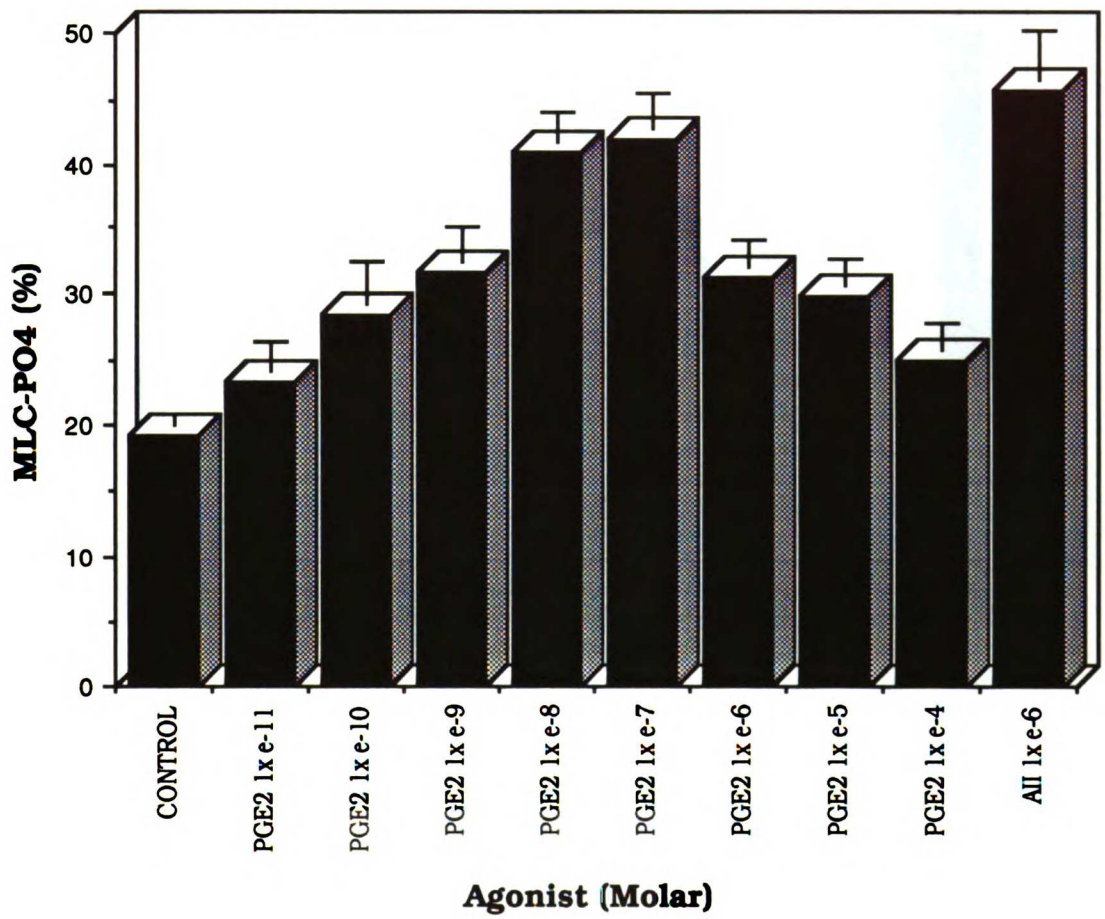


FIGURE 7: PGE2 stimulation: myosin light chain phosphorylation dose response. Myocytes were treated with prostaglandin E2 at concentrations from 10 pM to 100 μM or 1 μM angiotensin II. Reaction was stopped at 2 min. Data are from 5 experiments in triplicate. (PGE2 = prostaglandin E2, AII = angiotensin II)

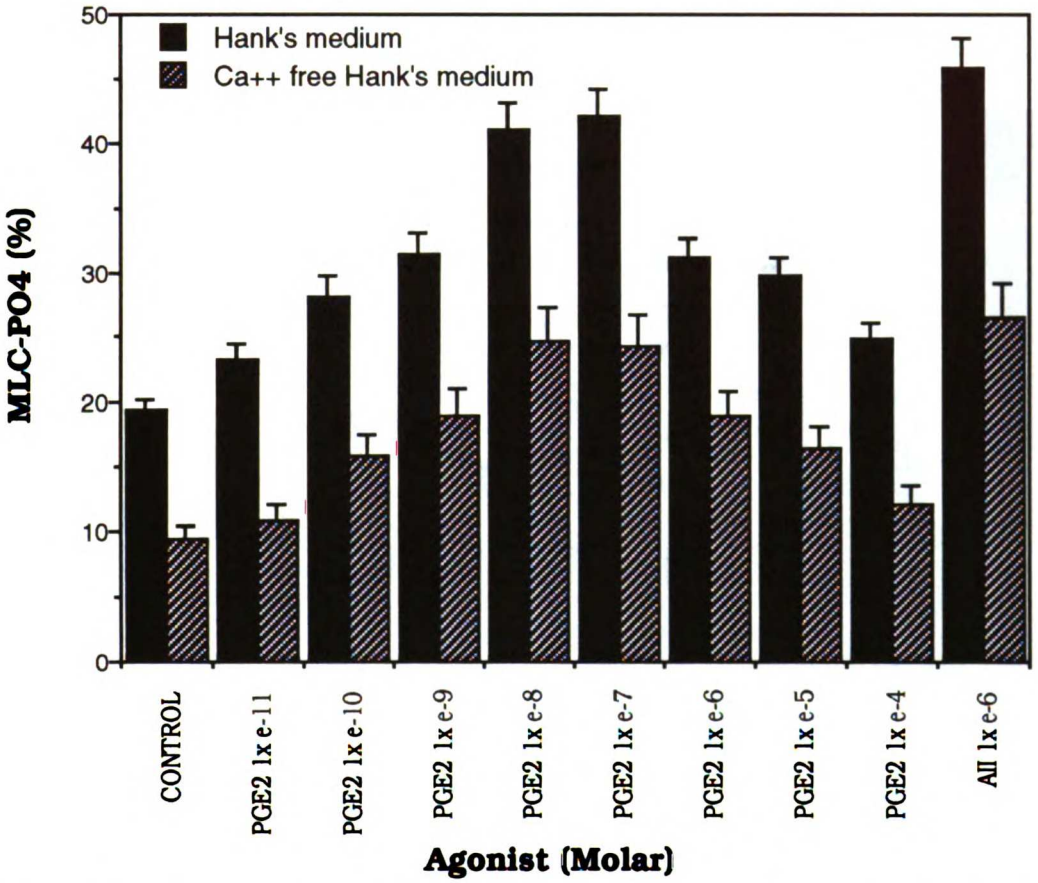


FIGURE 8: PGE2 stimulation: myosin light chain phosphorylation in Ca^{2+}-free medium. In experimental group, reaction media were changed to Ca^{2+}-free Hank's media 30 minutes before exposed to concentrations of PGE2 or 1 μM angiotensin II. Reaction was stopped at 2 minutes. Percentage myosin light chain phosphorylation was significantly lower in group with stimulation in Ca^{2+}-free Hank's medium than in control group (in normal Hank's medium) ($P < 0.05$, one way ANOVA). In fold stimulation (fold of control in each group), no significant difference was found ($P > 0.05$, two way ANOVA). Data are the mean determinations from three experiments in triplicate.

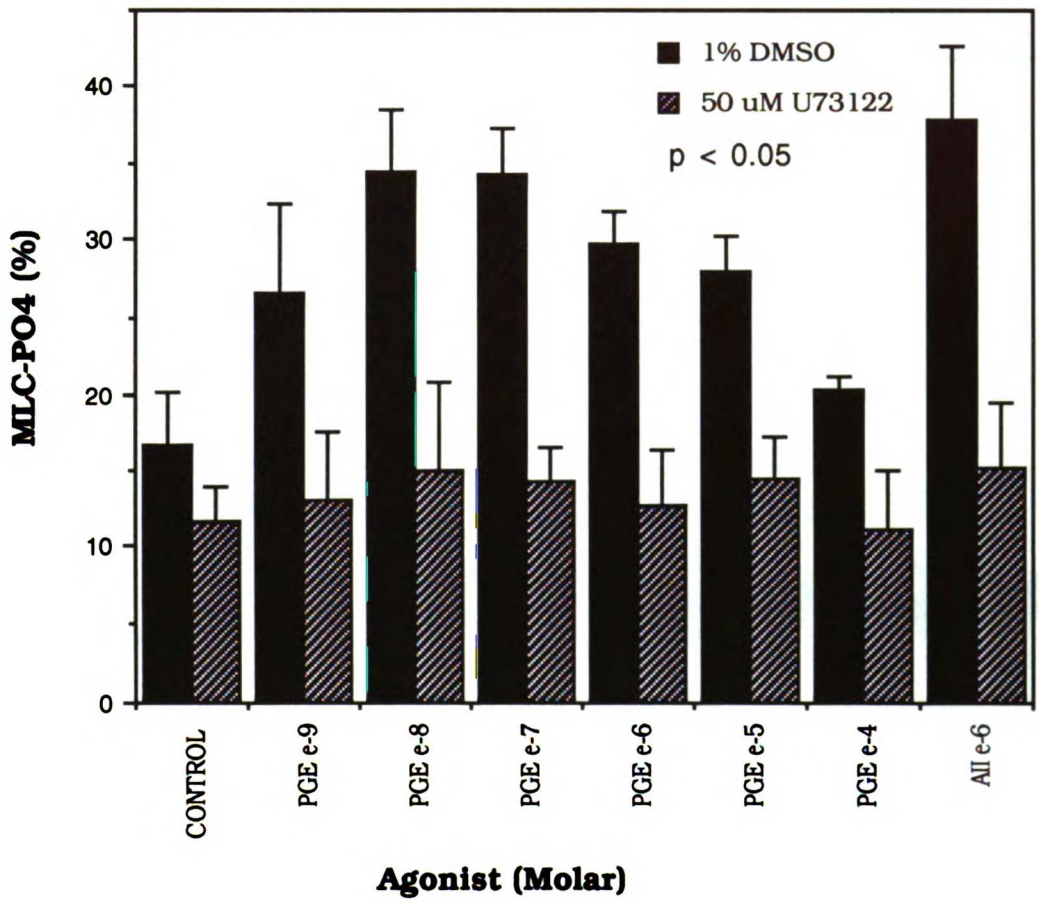


FIGURE 9: Effect of PLC antagonist U73122 on PGE2-stimulated MLC phosphorylation. Myocytes were pretreated with 50 μM U73122 10 minutes before exposed to PGE2 or AII. Control group was pretreated with vehicle (1% DMSO). Reaction was stopped at 2 minutes. Statistical analysis, done by one way ANOVA, revealed significant decrease of percentage myosin light chain phosphorylation with pretreatment with U73122 ($P < 0.05$). Data are from two experiments done in triplicate. (PGE = prostaglandin E2; AII = angiotensin II)

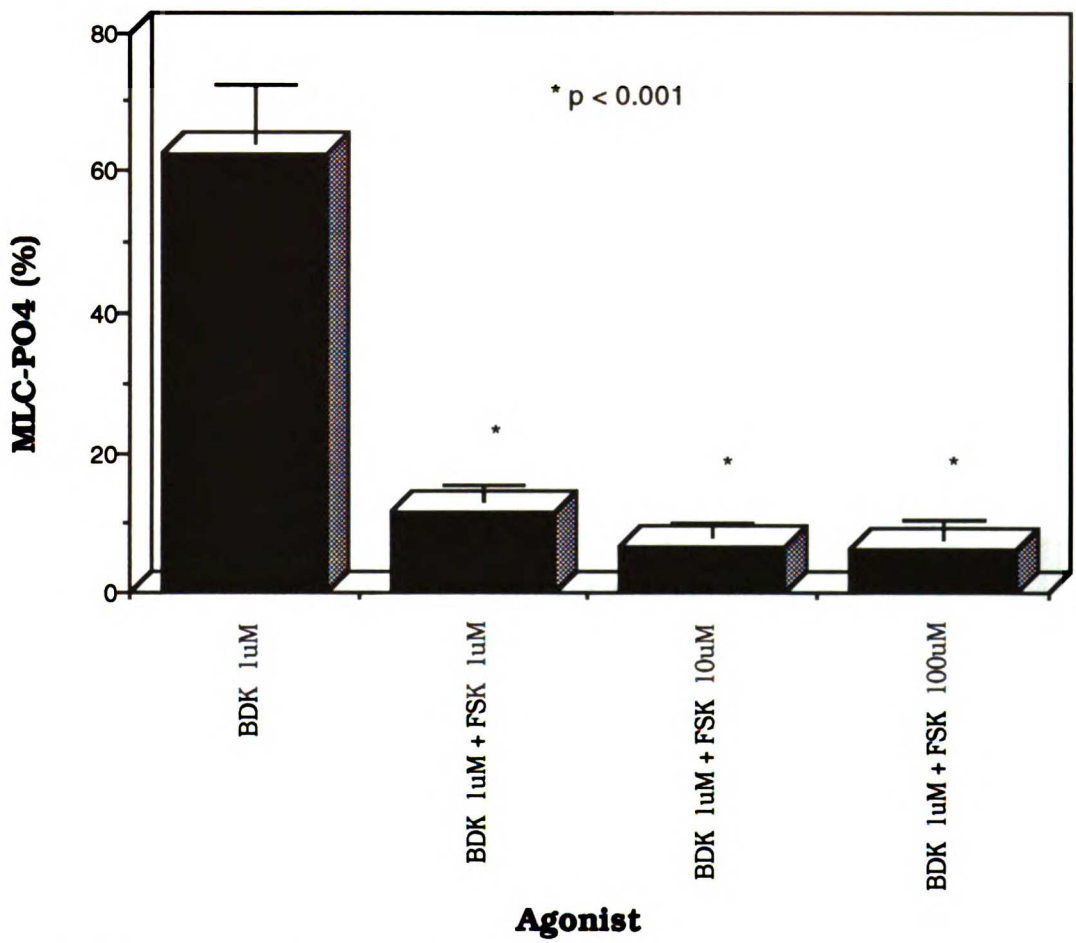


FIGURE 10: Forskolin antagonizes bradykinin-stimulated myosin light chain phosphorylation. Myocytes were treated with 1 μM bradykinin alone or with different concentrations of forskolin (1 μM to 100 μM). Reaction was stopped at 2 minutes. Compared to the control (bradykinin treatment), forskolin treatment significantly inhibited myosin light chain phosphorylation ($P < 0.001$). Data are from 3 experiments done in triplicate. (BDK = bradykinin, FSK = forskolin)

