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Mitogen-activated protein kinase inhibitors suppress prostaglandin $F_{2\alpha}$-induced myosin-light chain phosphorylation and contraction in iris sphincter smooth muscle

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Abstract

The purpose of this study was to investigate the potential role of mitogen-activated protein (MAP) kinase in contraction by monitoring MAP kinase phosphorylation (activation) and contraction during agonist stimulation of cat iris sphincter smooth muscle. Changes in tension in response to prostaglandin $F_{2\alpha}$, latanoprost, a prostaglandin $F_{2\alpha}$ analog used as an anti-glaucoma drug, and carbachol were recorded isometrically, and MAP kinase activation was monitored by Western blot using a phosphospecific p42/p44 MAP kinase antibody. We found that treatment of the muscle with 2′-Amino-3′-methoxyflavone (PD98059) (10 μM), a specific inhibitor of MAP kinase kinase (MEK), inhibited significantly prostaglandin $F_{2\alpha}$- and latanoprost-induced phosphorylation and contraction, but had little effect on those evoked by carbachol. Prostaglandin $F_{2\alpha}$ increased MAP kinase phosphorylation in a concentration-dependent manner with EC_{50} value of 1.1×10^{-8} M and increased contraction with EC_{50} of 0.92×10^{-9} M. The MAP kinase inhibitors PD98059, Apigenin and 1,4-Diamino-2,3-dicyano-1,4bis(2-aminophenylthio)butadiene (UO126) inhibited prostaglandin $F_{2\alpha}$-induced contraction in a concentration-dependent manner with IC_{50} values of 2.4, 3.0 and 4.8 μM, respectively. PD98059 had no effect on prostaglandin $F_{2\alpha}$- or on carbachol-stimulated inositol-1,4,5-trisphosphate (IP_3) production. In contrast, the MAP kinase inhibitor inhibited prostaglandin $F_{2\alpha}$-induced myosin-light chain (MLC) phosphorylation, but had no effect on that of carbachol. *N*-[2-(*N*-(4-Chloro-cinnamyl)-*N*-methyl-aminomethyl)phenyl]-*N*-[2-hydroxyethyl]-4-methoxybenzenesulfonamide (KN-93) (10 μM), a Ca^{2+}-calmodulin-dependent protein kinase inhibitor, and Wortmannin (10 μM), an MLC kinase inhibitor, inhibited significantly (by 80%) prostaglandin $F_{2\alpha}$- and carbachol-induced contraction. It can be concluded that in this smooth muscle p42/p44 MAP kinases are involved in the mechanism of prostaglandin $F_{2\alpha}$-, but not in that of carbachol, induced contraction. In addition, these data clearly indicate that the stimulation of the iris sphincter with prostaglandin $F_{2\alpha}$ and carbachol activate two distinct pathways, the MAP kinase pathway and the Ca^{2+} mobilization pathway.

Keywords: Nonvascular smooth muscle; Mitogen-activated protein kinase; Contraction; Prostaglandin $F_{2\alpha}$; Carbachol

1. Introduction

Prostaglandins exert a broad range of physiological and pharmacological actions in a wide variety of tissues through interaction with specific cell surface receptors (Coleman et al., 1990). In ocular tissues, responses to prostaglandins are due to the activation of specific receptors which are located on the iris–ciliary body, cornea and retina (Abdel-Latif, 1991; Ohia and Jumblatt, 1991; Bhatacherjee et al., 1993; Fujimoto et al., 1995; Ocklind et al., 1996; Krauss et al., 1997; Anthony et al., 1998). In general, the prostaglandin receptors are coupled through different types of G-proteins to the stimulation of adenylyl cyclase (IP, DP, EP_2, EP_3 and EP_4 subtypes), the inhibition of adenylyl cyclase (EP_3) or to the stimulation of phospholipase C (FP, EP_1, EP_3) (Coleman et al., 1990). The prostaglandin $F_{2\alpha}$ receptor has been cloned from cDNA libraries of mouse, rat, cow and sheep corpora lutea, human myometrium and human ciliary body and is linked to phosphoinositide turnover, as evaluated by measurements of radiolabelled inositol phosphates formation or increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$) in cells transfected with different prostaglandin $F_{2\alpha}$ receptor cDNAs (Abramowitz et al., 1994; Sugimoto et al., 1994; Lake et al., 1994; Sakamoto et al., 1994; Kunapuli et al., 1997; Pierce et al., 1997).