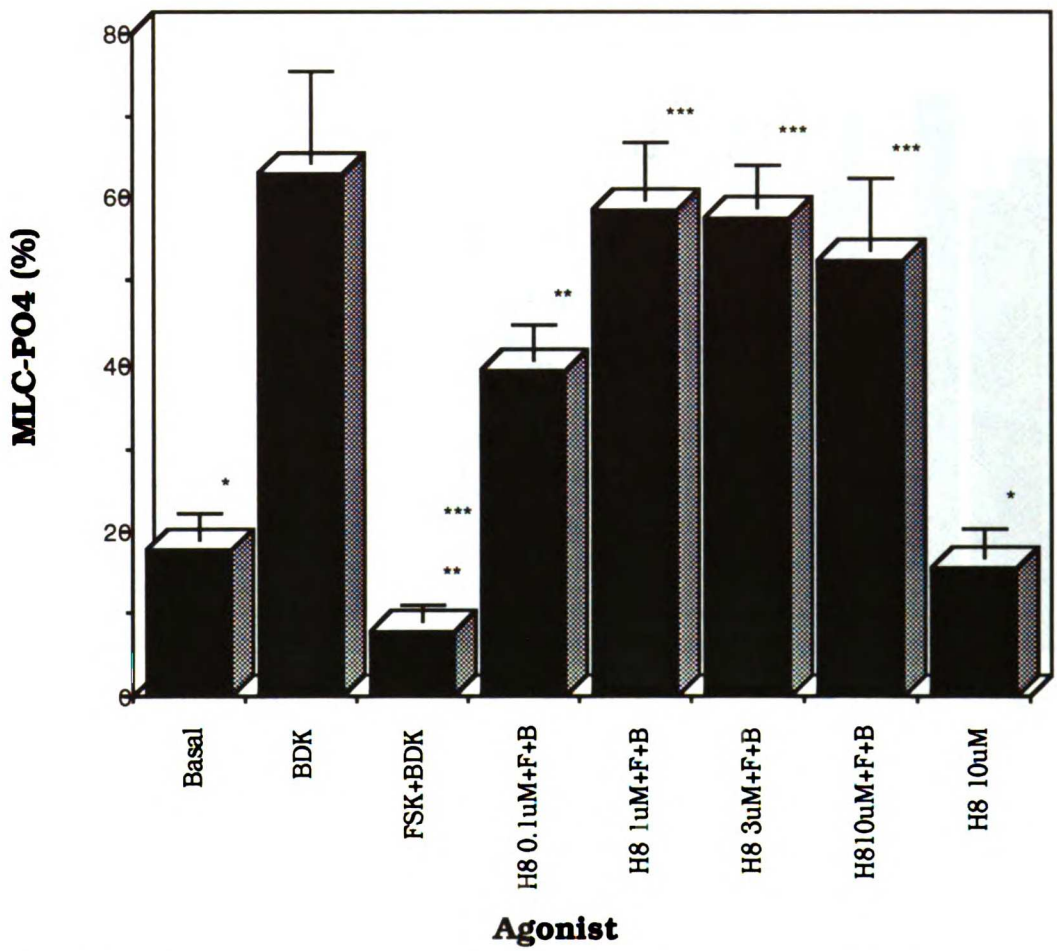


FIGURE 11: H8 reversal of forskolin inhibition of MLC-PO4. Myocytes were pretreated with or without H8 for 10 minutes and were exposed to bradykinin, forskolin plus bradykinin or Hank's solution. No statistical significant difference between basal and H8 treatment (* $P > 0.05$). Pretreatment with H8 significantly increased percentage MLC phosphorylation after exposure to forskolin and bradykinin (** $P < 0.005$, *** $P < 0.001$). Data are from two experiments conducted in triplicate. (B, BDK = bradykinin; F, FSK = forskolin)

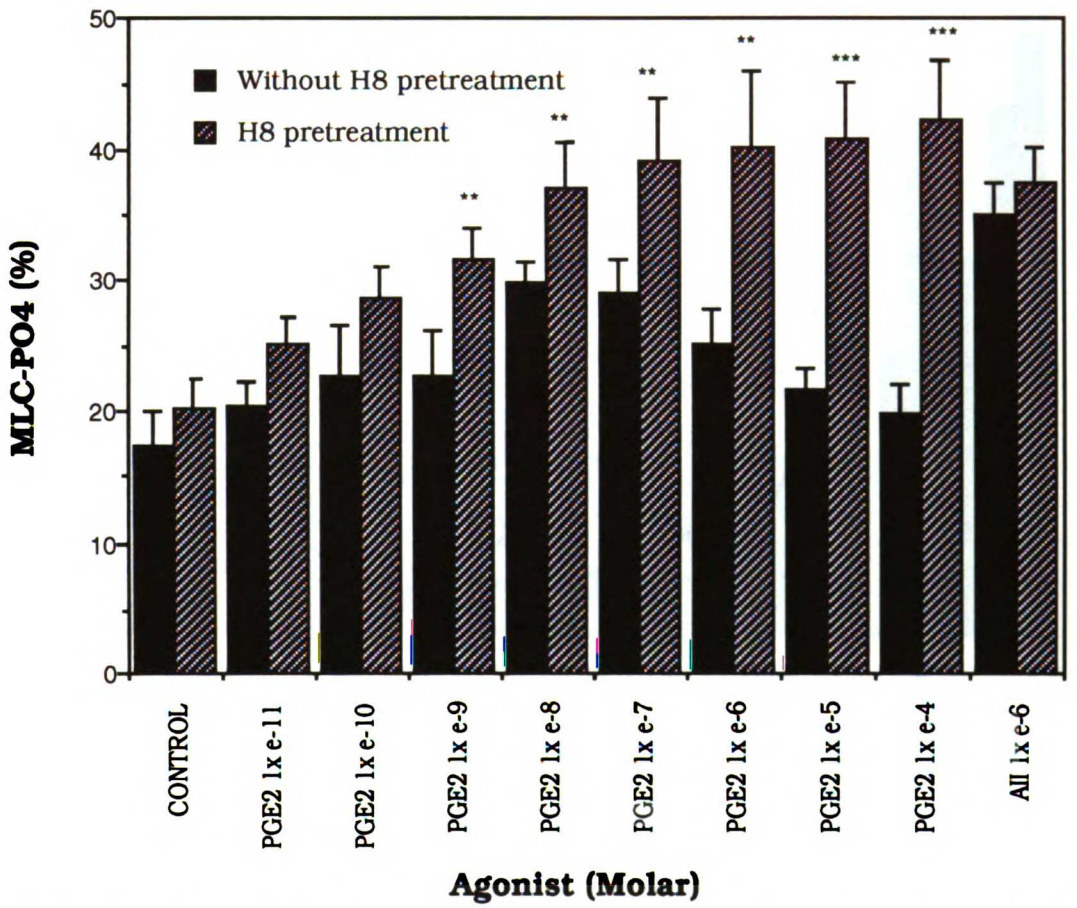


FIGURE 12: PGE2 stimulation: myosin light chain phosphorylation with H8 (1 μM) pretreatment. Myocytes were pretreated with or without H8 for 10 minutes and then exposed to different concentrations of PGE2 or 1 μM angiotensin II. No significant difference was found with H8 pretreatment in control or angiotensin II treated group ($P > 0.05$). Pretreatment with H8 significantly increased PGE2 stimulated myosin light chain phosphorylation at PGE2 concentrations 10^{-9} to 10^{-4} M (** $P < 0.05$, *** $P < 0.01$). Data are from 3 experiments done in triplicate. (AII = angiotensin II)

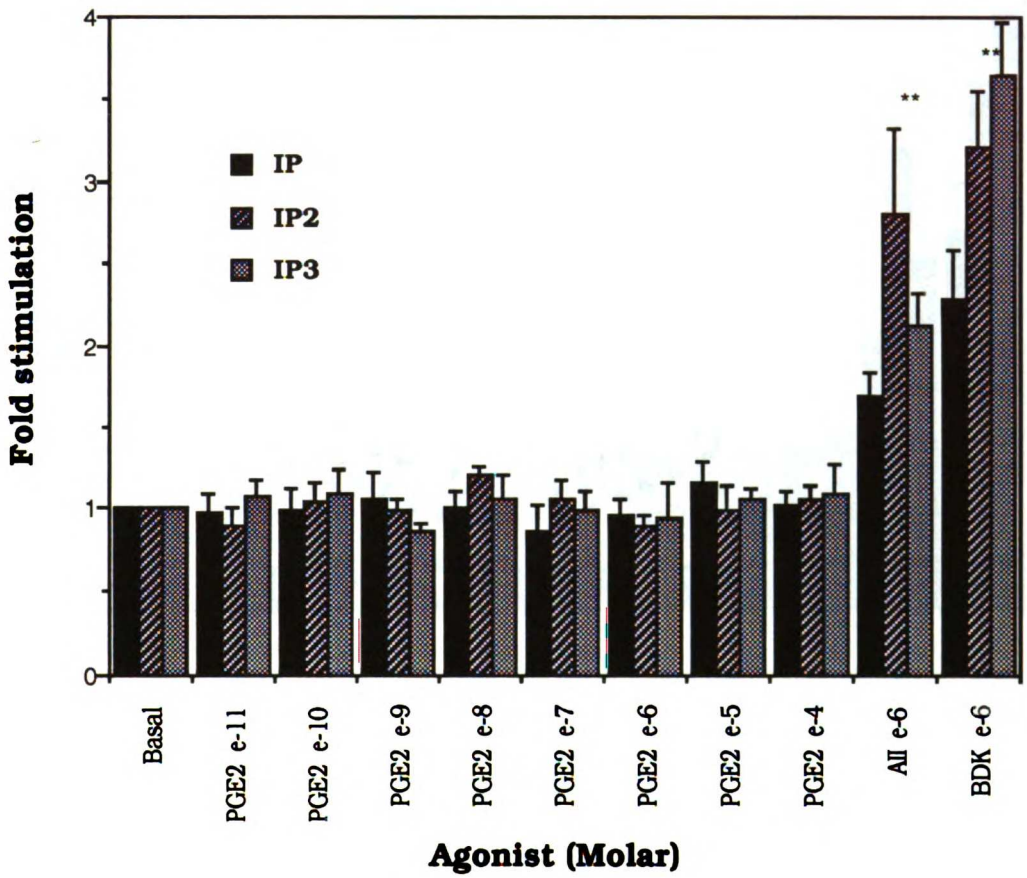


FIGURE 13: PGE2 stimulation: time course of inositol phosphates generation. Myocytes were radiolabelled with [^{3}H] inositol for 24 hours, pretreated with LiCl for 5 minutes and followed by 10 nM PGE2 for designated time or 1 μM angiotensin II for 15 minutes. Data are from 3 experiments in triplicate and expressed as fold stimulation of basal stimulation (Hank's medium). Statistical analysis was done by one-sided t test. No stimulation of IP generation was found in PGE2 treatment group at any time points tested (* $P > 0.05$). Significant IP generation was found in angiotensin treatment group (** $P < 0.0$ 1). Data are from 3 experiments done in triplicate. (AII = angiotensin)

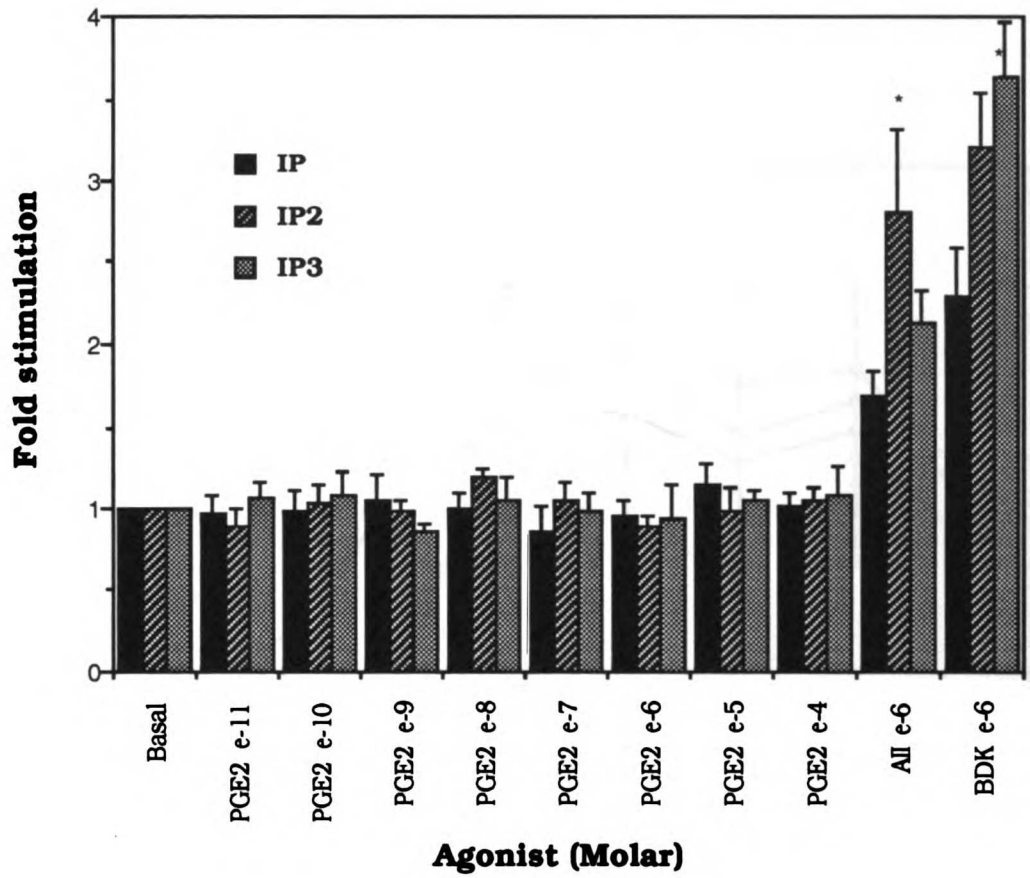


FIGURE 14: PGE2 stimulation: dose response of inositol phosphates generation. Myocytes were radiolabelled for 24 hours, pretreated with LiCl and exposed to prostaglandin E2, angiotensin II or bradykinin for 15 minutes. Data are from 3 experiments in triplicate. No significant IP generation was found in PGE2 treated group ($P > 0.05$, one tailed test). Significant IP generation was found in groups treated with angiotensin or bradykinin (* $P < 0.01$). Data are from 3 experiments done in triplicate. (AII = angiotensin II; BDK = bradykinin)