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In the eye, prostaglandin $F_{2\alpha}$ and its analog PhXA41 (latanoprost) mediate, through prostaglandin $F_{2\alpha}$ receptors, a broad range of biological effects including smooth muscle contraction (for review, see Abdel-Latif, 1991) and reduction of intraocular pressure in glaucoma patients (for review, Bito et al., 1993). While the hypotensive effects of prostaglandin $F_{2\alpha}$ and latanoprost are mediated through an increase in uveoscleral outflow of aqueous humor (Crawford and Kaufman, 1987; Stjernschantz et al., 1995), the molecular mechanism underlying this effect is not known. Prostaglandin $F_{2\alpha}$ receptors are widely distributed in various regions of mammalian ocular tissues including iris sphincter (Ocklind et al., 1996). In the iris sphincter, prostaglandin $F_{2\alpha}$ binds to prostaglandin $F_{2\alpha}$ receptors to stimulate phospholipase C activity, this leads to the generation of the two second messengers inositol-1,4, 5-trisphosphate (IP_3) and 1, 2-diacylglycerol from phosphatidylinositol-4,5-bisphosphate, and to muscle contraction (Yousufzai et al., 1988). Diacylglycerol activates protein kinase C, and IP_3 stimulates the release of intracellular Ca^{2+} (Abdel-Latif, 1996). In non-contractile tissues, prostaglandin $F_{2\alpha}$ has been reported to enhance tyrosine phosphorylation and DNA synthesis through phospholipase C-coupled receptor via Ca^{2+}-dependent intracellular pathway in NIH-3T3 cells (Watanabe et al., 1994), and to stimulate mitogenesis (Quarles et al., 1993) and tyrosine phosphorylation and mitogen-activated protein kinase (MAP kinase) in osteoblastic MC3T3-E1 cells (Hakeda et al., 1997).

Stimulation of smooth muscle results in an increase in cellular free Ca^{2+} and the activation of several signaling pathways. The most widely studied pathway in smooth muscle is the Ca^{2+} and calmodulin-dependent myosin-light chain (MLC) kinase which catalyzes phosphorylation of the 20-kDa MLC and initiates contraction (Kamm and Stull, 1985). There is accumulating evidence which indicates that in addition to the MLC kinase pathway, tyrosine kinase pathways play an important role in the regulation of smooth muscle contraction (Di Salvo et al., 1997). Protein kinase C and MAP kinase are two families of kinases that are present in smooth muscle cells and are believed to play important roles in smooth muscle regulation. MAP kinases are serine/threonine kinases that are activated by phosphorylation on both threonine and tyrosine residues through an upstream MAP kinase, MEK (MAP kinase kinase). Stimulation of MAP kinase in response to mechanical and pharmacological stimulation has been reported in vascular smooth muscle (Katoch and Moreland, 1995; Adam et al., 1995; Dessy et al., 1998) and in nonvascular smooth muscle (Gerthoffer et al., 1996, 1997; Nohara et al., 1996). Prostaglandin $F_{2\alpha}$ was reported to activate MAP kinase and MAP kinase kinase in cultured rat puerperal uterine myometrial cells (Ohmichi et al., 1997).

In cat iris sphincter, protein tyrosine phosphorylation is involved in the mechanism of prostaglandin $F_{2\alpha}$-but not carbachol-induced contraction, IP_3 accumulation, and Ca^{2+} mobilization (Yousufzai and Abdel-Latif, 1998). The signaling pathways of prostaglandin $F_{2\alpha}$ receptor to smooth muscle contraction remain unclear. To throw more light on the mechanism of action of prostaglandin $F_{2\alpha}$ and its analog, latanoprost, in the eye, in the present study we have asked the question as to whether MAP kinase phosphorylation is involved in prostaglandin $F_{2\alpha}$- and carbachol-induced MLC phosphorylation and contraction in the cat iris sphincter. We found that MAP kinase phosphorylation is involved in the mechanism of prostaglandin $F_{2\alpha}$-induced MLC and contraction but not in IP_3 accumulation, however, its role in carbachol-induced MLC phosphorylation and contraction was considerably less pronounced.

2. Materials and methods

2.1. Materials

Reagent sources were as follows: prostaglandin $F_{2\alpha}$ and latanoprost from Cayman (Ann Arbor, MI); 2′-Amino-3′-methoxyflavone (PD98059), 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB-203580), and 1,4-Diamino-2,3-dicyano-1,4bis(2-aminophenylthio)butadiene (UO126) from Biomol Research Laboratory (Plymouth Meeting, PA); Wortmannin and Carbachol from Sigma (St. Louis, MO); Apigenin and *N*-[2-(*N*-(4-Chlorocinnamyl)-*N*-methylaminomethyl)phenyl]-*N*-[2-hydroxyethyl]-4-methoxybenzenesulfonamide (KN-93) from Calbiochem (La Jolla, CA); polyclonal anti-MAP kinase (P-Erk and Erk-1) antibodies recognizing p42/p44 MAP kinases from Santa Cruz Biotechnology (Santa Cruz, CA); *myo*-[^{3}H]inositol (18 Ci/mmol) from Amersham (Arlington Heights, IL); and Dowex AG1-X8 anion-exchange resin (200–400 mesh, formate form) from BioRad (Melville, NY).

The MAP kinase inhibitors were dissolved in dimethylsulfoxide, and prostaglandin $F_{2\alpha}$ was dissolved in absolute ethanol. The final concentrations of the solvents in the reaction mixtures were $< 0.1\%$, concentrations which had no effect on the basal levels of MAP kinase phosphorylation, IP_3 formation, MLC phosphorylation and contraction in the cat iris sphincter.

2.2. Preparation of the iris sphincter

Cat eyes were obtained through the courtesy of Richmond County Animal Control (Augusta, GA). Eyes were removed immediately after death and were transported to the laboratory packed in ice. The iris sphincter was dissected out and placed in Krebs–Ringer bicarbonate buffer (pH 7.4) containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 10 mM D-glucose and 1.25 mM $CaCl_2$. Indomethacin (1 μM), a cyclooxygenase inhibitor, was added to the incubation medium in all of the experiments in order to prevent

formation of endogenous prostaglandins. The Krebs–Ringer bicarbonate buffer was used as the incubation medium in the following studies. pH of the buffer was adjusted and maintained at 7.4 with 97% O_2–3% CO_2. In general, each sphincter muscle was cut into two equal strips: one strip served as control, and the other was used as experimental. To test the viability of the tissue we routinely monitor the contractility of this cholinergically innervated smooth muscle with carbachol.

2.3. Measurement of agonist-induced tension response in iris sphincter

For measurements of the contraction response, the sphincters were mounted individually in separate organ baths (10 ml) containing buffer. A mixture of 97% O_2–3% CO_2 was bubbled continuously through the buffer, which was maintained at 37°C. The tissue was allowed to equilibrate for 90 min under a resting tension of 50 mg. During this period, the medium was replaced with fresh buffer every 30 min. After equilibration of the tissue, the agonist was added and changes in tension were monitored continuously with a Grass FT-03 force transducer connected to a Grass DC amplifier (Grass Medical Instruments, Quincy, MA) as previously described (Howe et al., 1986).

2.4. Incubation of sphincter muscle with myo-[^{3}H]inositol and analysis of inositol phosphates

To prelabel the tissue with *myo*-[^{3}H]inositol, the two halves of sphincter muscle (from the same eye) were incubated at 37°C for 90 min in 1 ml of buffer that contained 10 μCi of *myo*-[^{3}H]inositol. The muscles were washed four times with 4 ml of nonradioactive buffer and then suspended singly (one half was used as control and the other as experimental) in 1 ml of fresh nonradioactive buffer. Lithium Chloride, to a final concentration of 10 mM, was added to each incubation. After 10 min, carbachol or prostaglandin $F_{2\alpha}$ was added as indicated, and incubation continued for another 5 min. When used, the MAP kinase inhibitors were added 10 min before the addition of the agonist. The incubations were stopped by the addition of 1 ml of 10% (w/v) trichloroacetic acid. The trichloroacetic acid extract was analyzed for ^{3}H-labeled *myo*-inositol phosphates. The method used to extract and separate the [^{3}H]inositol phosphates was as described previously (Howe et al., 1986). Briefly, the tissues were homogenized in 5% (w/v) trichloroacetic acid, and the homogenate was centrifuged at $3000 \times g$ for 15 min. The supernatant was extracted four times with 4 ml of water-saturated diethylether and then neutralized with 0.1 M NaOH. The inositol phosphates were analyzed by anion-exchange chromatography with BioRad Dowex AG1-X8 resin (formate form, 200–400 mesh). The pelleted material was solubilized overnight in 1 M NaOH, and suitable aliquots were removed for determination of proteins according to the method of Lowry et al. (1951) with bovine serum albumin as standard. Data were normalized to the amount of tissue proteins.