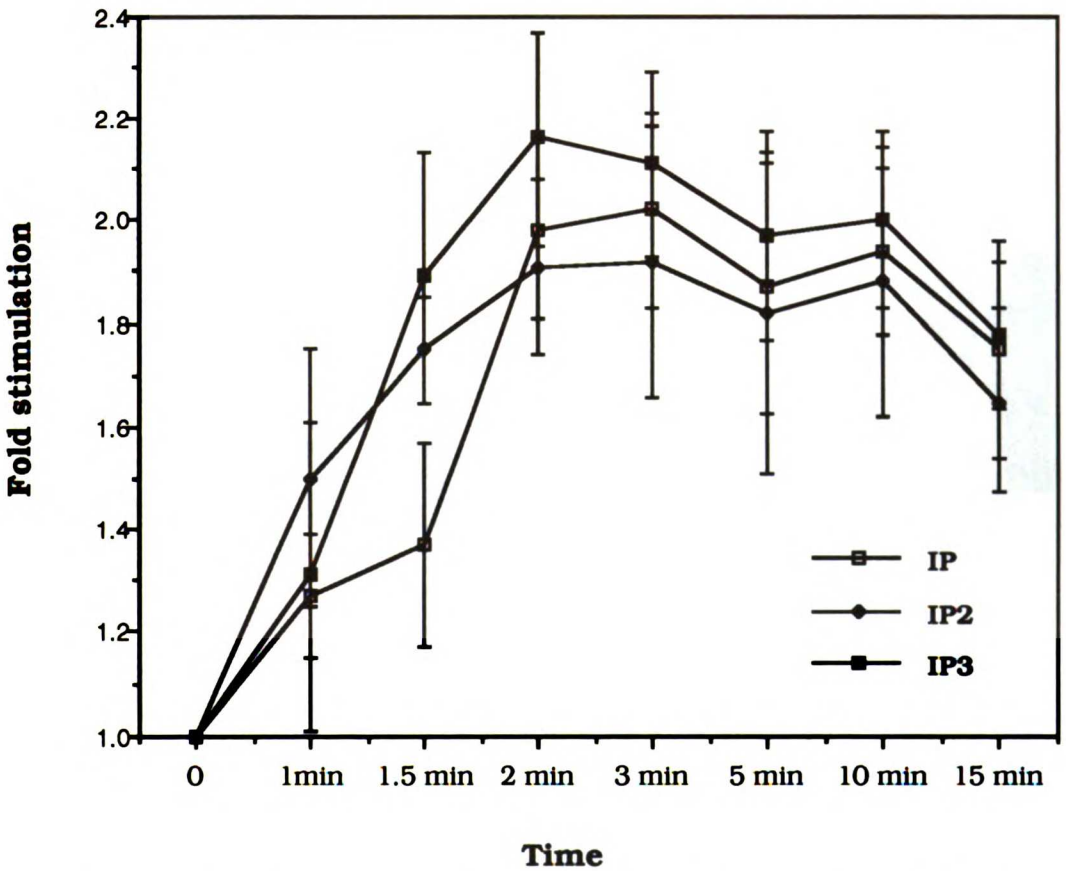


FIGURE 15: PGE_2 stimulation: time course of angiotensin II stimulation of inositol phosphate generation. Myocytes were radiolabelled with [^{3}H] inositol for 24 hours, pretreated with LiCl for 5 minutes and followed by 1 μM angiotensin II. Reactions were stopped at designated time points. More IP_3 was generated at 2 minutes than at 15 minutes ($P < 0.05$). Data are from 2 experiments conducted in triplicate.

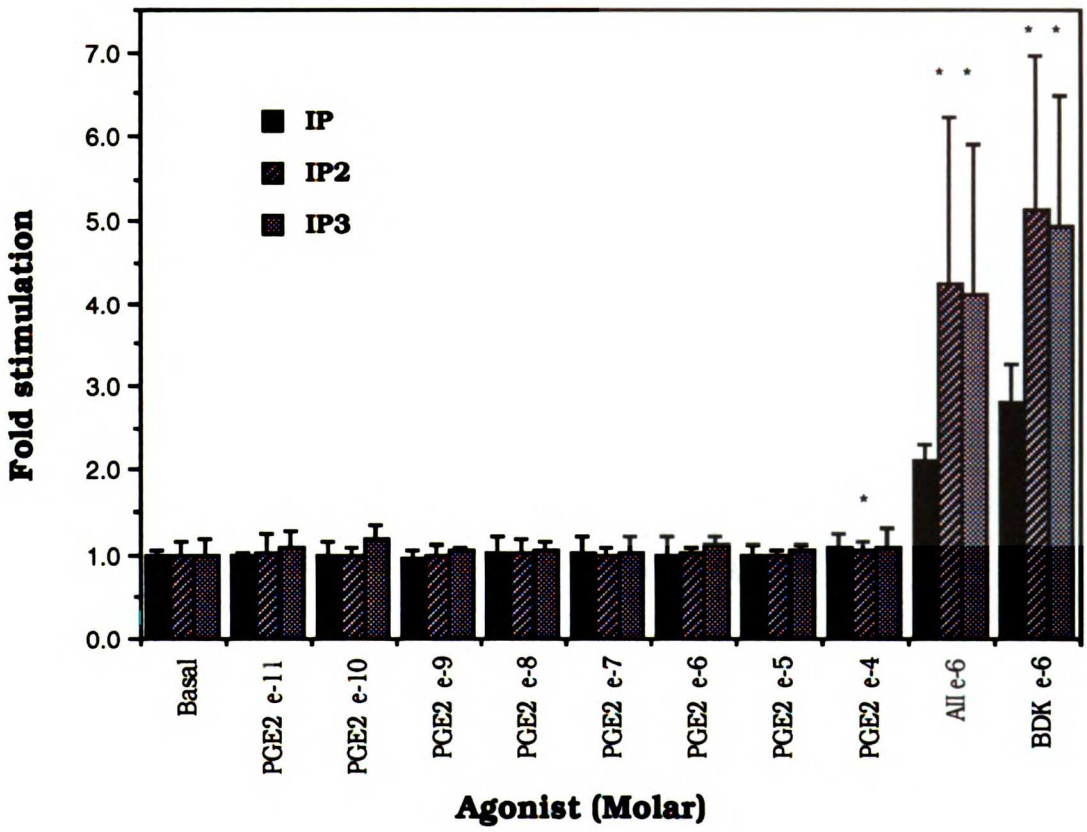


FIGURE 16: The effect of H8 pretreatment on inositol phosphates generation in response to prostaglandin E2. Myocytes were pretreated with 1 μM H8 for 10 minutes and LiCl. Then they were exposed to agonists for 2 minutes. No significant IP generation was found in PGE2 treated group ($P > 0.05$, one tailed test). Significant IP generation was found in groups treated with angiotensin II or bradykinin (** $P < 0.01$). Compared to the data without 1 μM H8 pretreatment, no significant difference was found in H8 pretreatment and AII or BDK treated group ($P > 0.05$). Data are from 3 experiments done in triplicate. (AII = angiotensin II; BDK = bradykinin)

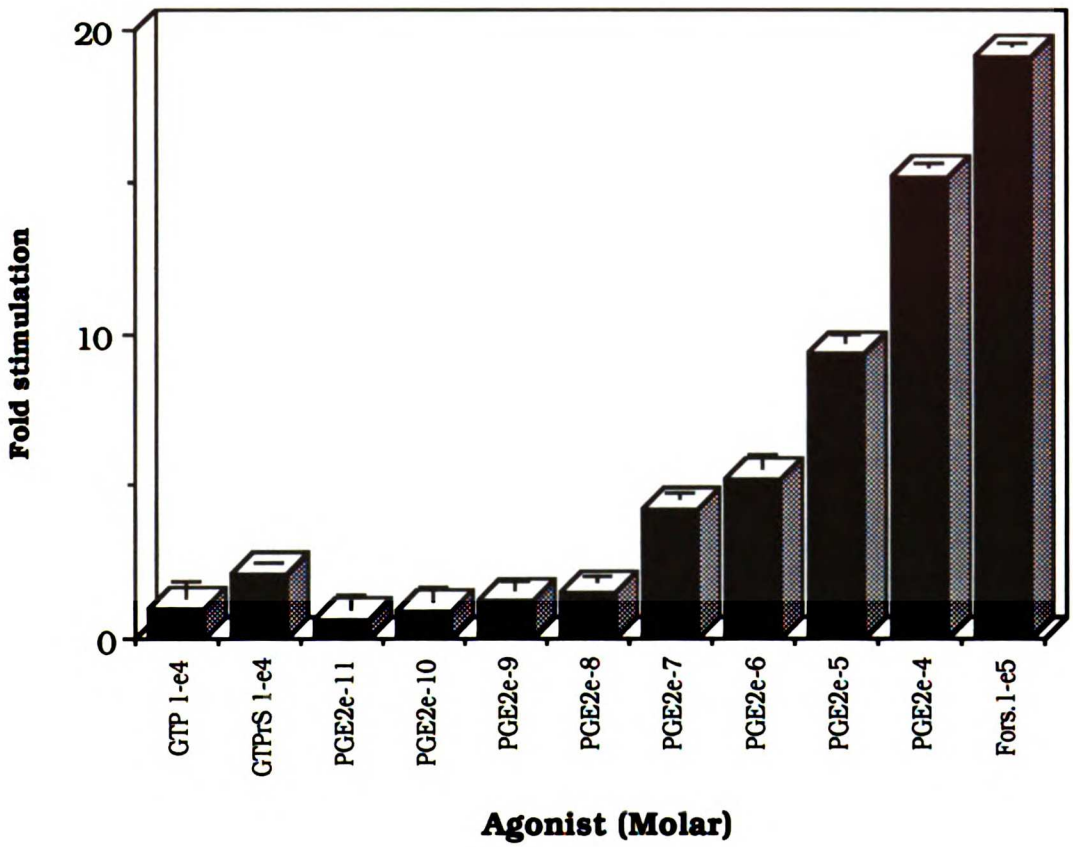


FIGURE 17: PGE_2 stimulation: dose response of PGE_2 to activate adenylyl cyclase. Myocytes were permeabilized with saponin 30 μg/ml for 10 minutes. They were exposed to 100 μM GTP, GTPγS, different concentrations of prostaglandin E_2 or 10 μM forskolin in reaction mixture prepared from permeabilization medium. Stimulation was stopped at 20 minutes with perchloric acid (0.33N). The adenylyl cyclase activity was measured as femtomole/min/well and expressed as fold stimulation of 100 μM GTP. Data are from 3 experiments conducted in duplicates. (Fors = forskolin)

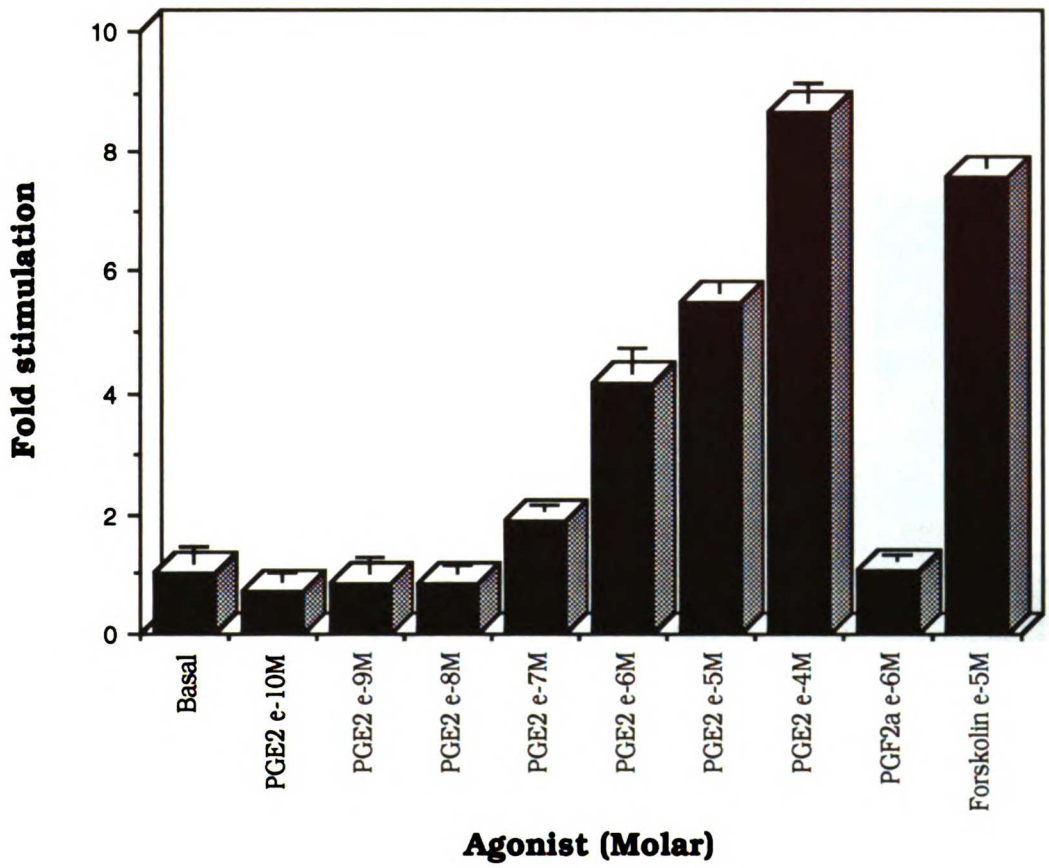


FIGURE 18: PGE2 stimulation: dose response of PGE2 to activate adenylyl cyclase in myocytes radiolabeled with [^{3}H]adenine. Myocytes were radiolabeled with [^{3}H]adenine for 3 hours before stimulation. Reaction was stopped at 20 minutes. The adenylyl cyclase activity was expressed as fold stimulation of basal stimulation (Hank's medium). Compared to the adenylyl cyclase activity in permeabilized cells, the fold stimulation in [^{3}H]adenine radiolabelled group was significantly lower ($P < 0.05$, PGF2α was excluded.). Data are from 2 experiments done in triplicate. (PGF2α = prostaglandin F2α)

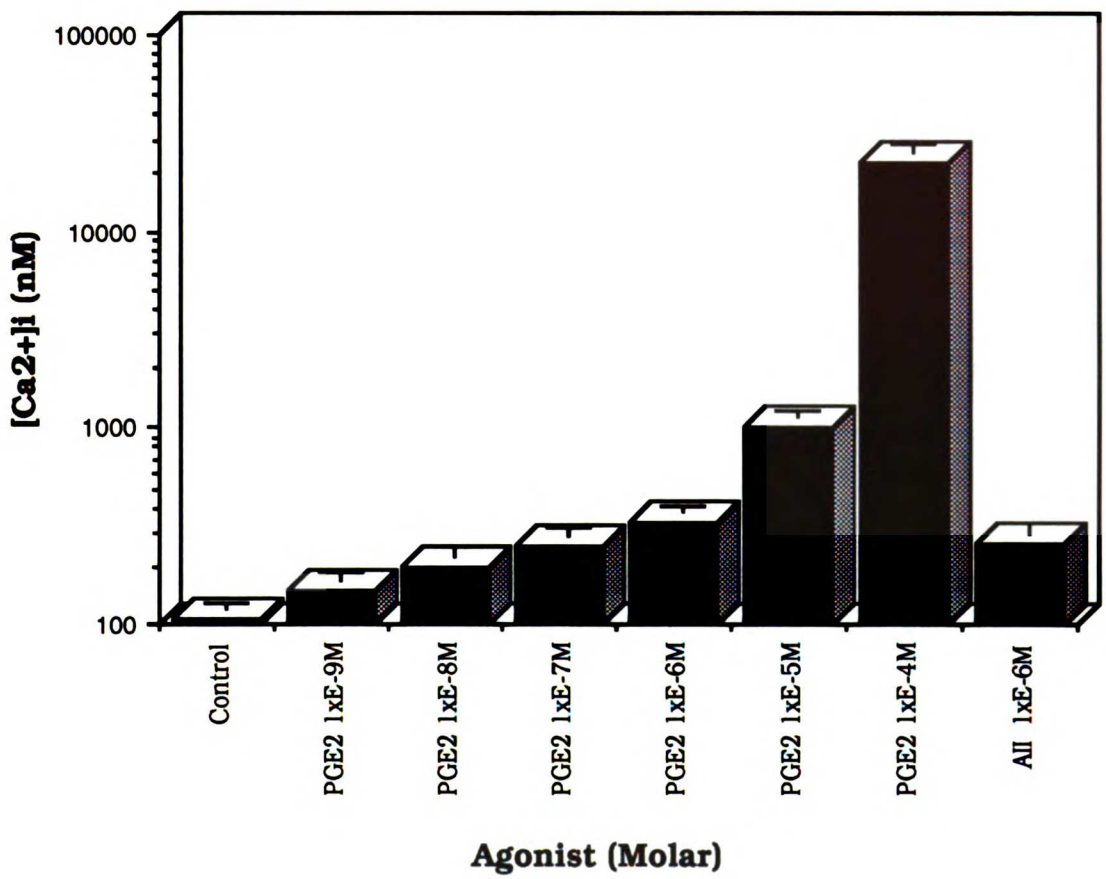


FIGURE 19: PGE2 stimulation: dose response of PGE2 to increase intracellular [Ca^{2+}]. Myocytes grown on coverslips were loaded with fura-2 for 1 hour at room temperature before experiment. Myocytes with good fura-2 loading were selected and framed for fluorescence signal recording. Stimulation was done at 37° C. The intracellular $[Ca^{2+}]_i$ was calculated according to the ratio method using the equation: $[Ca^{2+}]_i = KdS_{f380/b380}(R - R_{min})/(R_{max} - R)$, where Kd is 224 nM, $S_{f380/b380}$ is the ratio of the intensities of the free and bound dye forms at 380 nm, R is the fluorescence ratio (340 nm/380 nm) of the intracellular fura-2, and R_{min} and R_{max} are the minimal and maximal fluorescence. Data are the mean of 3 experiments. (AII = angiotensin II)

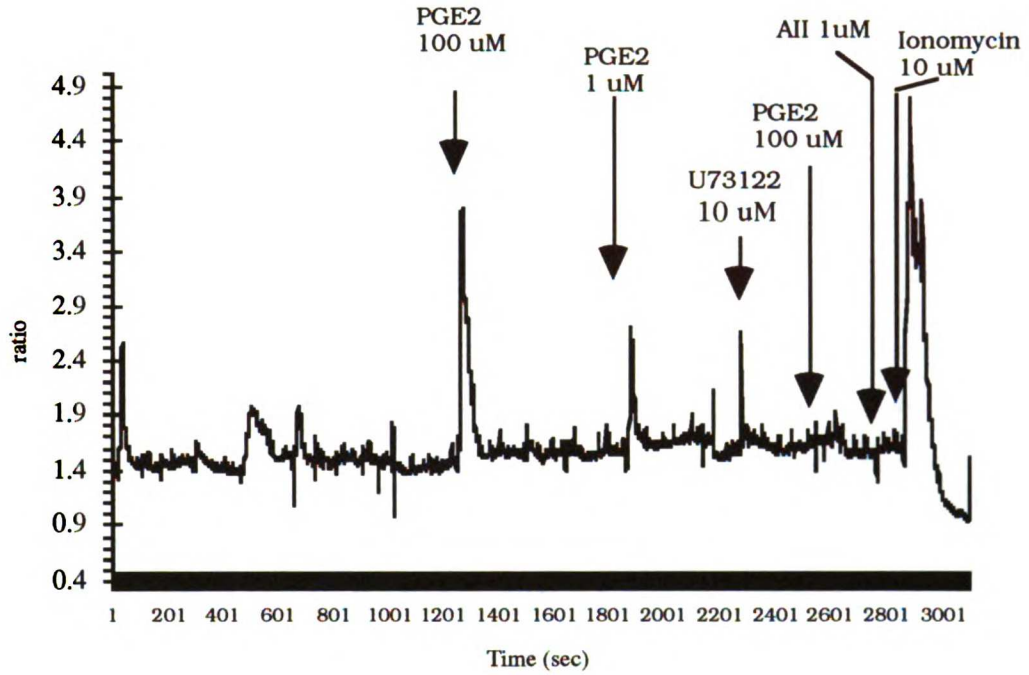


FIGURE 20: Inhibition of prostaglandin E2-stimulated increase of intracellular [Ca^{2+}] by phospholipase C inhibitor U73122. Myocytes were loaded with fura-2 for one hour before experiment. Washings were done until the fluorescence ratio was stable. Elevated ratios were found when cells were exposed to 1 nM or higher concentrations of prostaglandin E2 (not labeled in this figure). A marked elevation of ratio when exposed to 100 μM PGE2 and subsequently a moderate one to 1 μM PGE2 were marked in this figure. Then the myocytes were treated with 10 μM U73122. Ten minutes later, no response was found when myocytes were exposed to 100 μM PGE2 or later 1 μM angiotensin II. The responsiveness of myocytes from dose response experiments excluded the possibility that this inhibition is due to homologous desensitication. At the end of the experiment, maximal elevation of ratio was stimulated by 10 μM ionomycin and was reversed by addition of 25 mM EGTA. Representative of 2 experiments. (AII = angiotensin II)

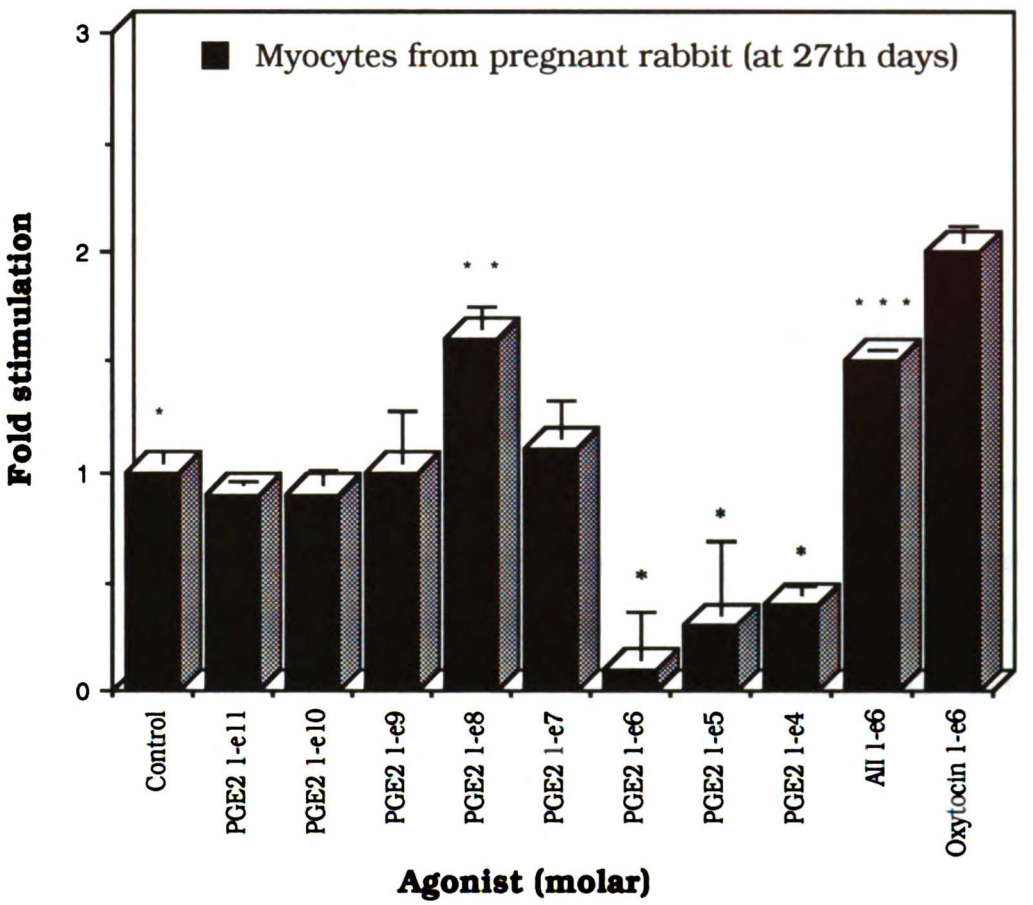


FIGURE 21 : PGE2 stimulation: myosin light chain phosphorylation dose response in pregnant rabbit uterus (27th day). Pregnant rabbit uteri were removed and myocytes dispersed. The myocytes were cultured in the same DME H-21 d-Valine medium for 3 days then in Q medium for one day. Cells were exposed to prostaglandin E2, angiotensin II or oxytocin. Reaction was stopped at 2 minutes. As compared to the data in estrogen treated non-pregnant uterus (the data in FIGURE 9), the basal myosin light chain phosphorylation was not significantly different (*P >0.05). However, in response to 10 nM PGE2, the percentage myosin light chain phosphorylation in myocytes from pregnant uterus was significantly less than that in those from estrogen treated nonpregnant uterus (** $P < 0.05$). As was the response to 1 μM angiotensin II (*** $P <$ 0.01). The response to high concentration of PGE2 (1 μM to 100 μM) in myocytes from pregnant uterus was much less than that of myocytes form estrogen treated nonpregnant uterus (* $P < 0.005$). Data are from two experiments done in triplicate.

Chapter 4 Discussion

4.1. Prostaglandin E_2 on rabbit uterine contraction

4.1.1. Biphasic response of PGE_2

4.1.1.1. Biphasic effect of prostaglandin E_2 on uterine contraction in estrogen treated rabbit

My experiments examining uterine contraction with PGE_2 confirmed the biphasic response previously reported by Bygdeman [Lundstrom, 1986]. The myosin light chain phosphorylation data with prostaglandin E_2 were consistent with this finding. There was a high percentage of myosin light chain phosphorylation at 10 nM PGE_2 and a lower percentage at concentrations of PGE_2 greater than 1 μM. Reduced MLC phosphorylation at PGE_2 concentrations of 100 μM corresponded to a 15 fold activation of adenylyl cyclase. This suggestion that cyclic AMP at high concentration might be inhibitory to MLC phosphorylation was supported by the finding that pretreatment with the protein kinase A inhibitor, H8, eliminated the biphasic response. This indicates that PGE_2 stimulates two signal transduction pathways with opposite effects on contraction. At low PGE_2 concentration, the stimulatory pathway predominates and results in a high percentage of myosin light chain phosphorylation and uterine contraction. Conversely, at high concentrations, the inhibitory pathway predominates, the percentage of MLC phosphorylation is lower and contractions cease. This also suggests that these two opposing pathways converge at the level of myosin light chain kinase or at proximal steps.