2.5. Immunoblot analysis of MAP kinase and phospho-MAP kinase in iris sphincter

Phosphorylation of MAP kinase was determined by using Western blot and immunodetection as previously described (Husain and Abdel-Latif, 1998; Katoch et al., 1999). Briefly, the muscles were first pre-incubated for 75 min at 37°C in 1 ml of Krebs–Ringer bicarbonate buffer (pH 7.4). At this time the tissues were transferred to 1 ml of fresh buffer and incubation continued for an additional 15 min to give a total pre-incubation time of 90 min. The tissues were then incubated in the absence or presence of PD98059 (10 μM) for 30 min, followed by incubation with prostaglandin $F_{2\alpha}$ (1 μM) or carbachol (10 μM) for 2 min. The reaction was stopped by immersing the tissue in a methanol–dry ice slurry at −80°C. The dehydrated tissue was homogenized in buffer containing 50 mM Tris (pH 8), 40 mM $Na_4P_2O_7$, 50 mM NaF, 5 mM $MgCl_2$, 0.1 mM Na_2VO_4, 10 mM EGTA, 2 mM phenyl methylsulfonate fluoride, 1% (V/V) Triton x-100, 10 μg/ml aprotinin and 10 μg/ml leupeptin. Sample homogenates were then clarified by centrifugation at 4°C for 5 min at 6000 rpm. Protein concentrations in the supernatants were determined according to the method of Lowry et al. (1951) employing bovine serum albumin as a standard. The supernatants from the samples were mixed with equal amounts of Laemmli buffer containing 0.5 M Tris (pH 6.8), 10% glycerol, 10% sodium dodecylsulfate, 0.3% β-mercaptoethanol, 0.05% bromophenol blue and boiled for 3 min. Sodium dodecylsulfate gel electrophoresis was performed using 0.75 mm thick mini-gels as described by Laemmli (1970). Samples of equal protein content were loaded on to 10% gel and subjected to 200 V constant voltage for 45 min. The electrophoresed proteins were immediately transferred to nitrocellulose membranes and probed with antibodies specific to p42/p44 MAP kinases and phospho-p42/p44 MAP kinases, followed by an incubation with secondary antibodies (horseradish peroxidase conjugated goat anti-rabbit immunoglubulin G at 1:4000 dilution) for 1 h at 20°C as described previously (Husain and Abdel-Latif, 1998). For chemiluminescent detection, the membranes were treated with enhanced chemiluminescent (ECL) reagent (Amersham) for 1 min and subsequently exposed to ECL hyperfilm for 1–2 min. Relative band intensities were determined by densitometric analysis.

2.6. Measurement of ^{32}Pi incorporation into iris sphincter phosphoproteins

The method used to determine the phosphorylation of sphincter muscle proteins was as previously described (Howe et al., 1986). Briefly, the muscles were first pre-in-

cubated for 75 min at 37°C in 1 ml of Krebs–Ringer bicarbonate buffer (pH 7.4) containing 100 μCi of ^{32}Pi. At this time the tissues were transferred to 1 ml of fresh Krebs–Ringer bicarbonate buffer containing 100 μCi of ^{32}Pi and incubation continued for an additional 15 min to give a total pre-incubation time of 90 min. After this incubation, the tissues were incubated in the absence or presence of PD98059 (10 μM) for 30 min, followed by incubation with prostaglandin $F_{2\alpha}$ (1 μM) or carbachol (10 μM) for 2 min. The reaction was stopped by immersing the tissue in a methanol–dry ice slurry at −80°C. The dehydrated tissue was homogenized in ice-cold 5% (w/v) trichloroacetic acid and the homogenate centrifuged at 6000 g for 15 s. The supernatant was discarded and the pelleted material was solubilized in 200 μl of sodium dodecylsulfate-containing buffer by boiling for 30 min in sealed tubes. Aliquots containing 100 μg proteins were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis. The gel was fixed, stained with Coomassie Brilliant Blue and then dried under vacuum. Autoradiography was performed by exposing the dried gel to Kodak XRP-5 medical X-ray film. The molecular weights of various protein bands were estimated by comparing their mobilities during electrophoresis with the mobilities of standard proteins of known molecular weights, including MLC isolated from the iris muscle, run in the same gel. The radioactive band corresponding to MLC was cut from the dried gel and counted in a Beckman liquid scintillation counter.

2.7. Calculation of data

All values are presented as the mean ± S.E.M. $[^3H]IP_3$ data are reported as dpm/mg protein. Dose–response curves for muscle contraction were constructed by cumulative addition of the agonist in the organ bath. The concentration of the agonist was increased only after the response to the previous concentration had stabilized. EC_{50} value is defined as that concentration of the agonist that produces 50% of the maximum response. Statistical significance between means was determined using the Student's *t*-test for unpaired values. A $P < 0.05$ was taken as significant.

3. Results

3.1. Representative recordings of the effects of PD98059 on prostaglandin $F_{2\alpha}$-, latanoprost-, and carbachol-induced contraction

Previously, we reported that prostaglandin $F_{2\alpha}$ and carbachol induced contraction in this smooth muscle in a concentration-dependent manner with EC_{50} values of 0.92×10^{-9} and 1.75×10^{-8} M, respectively (Yousufzai and Abdel-Latif, 1998). To explore the involvement of MAP kinase in agonist-induced contraction of the iris sphincter, we used PD98059. This compound prevents the activation

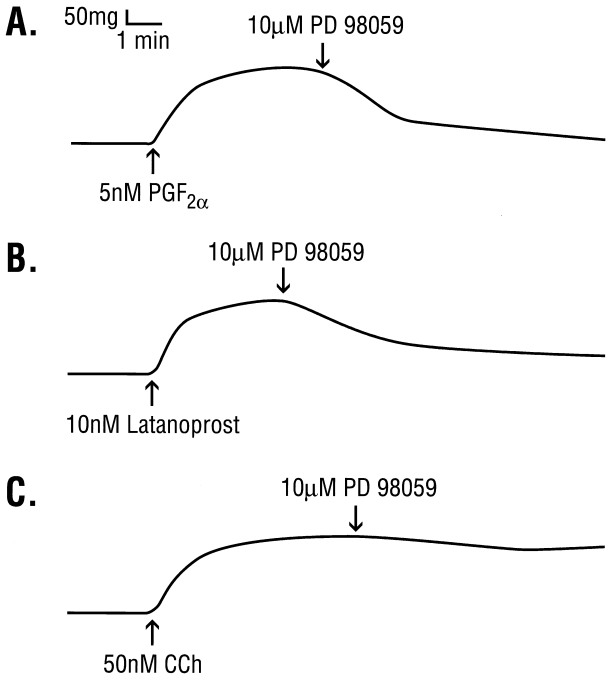

Fig. 1. Representative recordings of mechanical responses of cat iris sphincter muscle to prostaglandin $F_{2\alpha}$ (A), latanoprost (B) and carbachol (C) in the absence and presence of PD98059. The muscles were pre-equilibrated in Krebs–Ringer bicarbonate containing 1 μM indomethacin for 90 min, prostaglandin $F_{2\alpha}$ (5 nM), latanoprost (10 nM) and carbachol (50 nM) were then added as indicated for 3–5 min followed by addition of PD98059 (10 μM).