4.1.2. Ca^{2+} mobilization -- contractile pathway

Although the PGE_2 dose response for contraction and MLC phosphorylation were biphasic, at all concentrations prostaglandin E_2 increased intracellular $[Ca^{2+}]$. Thus the second message responsible for activating myosin light chain kinase to phosphorylate myosin light chains leading to muscle contraction increased with PGE_2 concentration. It did not appear that the effect of cAMP was to reduce $[Ca^{2+}]_i$. This favors the alternate mechanism suggested for cAMP, perhaps a direct effect on MLCK.

An unexpected finding was that PGE_2 did not stimulate 1,4,5-inositol triphosphate generation, usually responsible for calcium mobilization. This raised the question as to what was the message via which PGE_2 increased intracellular $[Ca^{2+}]$.

4.1.2.1. Messenger for Ca^{2+} mobilization

From the literature, intracellular $[Ca^{2+}]$ increases due to release of calcium from intracellular calcium stores or extracellular calcium entry through membrane calcium channels. In this study, 1,4,5-IP_3, the usual trigger for release of intracellular store, was not detectable at concentrations of PGE_2 which increased $[Ca^{2+}]_i$. Phosphorylation of myosin light chain by PGE_2 was not completely blocked by depletion of extracellular Ca^{2+}. The observation that the PLC inhibitor U73122 inhibited elevation of $[Ca^{2+}]_i$ and prevented MLC phosphorylation suggests a role for PLC on PGE_2 effect on uterine contraction.

4.1.2.1.1. Possible role of calcium channels in PGE2-stimulated myosin light chain phosphorylation

If PGE2 increased $[Ca^{2+}]_i$ by calcium entry through calcium channels, its effect on increasing MLC phosphorylation would be blocked when the extracellular calcium source was depleted. In my study, the stimulatory effect persisted in the absence of extracellular calcium. In human uterine myocytes, oxytocin, which induced IP3 generation and increased $[Ca^{2+}]_i$, had the same potency in the absence of extracellular Ca^{2+}, although the extent of increase of $[Ca^{2+}]_i$ was markedly reduced. However, for PGF2α, which increased $[Ca^{2+}]_i$ by opening calcium channels, depletion of extracellular calcium abolished the increase of $[Ca^{2+}]_i$. Thus the hypothesis that PGE2 opens calcium channels on membranes to increase intracellular $[Ca^{2+}]$ is not consistent with our findings.

4.1.2.1.2. Role of cyclic AMP to mediate PGE2-stimulated MLC-PO4

The stimulation of adenylyl cyclase activity by PGE2 raised the possibility that cyclic AMP may mediate increased $[Ca^{2+}]_i$ [Kelley, 1990]. In cardiac myocytes, cyclic AMP opens calcium channels [Bean, 1989]. In rabbit aorta, there is a report that forskolin and caffeine induce Ca^{2+} release from intracellular stores [Hai, 1989]. If this did occur in rabbit uterine myocytes, cyclic AMP dependent protein kinase inhibitor H8 would block calcium entry, inhibit increased $[Ca^{2+}]_i$ and thus prevent myosin light chain phosphorylation. However my finding that the cyclic AMP dependent protein kinase inhibitor, H8, increased MLC-PO4, was not consistent with this hypothesis.

4.1.2.1.3. The possible role of PLC

4.1.2.1.3.1. PLC inhibitor -- U73122

U73122, a steroidal amine, was a phospholipase C inhibitor used in recent years. It inhibits hydrolysis of [^{3}H]phosphatidylinositol in human amnion cells [Bleasdale, 1989]. Unlike other steroidal amines, it is not an inhibitor of phospholipase A2 enzymes. Although the specificity of U73122 for PLC is not yet extensively tested, there is report to support this [Yule, 1992]. Yule *et al.* reported that U73122 is a relatively specific inhibitor of G-protein-mediated phospholipase C and it does not involve an interference of Ca^{2+} metabolism.

In this study, pharmacological inhibitors were used to study the signal transduction pathway. The conclusion I reached from those data with inhibitors based on the relative specificity for the target enzymes within the inhibitor concentrations used.

4.1.2.1.3.2. U73122 blocked Ca^{2+} mobilization and increase of MLC-PO4

The possible role of PLC to mediate increased $[Ca^{2+}]_i$ was supported by my finding that U71322, a putative PLC inhibitor, prevented both prostaglandin E2-elicited increase of intracellular $[Ca^{2+}]$ and MLC phosphorylation [Bleasdale, 1989].

4.1.2.1.3.3. No detectable increase of IP3

Despite extensive efforts (different time, temperature, etc.), I could detect no PGE2 effect on IP3 accumulation. If PGE2 did elicit increase of

$[Ca^{2+}]_i$ via 1,4,5-IP3 in rabbit uterine myocytes, the dose response of 1,4,5-IP3 to increase $[Ca^{2+}]_i$ is different from that of most agonists. Based on my findings, PGE2 would only require a non-detectable increase of IP3 to elicit a $[Ca^{2+}]_i$ increase response similar to that in other systems with other agonists for which IP3 generation is measurable. This is opposite to the usual reported finding in which the concentration of agonist necessary to cause contraction is frequently less than one-tenth that required for a comparable effect of IP3 generation [Fain, 1979; Marc, 1986; Howe, 1986; Best, 1985]. However, Babich *et al.* reported that parathyroid hormone increased intracellular $[Ca^{2+}]_i$ without detectable IP generation in rat osteosarcoma cell [Babich, 1991]. Yule *et al.* reported that stimulation of rat pancreatic acinar cells with low concentrations of phosphatidylinositol-linked secretagogues induced $[Ca^{2+}]_i$ oscillations, without measurable changes in the formation of inositol 1,4,5-trisphosphate [Yule, 1992]. The following are two possible interpretations for the non parallel relationship between the potencies for alternation of $[Ca^{2+}]_i$ and for the generation of IP3.

4.1.2.1.3.3.1. Spatial or compartmental uniqueness of phosphoinositide [Babich, 1991]

The unique PI compartment, which couples to PGE2 stimulation, elicits a very effective $[Ca^{2+}]_i$ increase, e.g., it is closely associated with the calcium pool (calciosome), while this IP3 generation is not detectable by the conventional method of IP3 measurement [Parker, 1990]. However, it still responds in a dose dependent fashion.

4.1.2.1.3.3.2. Sensitive calcium pool

The other explanation for an undetectable IP_3 generation to induce an intracellular $[Ca^{2+}]$ increase is that the intracellular calcium pools that respond to PGE_2 stimulation are unusually sensitive to IP_3, hence a small amount of IP_3 generation, which is not detectable by the method currently used in this study, can cause a calcium mobilization. However, this is not consistent with the general concept of Ca^{2+} release which occurs in a conventional dose response fashion [Babich, 1991; Irvine, 1990; Muallem, 1989].

4.1.2.1.3.3.3. Inositol phosphate generation was inhibited due to crosstalk inhibition by cyclic AMP.

Pathway crosstalk has been shown in signal transduction systems [Fain, 1988; Bianca, 1986; Watson, 1984]. A third possibility would be that inositol phosphate generation could not be detected after exposing myocytes to PGE_2 because of inhibition by cyclic AMP. Contrary to this, I found that pretreatment with cyclic AMP dependent protein kinase inhibitor H8 increased myosin light chain phosphorylation, and did not unmask inositol phosphate generation. This excluded the possibility that failure to detect IP generation was because inositol phosphates generation was inhibited by cyclic AMP concomitantly generated by PGE_2.

4.1.2.1.3.4. PLC mediates other second messenger to mobilize $[Ca^{2+}]_i$.

An alternative interpretation of my finding that PGE_2 does not cause measurable IP_3 generation yet a PLC inhibitor abolishes the PGE_2-elicited increase of $[Ca^{2+}]_i$, is that PLC generates another second messenger that releases $[Ca^{2+}]_i$ from the IP_3-independent calcium pool. The support for this hypothesis comes from the observation that less than half of the releasable non-mitochondrial calcium store is mobilized by 1,4,5 -IP_3 in other systems [Babich, 1991].

4.1. 2. 2. Proposed test in the future

4.1. 2. 2.1. Measure PLC activity in permeabilized cell

In future experiments a more sensitive assay for PGE_2 stimulated IP3 release would be used. It is possible that PGE_2 activated PLC activity determinators in permeabilized cells and would be more sensitive. I would permeabilize cells and exposed them to radiolabelled PIP_2 and then treat with PGE_2. Increased radiolabeled inositol phosphates or 1,2-diacylglycerol signal (compared to control) would indicate increased PLC activity by PGE_2 activation.

4.1.2.2.2. Other PLC target (phosphatidylcholine)

Another explanation for the ability PGE_2 to activate PLC without measurable IP_3 is that PLC hydrolyzes phosphatidylcholine (PC) rather than phosphoinositide phosphate to generate phosphocholine and diacylglycerol which mediates the signal [Exton, 1990]. In rat tail arterial smooth muscle, Gu *et al.* reported that norepinephrine stimulated phosphatidylcholine hydrolysis by phospholipase C and phospholipase D

(PLD) [Gu, 1992]. Also, PC hydrolysis can generate much more DAG than PIP_2 hydrolysis because there is at least 40-fold more PC in plasma membrane than there is PIP_2 [Gu, 1992]. Since in this pathway kinase-C is the purported effector, blockade of this kinase would test this possibility.

4.1.2. 2.3. Phospholipase D (PLD) activity

It is also possible that PGE_2 mobilize calcium ion through PLD, which generates phosphatidic acid (PA). There are reports that PA mobilizes intracellular Ca^{2+} [Murayama, 1985; Moolenaar, 1986; Gu, 1992]. Phosphatidic acid can be dephosphorylated to form DAG, which can stimulate protein kinase C activity. Although not as clearly established, it is also likely that PLD effects are transduced by DAG, activator of kinase-C. Kinase-C blockade would also be an initial test of this pathway.

4.1.3. Adenylyl cyclase stimulation -- relaxation pathway

4.1.3.1. Adenylyl cyclase activity for relaxation and protein kinase A inhibitor (Hs)

Adenylyl cyclase activity may not be the only pathway responsible for uterine relaxation. However, my findings that kinase A inhibitor Hs restored the arrested contraction of muscle strips by 100 μM PGE_2, increased myosin light chain phosphorylation response to PGE_2 and reversed the inhibitory phase of biphasic response clearly demonstrate that adenylyl cyclase activity mediated rabbit uterine smooth muscle

relaxation. Other pathways in addition to this are of course still be possible, e.g., cyclic GMP pathway.

Although H8 is not specific for protein kinase A [Hidaka, 1984], the data from my experiments did not show its inhibition on myosin light chain kinase with the concentrations I used in the experiments.

Likewise although we cannot exclude inhibition of protein kinase C as part of the effect of H8, the fact that it inhibited forskolin effect indicates it did block protein kinase A.

4.1.3.1.1. Kinase A inhibitor -- important role in studying the role of cyclic AMP

In biological system, different types of cells may share the same basic signalling mechanism to effect different biological response. Although correlation of signal generation to biological response is important in studying signal transduction of effectors, the definite proof comes from blockade of that signal pathway and the resulting loss of biological function [Gustafson, 1983; Hoffman, 1983; Babich, 1991]. I have found H8 to be very useful in that regard.

4.1.3.1.2. Cell permeability offers a sensitive adenylyl cyclase assay.

For many years, the role of adenylyl cyclase activity on uterine relaxation has been controversial because of inconsistent findings. One report claims no detectable cyclic AMP generation was measured when uterine smooth muscle was relaxed by exposure to some tocolytic agonists [Polacek, 1971]. The adenylyl cyclase assay on permeabilized

cells increases the sensitivity to determine adenylyl cyclase activity. Another advantage of adenylyl cyclase activity assay on permeabilized cells is that it allows manipulations of the receptor-guanine nucleotide binding protein-adenylyl cyclase system without losing the sensitivity of the assay. This increased sensitivity also allows the assay to be done in a small amount of cells or tissue.

4.1.3.2. The possibility of cyclic AMP compartmentalization

There are other reports of inconsistency of cyclic AMP generation and smooth muscle relaxation which indicate smooth muscle relaxation without detectable cyclic AMP or contraction in the setting of elevated cyclic AMP level [Verma, 1976; Diamond, 1975]. Hei reported that dibutyryl cyclic AMP but not 8-bromo cyclic AMP inhibited smooth muscle contraction of rat vas deferens -- a disturbing differential effect of cyclic AMP analogs [Hei, 1991]. If no error was involved in measurement of cyclic AMP level, one possible interpretation of the above observations is that there exist different compartments of cyclic AMP which couple to different cellular function or different compartment of cyclic AMP with different efficacy to elicit cellular function.

4.2. Signal pathway crosstalk

4.2.1. Importance of a functional assay to study agonist-stimulated signal transduction

4.2.1.1. MLC phosphorylation proved to be a useful guide to study muscle contraction in uterine myocytes.

The myosin light chain phosphorylation assay is technically demanding, however, it proved invaluable in my study. In previous studies, it had been used in the study of tonic and phasic smooth muscle contraction. The concern about its usefulness came from the observation that in tetanic contraction (e.g., in tonic vascular smooth muscle), myosin light chain phosphorylation decreased without losing contraction force. However, in phasic smooth muscle such as uterine smooth muscle, Janis reported that myosin light chain phosphorylation is a useful indicator of uterine smooth muscle contraction [Janis, 1980].

4.2.1.2. MLC phosphorylation proved to be a useful guide to Ca^{2+} pathway and cyclic AMP pathway crosstalk.

Although activation of calmodulin-myosin light chain kinase and myosin light chain phosphorylation pathway was demonstrated to be both necessary and sufficient to trigger contraction of smooth muscle [Itoh, 1989], it has not previously been reported as a functional assay to study signal transduction. In my study, the Ca^{2+}-contraction pathway and the adenylyl cyclase - relaxation pathway interacted and the net response was integrated as myosin light chain phosphorylation in response to PGE_2 stimulation. With downstream pathway crosstalk, the interaction would not be detected without a downstream functional assay. Also, in signal transduction study, without a functional assay for agonist stimulation, it would be difficult to be sure the functional role of a second message generated by the agonist. This need for a functional assay will be even more compelling if compartmental specificity of signal is considered.

4.3. Conclusion and summary

4.3.1. Conclusion

Prostaglandin E2 mobilized intracellular calcium ion stores in rabbit uterine myocytes. This increased intracellular Ca^{2+} bound with calmodulin and activated myosin light chain kinase, which resulted in myosin light chain phosphorylation. Phosphorylation of myosin light chain activated actinomyosin ATPase which allowed actin to bind with myosin and triggered a muscle contraction. Myosin light chain phosphorylation was a useful indicator of muscle contraction in cultured myocytes. The increase of intracellular [Ca^{2+}] after exposure to prostaglandin E2 was not through opening of calcium channels. No detectable inositol phosphates generation was found even if myocytes were exposed to 100 μM PGE2, which massively increased intracellular [Ca^{2+}], which I propose was most likely due to mobilizing intracellular calcium stores. The observation that U73122, a putative phospholipase C inhibitor, inhibited PGE2 induced increase of intracellular [Ca^{2+}] and PGE2 stimulated myosin light chain phosphorylation suggested a role for phospholipase C. At high concentrations, PGE2 activated adenylyl cyclase, which I propose decreased myosin light chain phosphorylation and inhibited muscle strip contraction. This inhibition of both myosin light chain phosphorylation and muscle strip contraction at high PGE2 concentration was reversed with pretreatment with the cyclic AMP dependent protein kinase inhibitor, H8. H8 resulted in resumption of muscle contraction and increased MLC phosphorylation. Nevertheless, pretreatment with H8, did not result in detectable inositol phosphate

generation. This indicated that failure to detect IP generation was not due to inhibition via pathway crosstalk by cyclic AMP.

4.3.2. Summary

In summary, in uterine myocytes from rabbits treated with estradiol, prostaglandin E_2, at low concentration (100 pM to 10 nM) stimulated myosin light chain phosphorylation and muscle strip contraction. This contraction stimulation became attenuated as PGE_2 concentration further increased, which I propose is due to the inhibition by PGE_2 stimulated adenylyl cyclase activity. This same effect resulted in inhibition of contraction at 100 μM PGE_2 in intact uterine strips. The integration of these two different pathways leading to 2 opposite biological functions explained the biphasic response paradox of rabbit uterine smooth muscle contraction in response to prostaglandin E_2 stimulation.

4.4. Speculation

4.4.1. PGE_2 receptor subtype

Dong *et al.* reported in binding studies of smooth muscle preparations that there might be two subtypes of PGE receptors [Dong, 1986]. The different pathways coupled to PGE_2 stimulation, further suggest different PGE_2 receptors subtypes coupled to different signal pathway.

4.4. 2. Hormonal modulation of uterine response

Uterine responses to agonists are affected by its hormonal status and uterine contractile response to agonist stimulation differed at different stages of estrous cycle [Martin, 1975]. The understanding of the pathway crosstalk in the mechanism of action by which PGE2 affects rabbit uterine contraction allows us to speculate that this hormone modulation might act through different signal transduction pathways to effect a different contractile response in different hormonal status [Riemer, 1987].

4.5. Future direction

1) The origin of increased intracellular Ca^{2+} by PGE2 stimulation should be sought for.

This would include the role of PKC, PLD and possible role of phosphatidylcholine hydrolysis. Experiments would include

(a) measurement of DAG

(b) assay of PKC activity

(c) assay of PLD activity

(d) blockade of PKC

2) PGE2 receptor classification and search for selective antagonists

3) The possible role of cyclic GMP pathway in mediating PGE2 effect on uterine smooth muscle.

Bibliography

Abdel-Latif, A. A. (1986). Calcium-mobilizing receptors, polyphosphoinositides, and the generation of second messengers. Pharmacol. Rev., 38, 227-272.

Adelstein, R. S., Conti, M. A., Hathaway, D. R., & Klee, C. B. (1978). Phosphorylation of smooth muscle myosin light chain kinase by the catalytic subunit of adenosine 3' : 5' -monophosphate-dependent protein kinase. J. Biol. Chem., 253, 8347-8350.

Adelstein, R. S., & Eisenberg, J. (1980). Regulation and kinetics of the actin-myosin-ATP interaction. Annu. Rev. Biochem., 49, 921-956.

Anwer, K., Hovington, J. A., & Sanborn, B. M. (1989). Antagonism of contractants and relaxants at the level of intracellular calcium and phosphoinositide turnover in the rat uterus. Endocrinology, 124(6), 2995-3002.

Ashton, F. T., Somlyo, A. V., & Somlyo, A. P. (1975). The contractile apparatus of vascular smooth muscle: Intermediate high voltage stereo electron microscopy. J. Mol. Biol., 98, 17-29.

Babich, M., Choi, H., Johnson, R. M., King, K. L., Alford, G. E., & Nissenson, R. A. (1991). Thrombin and parathyroid hormone mobilize intracellular calcium in rat osteosarcoma cells by distinct pathways. Endocrinology, 129(3), 1463-1470.

Bagby, R. M. (1983). Organization of contractile/cytoskeletal elements, in Biochemistry of Smooth Muscle, Vol. I (N. L. Stephens, ed.), CRC Press, Boca Raton, FL, pp. 1-85.

Bean, B. P. (1989). Multiple types of calcium channels in heart muscle and neurons. Modulation by drugs and neurotransmitters. Ann NY Acad Sci, 560, 334-345.

Berg, G., Anderson, R. G. G., & Ryden, G. (1986). α–adrenergic receptors in human myometrium during pregnancy. Am. J. Obstet. Gynecol., 154, 601-606.

Berridge, M. J. (1983). Rapid accumulation of inositol triphosphate reveals that agonists hydrolyze polyphosphoinositides instead of phosphatidylinositol. Biochem. J., 212, 849-858.

Berridge, M. J., & Irvine, R. (1989). Inositol phosphates and cell signaling. Nature, 341, 197-205.

Best, L., Brooks, K. J., & Bolton, T. B. (1985). Relationship between stimulated inositol lipid hydrolysis and contractility in guinea-pig visceral longitudinal smooth muscle. Biochem. Pharmacol., 34, 2297-2301.

Bianca, V. D., Togni, P. D., Grzeskowiak, M., Vicentini, L. M., & Di Virgilio, F. (1986). Cyclic AMP inhibition of phosphoinositide turnover in human neutrophils. Biochem. Biophys. Acta., 886, 441-447.

Bleasdale, J. E., Bundy, G. L., Bunting, S., Fitzpartick, F. A., Huff, R. M., Sun, F. F., & Pike, J. E. (1989). Inhibition of phospholipase C dependent processes by U-73122. Advances in prostaglandin, thromboxane, and leukotriene Research, 19, 590-593.

Bokoch, G. M., & Gilman, A. G. (1984). Inhibition of receptor-mediated release of arachidonic acid by pertussis toxin. Cell, 39, 301-308.

Bond, M., Kitazawa, T., Somlyo, A. P., & Somlyo, A. V. (1984). Release and recycle of calcium by the sarcoplasmic reticulum in guinea-pig vein smooth muscle. J. Physio. (London), 355, 677-695.

Bottari, S. P., Vokaer, A., Kaivez, E., Lescrainier, J. P., & Vauquelin, G. (1985). Regulation of alpha- and beta- adrenergic receptor subclasses by gonadal steroids in human myometrium. Acta Physiol. Hung., 65, 335-346.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72, 248-254.

Carsten, M. E. (1973). Prostaglandins and cellular transport in the pregnant human uterus. Am. J. Obstet. Gyneocl., 117, 824-832.

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., & Nishizuka, Y. (1982). Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J. Biol. Chem., 257, 7847-7851.

Chacko, S., Conti, M. A., & Aldelstein, R. S. (1977). Effect of phosphorylation of smooth muscle myosin on actin activation and Ca^{2+} regulation. Proc. Natl. Acad. Sci. U.S.A., 74, 129-133.

Chacko, S. (1981). Effects of phosphorylation, calcium ion, and tropomyosin on actin-activated adenosine 5'-triphosphatase activity of mammalian smooth muscle myosin. Biochemistry, 20, 702-707.

Chan, W. Y. (1977). Relationship between the uterotonic action of oxytocin and prostaglandins: oxytocin action and release of PG-activity in isolated nonpregnant and pregnant rat uteri. Biol. Repro., 17, 541-548.

Chatterjee, M., & Tejada, M. (1986). Phorbol ester-induced contraction in chemically skinned vascular smooth muscle. Am. J. Physiol., 251, C356-C361.

Chow, E. H. M., & Marshall, J. M. (1981). Effects of catecholamines on circular and longitudinal muscle of the rat. Eur. J. Pharmacol., 76, 157-165.

Cohen, D. M., & Murphy, R. A. (1979). Cellular thin filament protein contents and force generation in porcine arteries and veins. Circ. Res., 45, 661-665.

Conti, M. A., & Adelstein, R. S. (1981). The relationship between calmodulin binding and phosphorylation of smooth muscle myosin kinase by the subunit of 3':5' cAMP-dependent protein kinase. J. Biol. Chem., 256, 3178-3181.

Csapo, A. I., Puri, C. P., Tarro, S., & Henzl, M. R. (1982). Deactivation of the uterus during normal and premature labor by the calcium antagonist nicardipine. Am. J. Obstet. Gynecol., 142, 483-491.

Dabrowska, R., Hinkins, S., & Walsh, M. P. (1982). The binding of smooth muscle myosin light chain kinase to actin. Biochem. Biophys. Res. Commun., 107, 1524-1531.

Daimond, J. (1983). Lack of correlation between cyclic GMP elevation and relaxation of nonvascular smooth muscle by nitroglycerin, nitroprusside, hydroxylamine and sodium azide. J. Pharmacol. Exp. Ther., 225, 422-426.

Dale, M. M., & Obianime, W. (1985). Phorbol myristate acetate causes in guinea-pig lung parenchyma strip a maintained spasm which is relatively resistant to isoprenaline. FEBS Lett., 190, 6-10.

Danthuluri, N. R., & Deth, R. C. (1984). Phorbol ester-induced contraction of arterial smooth muscle and inhibition of α-adrenergic response. Biochem. Biophys. Res. Commun., 125, 1103-1109.

Diamond, J., & Hartle, D. K. (1974). Cyclic nucleotide levels during spontaneous uterine contractions. Can. J. Physiol. Pharmacol., 52, 763-767.

Diamond, J., & Holmes, T. G. (1975). Effects of potassium chloride and smooth muscle relaxant on tension and cyclic nucleotides levels in rat myometrium. Can. J. Physiol. Pharmacol., 53, 1099-1107.

Diamond, J., & Holmes, T. G. (1975). Effects of potassium nitroprusside chloride and smooth muscle on tension and cyclic nucleotide levels in rat myometrium. Can. J. Physiol. Pharmacol., 53, 1099-1107.

DoKhac, L., Mokhtari, A., & Harbon, S. (1986). A re-evaluated role for cyclic AMP in uterine relaxation. Differential effect of isoproterenol and forskolin. J. Pharmacol. Exp. Ther., 239, 236-242.

Dong, J., Jones, R. L., & Wilson, N. H. (1986). Prostaglandin E receptor subtypes in smooth muscle: agonist activities of stable prostacyclin analogues. Br. J. Pharmac., 87, 97-107.

Doyle, V. M., & Ruegg, U. T. (1985). Vasopressin induced production of inositol trisphosphate and calcium influx in a smooth muscle cell line. Biochem. Biophys. Res. Commun., 131, 469-476.

Exton, J. H. (1990). Signaling through phosphatidylcholine breakdown. J. Biol. Chem., 265(1), 1-4.

Fain, J. N., & Berridge, M. J. (1979). Relationship between hormonal activation of phosphatidylinositol hydrolysis, fluid secretion, and calcium flux in the blowfly salivary gland. Biochem. J., 178, 45-58.

Fain, J. N., Wallace, M. A., & Wojickiewicz, R. J. H. (1988). Evidence for involvement of guanine nucleotide-binding regulatory proteins in the activations of phospholipases by hormones. FASEB, J 2, 2569-2574.

Fatigati, V., & Murphy, R. A. (1984). Actin and tropomyosin variants in smooth muscles. Dependence on tissue type. J. Biol. Chem., 259, 14383-14388.

Forder, J., Scriabine, A., & Rasmussen, H. (1985). Plasma membrane calcium flux, protein kinase C activation and smooth muscle contraction. J. Pharmacol. Exp. Ther., 235, 267-273.

Foyt, H. L., Guerriero, V. J., & Menas, A. R. (1985). Functional domains of chicken gizzard myosin light chain kinase. J. Biol. Chem., 260, 7765-7774.

Garfield, R. E., & Somlyo, A. P. (1985). Structure of smooth muscle, in Calcium and contractility (A. K. Grower, ed.) Futura Press, pp 1-36.

Godfraind, T. (1986). Calcium antagonism and calcium entry blockade. Pharmacol. Rev., 38, 321-416.

Graham, J. P. D., & Gurd, M. R. (1960). Effects of adrenaline on the isolated uterus of the cat. J. Physiol. (London), 152, 243-249.

Greenburg, D. A. (1987). Calcium channels and calcium channel antagonists. Ann. Neurol., 21, 317-330.

Grynkiewicz, G., Poenie, M., & Tsien, R. Y. (1985). A new generation of Ca^{+2} indicator with greatly improved fluorescence properties. J. Biol. Chem., 260, 3440-3450.

Gu, H., Trajkovic, S., & La Belle, E. F. (1992). Norepinephrine-induced phosphatidylcholine hydrolysis by phospholipase D and C in rat tail artery. Am. J. Physiol., 262, C1276-1383.

Guerriero, V. J., Russo, M. A., Olson, N. J., Putkey, J. A., & Means, A. R. (1986). Domain organization of chicken gizzard myosin light chain kinase deduced form a cloned cDNA. Biochemistry, 25, 8372-8381.

Gustafson, G. T., & Lerner, U. (1983). Thrombin, a stimulator of bone resorption. Biosci. Rep., 3, 255-261.

Haeberle, J. R., Holt, J. W., & Hathaway, D. R. (1985). Regulation of isometric force and isotonic shortening by phosphorylation of the 20,000 dalton myosin light chain of rat uterine smooth muscle. Pfuegers Arch, 403, 215-219.

Haeberle, J. R., Hathaway, D. R., & DePaoli-Roach, A. A. (1985). Dephosphorylation of myosin by the catalytic subunit of a type-2 phosphatase produces relaxation of chemically skinned uterine smooth muscle. J. Biol. Chem., 260, 9965-9968.

Haeberle, J. R., & Trockman, B. A. (1986). Two-site phosphorylation of the 20,000 dalton myosin light chain of glycerinated porcine carotid artery smooth muscle. Biophys. J., 49, 389a.

Hai, C. M., & Phair, R. (1989). Forskolin and caffeine induce Ca^{2+} release from intracellular stores in rabbit aorta. Am. J. Physiol., 257, C413-C418.

Hallcher, L. M., & Sherman, W. R. (1980). The effects of lithium and other agents on the activity of myo-inositol-1-phosphatase from bovine brain. J. Biol. Chem. 255, 10896-10901.

Hartshorne, D. J. (1987). Biochemistry of the contractile process in smooth muscle, in: Physiology of the Gastrointestinal Tract. (L.R. Johnson, ed.), Raven Press, New York, pp. 423-482.

Hathaway, D. R., & Haeberle, J. R. (1985). A radioimmunoblotting method for measuring myosin light chain phosphorylation levels in smooth muscle. Am. J. Physio., 249, C345-C351.

Hei, Y.-J., Macdonell, K. L., Mcneil, J. H., & Diamond, J. (1991). Lack of correlation between activation of cyclic AMP-dependent protein kinase and inhibition of contraction of rat vas deferens by cyclic AMP analogs. Mol. Pharmacol., 39, 233-238.

Hess, P., Lansman, J. B., & Tsien, R. W. (1986). Calcium channel selectivity for monovalent and divalent cations. H. Gen. Physiol., 88, 293-319.

Hidaka, h. M., Inagaki, S., Kawamoto, S., & Sasaki, Y. (1984). Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. Biochemistry, 23, 5036-5041.

Himpens, B., Matthijs, G., Somlyo, A. V., Butler, T. M., & Somlyo, A. P. (1988). Cytoplasmic free calcium, myosin light chain phosphorylation, and force in phasic and tonic smooth muscle. J. Gen. Physiol., 92, 713-729.

Hoffman, B. B., Lavin, T. N., Lefkowitz, R. J., & Ruffolo, R. R. (1981). Alpha adrenergic receptor subtypes in rabbit uterus: Mediation of myometrial contraction and regulation by estrogens. J. Pharmacol. Exp. Ther., 219, 290-295.

Hoffman, O., Klaushofer, K., Koller, K., Peterlik, M., Mavreas, T., & Stern, P. (1983). Indomethacin inhibits thrombin- but not thyroxin-stimulated resorption of fetal rat limb bones. J. Pharmacol. Exp. Ther., 31, 601-608.

Howe, P. H., Akhtar, R. A., & Naderi, S. (1986). Correlative studies on the effect of carbachol on myo-inositol triphosphate accumulation, myosin light chain phosphorylation and contraction in sphincter smooth muscle of rabbit iris. J. Pharmacol. Exp. Ther., 239, 574-583.

Hurwitz, L. (1986). Pharmacology of calcium channels and smooth muscle. Toxicology, 26, 225-258.

Ignarro, L. J., & Kadowitz, P. J. (1985). The pharmacological and physiological role of cyclic GMP in vascular smooth muscle relaxation. Annu. Rev. Pharmacol., 25, 171-191.

Ikebe, M., Ogihara, S., & Tonomura, Y. (1982). Nonlinear dependence of actin -activated Mg^{2+}-ATPase activity on the extent of phosphorylation of gizzard myosin and H-meromyosin. J. Biochem. (Tokyo), 91, 1809-1812.

Ikebe, M., & Hartshorne, D. J. (1985). Phosphorylation of smooth muscle myosin at two distinct sites by myosin light chain kinase. J. Biol. Chem., 260, 10027-10031.

Ikebe, M., Hartshorne, D. J., & Elzinga, M. (1986). Identification, phosphorylation, and dephosphorylation of a second site for myosin light chain kinase on the 20,000-dalton light chain of smooth muscle myosin. J. Biol. Chem., 261, 36-39.

Imai, A., & Gershengorn, M. C. (1987). Independent phosphatidylinositol synthesis in pituitary plasma membrane and endoplasmic reticulum. Nature, 325, 726-728.

Irvine, R. F. (1990). "Quantal Ca^{2+} release and the control of Ca^{2+} entry by inositol phosphates- a possible mechanism. FEBS Lett, 263, 5-9.

Ishizawa, M. (1988). Biphasic response of circular muscle to prostaglandin E_2 in Guinea-pig colon. Arch. Int. Pharmacodyn., 295, 282-290.

Itoh, T., Ikebe, M., Kargacin, G. J., Hartshorne, D. J., Kemp, B. E., & Fay, F. S. (1989). Effects of modulators of myosin light chain kinase activity in single smooth muscle cells. Arch. Int. Pharmacodyn., 338, 164-167.

Janis, R. A., Moats-Staats, B. M., & Gualtieri, R. T. (1980). Protein phosphorylation during spontaneous contraction of smooth muscle. Biochem. Biophys. Res. Commun., 96(1), 265-270.

Janis, R. A., Barany, K., Barany, M., & Sarmiento, J. G. (1981). Association between myosin light chain phosphorylation and contraction of rat uterine smooth muscle. Mol. Physiol., 1, 3-9.

Jeremy, J. Y., & Dandona, P. (1986). RU486 antagonizes the inhibitory action of progesterone on prostacyclin and thromboxane A_2 synthesis in cultured rat explants. Endocrinology, 119, 655-660.

Johansson, S. R. M., Anderson, R. G. A., & Wikberg, J. E. S. (1980). Comparison of β_1- β_2 receptor stimulation in oestrogen or progesterone dominated rat uterus. Acta Pharmacol. Toxicol., 47, 252-258.

Kamm, K. E., & Stull, J. T. (1985). The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. Annu. Rev. Pharmacol. Toxicol., 25, 593-620.

Kather, H., & Simon, B. (1979). Biphasic effects of prostaglandin E_2 on the human fat cell adenylate cyclase. J. Clin. Invest., 64, 609-612.

Kather, H., & Simon, B. (1981). Stimulatory and inhibitory effects of catecholamines and prostaglandin E_2 on human fat cell adenylyl cyclase. Adv. Cyc. Nucleo. Res., 14, 555-562.

Kelley, L. L., Blackmore, P. F., Graber, S. E., & Stewart, S. J. (1990). Agents that raise cAMP in human T lymphocyte release an intracellular pool of calcium in the absence of inositol phosphate production. J. Biol. Chem., 265, 17657-17664.

Kemp, B. E., Pearson, R. B., & Guerriero, V. J. (1987). The calmodulin binding domain of the chicken smooth muscle myosin light chain kinase contains a pseudosubstrate sequence. J. Biol. Chem., 262, 2542-2548.

Kerrick, W. G., & Hoar, P. E. (1981). Inhibition of smooth muscle tension by cyclic AMP-dependent protein kinase. Nature, 292, 253-255.

Kikkawa, U., & Nishizuka, Y. (1986). The role of protein kinase C in transmembrane signaling. Annu. Rev. Cell Biol., 2, 149-178.

Kim, D. H., Ohnishi, S. T., & Ikemoto, N. (1983). Kinetic study of calcium release from sarcoplasmic reticulum in vitro. J. Biol. Chem., 258, 9662-9668.

Kishikawa, T. (1981). Alterations in the properties of the rat myometrium during gestation and postpartum. Jpn. J. Physiol., 31, 515-536.

Klee, C. B. (1980). Calmodulin: Structure-function relationships, in Calcium and Cell function. Academic Press, New York, pp. 59-78.

Krebs, E. G., & Beavo, J. A. (1979). Phosphorylation-dephosphorylation of enzymes. Annu. Rev. Biochem., 48, 923.

Lands, A. M., Arnold, A., Mcauliff, J. P., Luduena, F. P., & Brown, T. G. (1967). Differentiation of receptors systems activated by sympathomimetic amines. Nature, 214, 597-598.

Lansman, J. B., Hess, P., & Tsien, R. W. (1986). Blockade of current through single calcium channels by Cd^{2+}, Mg^{2+} and Ca^{2+}. J. Gen. Physiol., 88, 321-347.

Law, P. Y. (1982). Comparison of opiate inhibition of adenylate cyclase activity in neuroblastoma N18TG2 and Neuroblastoma X glioma NG 108-15 hybrid cell lines. Mol. Pharmaco., 21, 483-491.

Lebowitz, E. A., & Cooke, R. (1979). Phosphorylation of uterine smooth muscle permits actin activation. J. Biochem. (Tokyo), 85, 1489-1494.

Leiber, D., Vesin, M.-F., & Harbon, S. (1978). Regulation of guanosine 3',5'-cyclic monophosphate levels and contractility in rat myometrium. FEBS Lett., 86, 183-187.

Lukas, T. J., Burgess, W. H., Prendergast, F. G., Lau, W., & Watterson, D. M. (1986). Calmodulin-binding domains: characterization of a phosphorylation and calmodulin binding site from myosin light chain kinase. Biochemistry, 25, 1458-1464.

Lundstrom, V. (1986). The uterus. In B. G. Bygdeman H Keith L. G. (Ed.), Prostaglandins and Their Inhibitors in Obstetrics and Gynecology Boston: MTP press., pp. 59-81.

Mackenzie, L. W., Word, R. A., Casey, M. L., & Stull, J. T. (1990). Myosin light chain phosphorylation in human myometrial smooth muscle cells. Am. J. Physiol., C92 - C98.

Marc, S., Leiber, D., & Harbon, S. (1986). Carbachol and oxytocin stimulate the generation of inositol phosphate in the guinea pig myometrium. FEBS Lett., 201, 9-14.

Marshall, J. M. (1970). Adrenergic innervation of the female reproductive tract: Anatomy, physiology and pharmacology. Ergeb. Physiol., 62, 6-67.

Marston, S. B., Trevett, R. M., & Walters, M. (1980). Calcium ion regulated thin filaments from vascular smooth muscle. Biochem. J., 198, 355-365.

Marston, S. B. (1982). The regulation of smooth muscle contractile proteins. Prog. Biophy. Mol. Biol., 41, 1-41.

Marston, S. B., & Smith, C. W. J. (1985). The thin filaments of smooth muscles. J. Muscle Res. Cell Motil., 6, 669-708.

Martin, J. N. J., Bygdeman, M., & Eneroth, P. (1978). The influence of locally administered prostaglandin E2 and $F_{2\alpha}$ on uterine motility in the intact nonpregnant human uterus. Acta. Obstet. Gynecol. Scand., 57, 141-147.

Martonosi, A. N. (1984). Mechanism of Ca^{2+} release from sarcoplasmic reticulum of skeletal muscle. Pharmacol. Rev., 64, 1240-1320.

May, W. S., Sahyoun, N., Wolf, M., & Custrecasas, P. (1985). Role of intracellular calcium mobilization in the regulation of protein kinase C-mediated membrane processes. Nature, 317, 549-551.

Miller, J. R., Silver, P. J., & Stull, J. T. (1983). The role of myosin light chain phosphorylation in beta-adrenergic relaxation of tracheal smooth muscle. Mol. Pharmacol., 24, 235-242.

Molnar, M., & Hertelendy, F. (1990). $PGF_{2\alpha}$ and PGE2 binding to rat myometrium during gestation, parturition, and postpartum. A. J. Physio., 258, E740-70.

Moolenaar, W. H., Kruijer, W., Tilly, B. C., Verlaan, I., Bierman, A. J., & de Laat, S. W. (1986). Growth factor-like action of phosphatidic acid. Nature, 323, 171-173.

Muallem, S., Merritt, B. S., Green, J., Kleeman, C. R., & Yamaguchi, D. T. (1989). Classification of prostaglandin receptors based on coupling to signal transduction systems. Biochem. J., 263, 769-774.

Muallem, S., Pandol, S. J., & Beeker, T. G. (1989). Hormone-evoked calcium release from intracellular stores is a quantal process. Biochem. J., 264, 205-212.

Murayama, T., & Ui, M. (1985). Receptor-mediated inhibition of adenylate cyclase and stimulation of arachidonic acid release in 3T3 fibroblasts. J. Biol. Chem., 260, 7226-7233.

Murphy, R. A. (1980). Mechanics of vascular smooth muscle, in Handbook of Physiology, section 2, volume 2, vascular smooth muscle. American Physiology Society, Bethesda, MD. pp. 325-353.

Murphy, R. A. (1990). Contraction in smooth muscle cells. Annu. Rev. Physiol, 51, 275-283.

Nakaki, T., Roth, B. L., Chuang, D.-M., & Costa, E. (1985). Phasic and tonic contraction in 5-HT2 receptor-mediated rat aorta contraction: Participation of Ca^{2+} channels and phospholipase C. J. Pharmacol. Exp. Ther., 234, 442-446.

Negishi, M., Ito, S., & Hayaishi, O. (1989). Prostaglandin E receptors in bovine adrenal medulla are coupled to adenylate cyclase via G_i and to phosphoinositide metabolism in a pertussis toxin-insensitive manner. J. Biol. Chem., 264, 3916-3923.

Nishikawa, M., Hidaka, H., & Adelstein, R. S. (1983). Phosphorylation of smooth muscle heavy meromyosin by calcium-activated, phospholipid-dependent protein kinase. J. Biol. Chem., 258, 14069-14072.

Nishikawa, M., Sellers, J. R., Adelstein, R. S., & Hidaka, H. (1984). Protein kinase C modulates in vitro phosphorylation of the smooth muscle meormyosin by myosin light chain kinase. J. Biol. Chem., 259, 8808-8814.

Nishizuka, Y. (1988). The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature, 334, 661-665.

Okazaki, T., Okita, J. R., MacDonald P. C. and Johnston, J. M. (1978). Initiation of human parturition: X. Substrate specificity of phospholipase A2 in human fetal membranes. Am. J. Obstet. Gynecol., 130, 432-438.

Park, S., & Rasmussen, H. (1986). Carbachol-induced protein phosphorylation changes in bovine tracheal smooth muscle. J. Biol. Chem., 261, 15734-15739.

Parker, I., & Ivorra, I. (1990). Localized all-or-none calcium liberation by inositol triphosphate. Science, 250, 977-979.

Pato, M. D., & Kerc, E. (1985). Purification and characterization of a smooth muscle myosin phosphatase from turkey gizzards. J. Biol. Chem., 260, 12359-12366.

Payne, M. E., Elzinga, M., & Adelstein, R. S. (1986). Smooth muscle myosin light chain kinase. Amino acid sequence at the site phosphorylated by adenosine cyclic 3',5'-phosphate-dependent protein kinase whether or not calmodulin is bound. J. Biol. Chem., 261, 16346-16350.

Pearson, R. B., Jakes, R., John, M., Kendrick-Jones, J. & Kemp, B. E. (1984). Phosphorylation site sequence of smooth muscle myosin light chain (Mr = 20,000). FEBS Lett., 168, 108-112.

Persechini, A., & Hartshorne, D. J. (1981). Phosphorylation of smooth muscle myosin: Evidence for cooperativity between the myosin heads. Science, 213, 1383-1385.

Persechini, A., Kamm, K. E., & Stull, J. T. (1986). Different phosphorylated forms of myosin in contracting tracheal smooth muscle. J. Biol. Chem., 261, 6293-6299.

Polacek, I., Bolan, J., & Daniel, E. E. (1971). Accumulation of adenosine 3': 5' monophosphate and relaxation in the rat uterus. Can. J. Pharmacol., 49, 999-1004.

Potter, J. D., Strang-Brown, P., Walker, P. L., & Iida, S. (1983). Ca^{2+} binding to calmodulin. Methods Enzymol., 102, 135-143.

Raeymaekers, L., & Hasselbach, W. (1981). Ca^{2+} uptake, Ca^{2+}-ATPase activity, phosphoprotein formation and phosphate turnover in a microsomal fraction of smooth muscle. Eur. J. Biochem., 116, 373-378.

Riemer, R. K., Jacobs, M. M., Wu, Y. Y., & Roberts, J. M. (1986). Progesterone-induced rabbit myometrial beta-adrenergic response in accompanied by increased concentration and expression of stimulatory adenylate cyclase coupling (Gs). 33th annual meeting, Society for Gynecologic investigation, Toronto, Canada. P. 171.

Riemer, R. K., Goldfien, A., & Roberts, J. M. (1987). Estrogen increases adrenergic but not cholinergic-mediated production of inositol phosphates in rabbit uterus. Mol. Pharmacol., 32, 663-668.

Rimele, T. J., & Vanhoutte, P. M. (1984). Differential effects of calcium entry blockers on vascular smooth muscle. Int. Angiol., 3, 17-23.

Roberts, J. M., Insel, P. A., Goldfien, R. D., & Goldfien, A. (1977). α-adrenoceptors but not β-adrenoceptors increase in rabbit uterus with estrogen. Nature, 270, 624-625.

Roberts, J. M., Insel, P. A., & Goldfien, A. (1981). Regulation of myometrial adrenoceptors and adrenergic response by sex steroids. Mol. Pharmacol., 20, 52.

Sakai, K., Yamaguchi, T., Morita, S., & Uchida, M. (1983). Agonist-induced contraction of rat myometrium in Ca^{2+} free solution containing Mn. Gen. Pharmacol., 14, 391-400.

Salomon, Y. (1979). Adenylate cyclase assay. In G. Brooker, P. Greegard, and G.A. Robins, eds. Adv. Cyclic Nucleo. Res., 10, 35-56.

Schultz, K. D., Bohmem, E., Kreye, V. A. W., & Schultz, G. (1979). Relaxation of hormonally stimulated smooth muscular tissues by the 8-bromo derivative of cyclic GMP. Naunyn Schmiedebergs Arch. Pharmacol., 306, 1-9.

Sellers, J. R., Chock, P. B., & Adelstein, R. S. (1983). The apparently negatively cooperative phosphorylation of smooth muscle myosin at low ionic strength is related to its filamentous state. J. Biol. Chem., 258, 14181-14188.

Sherry, J. M. F., Gorecka, A., Aksoy, M. O., Dabarowska, R., & Hartshorne, D. J. (1978). Roles of calcium and phosphorylation in the regulation of the activity of gizzard myosin. Biochemistry, 17, 4411-4418.

Sobieszek, A. (1982). Steady state kinetics of the actin activation of skeletal muscle heavy meromyosin subfragments. Effects of skeletal, smooth and non-muscle tropomyosins. J. Mol. Biol., 157, 275-286.

Somlyo, A. P., & Somlyo, A. V. (1968). Electromechanical and coupling in vascular smooth muscle. J. Pharmacol. Exp. Ther., 159, 129-145.

Somlyo, A. P., & Somlyo, A. V. (1969). Pharmacology of excitation-contraction coupling in vascular smooth muscle and in avian slow muscle. Fed. Proceed., 28, 1634-1642.

Somlyo, A. V., Vinall, P., & Somlyo, A. P. (1969). Excitation-contraction coupling and electrical events in two types of vascular smooth muscle. Microvascul. Res., 1, 354-373.

Sommerville, L. E., & Hartshorne, D. J. (1987). Intracellular calcium and smooth muscle contraction. Cell Calcium, 7, 353-364.

Storey, D. J., Shears, S. B., Kirk, C. J., & Michell, R. H. (1984). Stepwise enzymatic dephosphorylation of inositol 1,4,5-triphosphate to inositol in liver. Nature, 312, 374-376.

Stull, J. T., Nunnally, M. H., & Michnoff, C. H. (1986). Calmodulin-dependent protein kinase, in: The enzymes. Academic Press. New York. pp. 113-116.

Takai, Y., Kishimoto, A., kikawa, U., Mori, T., & Nishizuka, Y. (1979). Unsaturated diacylglycerol as a possible messenger for the activation of calcium-activated, phospholipid-dependent protein kinase system. Biochem. Biophys. Res. Commun., 91, 1218-1224.

Tanfin, Z., & Harbon, S. (1987). Heterologous regulations of cAMP response in pregnant rat myometrium. Evolution from a stimulatory to an inhibitory prostaglandin E2 and prostacyclin effect. Mol. Pharamacol., 32, 249-257.

Taylor, D. A., Bowman, B. F., & Stull, J. T. (1989). Cytoplasmic Ca^{2+} is a primary determinant for myosin phosphorylation in smooth muscle cells. J. Biol. Chem., 264, 6207-6213.

Trybus, K. M., & Lowey, S. (1985). Mechanism of smooth muscle myosin phosphorylation. J. Biol. Chem., 260, 15988-15995.

Tsai, T. H., & Fleming, W. W. (1964). The adrenotropic receptors of the cat uterus. J. Pharmacol. Exp. Ther., 143, 268-272.

Tsien, R. W., Hess, P., McClesky, E. W., & Rosenberg, R. L. (1987). Calcium channels: mechanisms of selectivity, permeation and block. Annu. Rev. Biophys. Biophys. Chem., 16, 265-290.

Umekawa, H., & Hidaka, H. (1985). Phosphorylation of caldesmon by protein kinase C. Biochem. Biophys. Res. Commun., 132, 56-62.

Van Breemen, C., Aaronson, P. I., Cauvin, C. A., Loutzenhizer, R. D., Mangel, A. W., & Saida, K. (1982). The calcium cycle in arterial smooth muscle, in Calcium Blockers: Mechanisms of Action and Clinical Applications (S. F. Flaim and R. Zelis, eds.) Urban & Sehwarzeng, Baltimore. pp: 53-63.

Vandekerchhove, J., & Weber, K. (1979). The complete amino acid sequence of actins from bovine aorta, bovine heart, bovine fast skeletal muscle, and rabbit slow skeletal muscle. A protein-chemical analysis of muscle actin differentiation. Differentiation, 14, 123-133.

Verma, S. C., & McNeill, J. H. (1976). Isoproterenol-induced relaxation, phosphorylase activation and cyclic adenosine levels in the polarized and depolarized rat uterus. J. Pharmacol. Exp. Ther., 198, 539-547.

Vesin, M.-F., & Harbon, S. (1974). The effect of epinephrine, prostaglandins and their antagonists on adenosine cyclic 3', 5' monophosphate concentration and motility of the rat uterus. Mol. Pharmacol., 10, 457-473.

Waldman, S. A., & Murad, F. (1987). Cyclic GMP synthesis and function. Pharmacol. Rev., 39, 163-196.

Walsh, M. P. (1985). Limited proteolysis of smooth muscle myosin light chain kinase. Biochemistry, 24, 3724-3730.

Watson, S. P., McDonnell, R. T., & Lapetina, E. G. (1984). The rapid formation of inositol phosphates in human platelets by thrombin is blocked by prostacyclin. J. Biol. Chem., 259, 13199-13203.

Weisbrodt, N. V., & Murphy, R. A. (1985). Myosin phosphorylation and contraction of feline esophageal smooth muscle. Am. J. Physiol., 249, C9-C14.

Wibo, M., Morel, N., & Godfraind, T. (1981). Differentiation of Ca^{2+} pumps linked to plasma membrane and endoplasmic reticulum in the microsomal fraction from intestinal smooth muscle. Biochem. Biophys., 649, 651-660.

Wolf, M., LeVine, H., May, W. S., Cuatrecasas, P., & Sahyoun, N. (1985). A model for intracellular translocation of protein kinase C involving synergism between Ca^{2+} and phorbol esters. Nature, 317, 546-549.

Word, R. A., Kamm, K. E., & Casey, M. L. (1992). Contractile effects of prostaglandins, oxytocin, and endothelin-1 in human myometrial *in vitro*: refractoriness of myometrial tissue of pregnant women to prostaglandins E2 and F2α. J. Clin. Endocrinol. Metab., 75(4), 1027-1032.

Worley, P. F., Baraban, J. M., Supattapone, S., Wilson, V. S., & Snyder, S. H. (1987). Characterization of inositol triphosphate receptor binding in brain. J. Biol. Chem., 262, 12132-12136.

Wu, H., James-Kracke, M. R., & Halenda, S. P. (1992). Direct relationship between intracellular mobilization and phospholipase D activation in prostaglandin E-stimulated human erythroleukemia cells. Biochemistry, 31(13), 3370-3377.

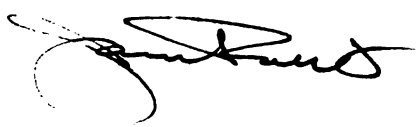
Wuytack, F., Raeymaekers, L., Verbist, J., De Smedt, H., & Casteels, R. (1984). Evidence for the presence in smooth muscle of two types of Ca^{2+}-transport ATPase. Biochem. J., 224, 445-451.

Wynn, R. M., & Jollie, W. P. (1989). Biochemistry of the myometrium and cervix., in Biology of the uterus. Plenum Medical. New York. pp. 355.

Yule, D. I., Williams, J. A. (1992). U73122 inhibits Ca^{2+} oscillation in response to cholecystokinin and carbachol but not to JMV-180 in rat pancreatic acinar cells. J. Biol. Chem., 267 (20),13830-13835.

Abstract

Prostaglandin E2 (PGE2), thought to be an important effector of uterine smooth muscle contraction presents an interesting and confusing paradox. Despite being a potent stimulator of uterine contraction, PGE2 also strikingly increases cAMP, the second messenger implicated in uterine relaxation. To study the mechanism of action for these responses, I examined the mechanism by which PGE2 stimulated myosin light chain phosphorylation in rabbit uterine smooth muscle cells. PGE2 at a concentration of 1 nM stimulated myosin light chain phosphorylation, a key regulatory step for uterine smooth muscle contraction, while at 100 μM myosin light chain phosphorylation was reduced. Intracellular free calcium increased at all PGE2 concentrations without significant change in phosphoinositide hydrolysis. PGE2-stimulated intracellular calcium increase was blocked by pretreatment with phospholipase C (PLC) inhibitor (U73122). The ability of PGE2 to accelerate myosin light chain phosphorylation was altered in calcium free medium. At 100 μM, PGE2 greatly stimulated adenylyl cyclase activity. Pretreatment of myocytes with a cAMP dependent protein kinase inhibitor (H8) potentiated PGE2-stimulated myosin light chain phosphorylation, but had no significant effect on phosphoinositide hydrolysis. In rabbit uterine smooth muscle strip PGE2 (100 μM) initially elicited contraction followed by a complete relaxation. With H8 pretreatment, PGE2 induced a consistent contractile response. In preliminary studies, myocytes prepared from uterus of pregnant rabbits (27th day) had a much greater reduction of myosin light chain phosphorylation at high concentrations of PGE2 than was present in myocytes from estrogen treated rabbits. In summary, PGE2, at low concentration (1 nM), stimulates myosin light chain phosphorylation and uterine smooth muscle contraction while at high concentration (100 μM), it induces muscle relaxation. Our data support the concept that this is due to PLC-stimulated increases of intracellular calcium predominating at low PGE2 concentration but adenylyl cyclase activation and cyclic AMP accumulation with inhibition of myosin light chain phosphorylation predominating at high PGE2 concentration. My preliminary data also supports hormonal modulation of the PGE2 response.

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