of MAP kinase through inhibition of activation of the upstream MAP kinase kinase (MEK) and has been widely used to investigate the role of ERK-MAP kinase (Dessy et al., 1998; Dudley et al., 1996; Servant et al., 1996). Fig. 1 shows typical recordings of mechanical responses to prostaglandin $F_{2\alpha}$, latanoprost and carbachol in the absence and presence of the specific p42/p44 MAP kinase inhibitor. At 10 μM, PD98059 completely blocked (relaxed) prostaglandin $F_{2\alpha}$- and latanoprost-induced contraction but it had little effect on that of carbachol. The inhibition was rapid, it began within 1 min and stabilized at 5–10 min. The inhibitory action of PD98059 was readily reversible upon washing the preparation free from the enzyme inhibitor. Pretreatment of the muscle with PD98059 inhibited the contraction induced by prostaglandin $F_{2\alpha}$ but not that induced by carbachol (data not shown). These results demonstrate that in cat iris sphincter, MAP kinase activity is involved in the signal transduction pathway by which muscle contraction is induced by prostaglandin $F_{2\alpha}$ and its analog latanoprost, but not by carbachol.

3.2. Effects of MAP kinase- and other protein kinase inhibitors on prostaglandin $F_{2\alpha}$- and on carbachol-induced contraction

To further investigate the role of MAP kinases in prostaglandin $F_{2\alpha}$-induced contraction in the iris sphincter

Table 1
Effects of MAP kinase and other protein kinase inhibitors on prostaglandin $F_{2\alpha}$- and on carbachol-induced contraction in cat iris sphincter

Inhibitor added	Concentration (μM)	Contractile response (mg tension per mg wet weight tissue)			
		Prostaglandin $F_{2\alpha}$ (5 nM)	% of Control	Carbachol (50 nM)	% of Control
PD98059	1	14.0 ± 1.2	75	17.2 ± 1.2	100
PD98059	10	1.1 ± 0.1	6	14.1 ± 1.0	82
Apigenin	1	14.5 ± 1.0	78	18.1 ± 1.0	100
Apigenin	10	2.4 ± 0.2	13	15.7 ± 1.0	87
U0126	1	16.5 ± 1.2	89	18.0 ± 1.2	100
U0126	10	3.6 ± 0.2	20	15.5 ± 1.3	86
SB203580	1	18.0 ± 1.3	97	17.6 ± 0.9	100
SB203580	10	11.2 ± 1.1	60	15.5 ± 1.2	88
KN-93	1	12.1 ± 1.4	65	11.6 ± 1.0	70
KN-93	10	3.7 ± 0.2	20	3.0 ± 0.1	18
Wortmannin	1	11.2 ± 1.0	60	9.4 ± 0.6	55
Wortmannin	10	3.7 ± 0.1	20	3.1 ± 0.1	18

Sphincter muscles were contracted by 5 nM prostaglandin $F_{2\alpha}$(18.6 mg tension per mg wet weight tissue) or by 50 nM carbachol (17.4 mg tension per mg wet weight tissue) for 3 min followed by addition of the inhibitors as indicated for 15 min. The data are the mean ± S.E.M. of three to five different experiments.

we investigated the effects of MAP kinase- and other protein kinase inhibitors (Table 1). PD98059, Apigenin, and UO126 are potent inhibitors of p42/p44 MAP kinase activity, whereas SB203580 is a potent inhibitor of p38 MAP kinase activity. At 1 μM concentrations, PD98059, Apigenin, UO126 and SB203580 inhibited prostaglandin $F_{2\alpha}$-induced contraction by 25%, 22%, 11% and 3%, respectively, and at 10 μM they inhibited the contractile response by 94%, 87%, 80% and 40%, respectively. At the same concentrations, these inhibitors had little effect on carbachol-induced contraction (Table 1). At 10 μM concentrations, KN-93, a calmodulin kinase II inhibitor, and Wortmannin, an MLC kinase inhibitor, inhibited by more than 80% both prostaglandin $F_{2\alpha}$- and carbachol-induced contraction (Table 1). These data demonstrate involvement of p42/p44 MAP kinase activity in prostaglandin $F_{2\alpha}$- but not in carbachol-induced contraction in this smooth muscle.

3.3. Concentration–contraction response curves of the effects of MAP kinase inhibitors on prostaglandin $F_{2\alpha}$-induced contraction

Results of concentration–response studies on the inhibitory effects of the MAP kinase inhibitors PD98059, apigenin, UO126 and SB203580 on prostaglandin $F_{2\alpha}$-induced contraction are given in Fig. 2. PD98059, apigenin, UO126 and SB203580 inhibited prostaglandin $F_{2\alpha}$-induced

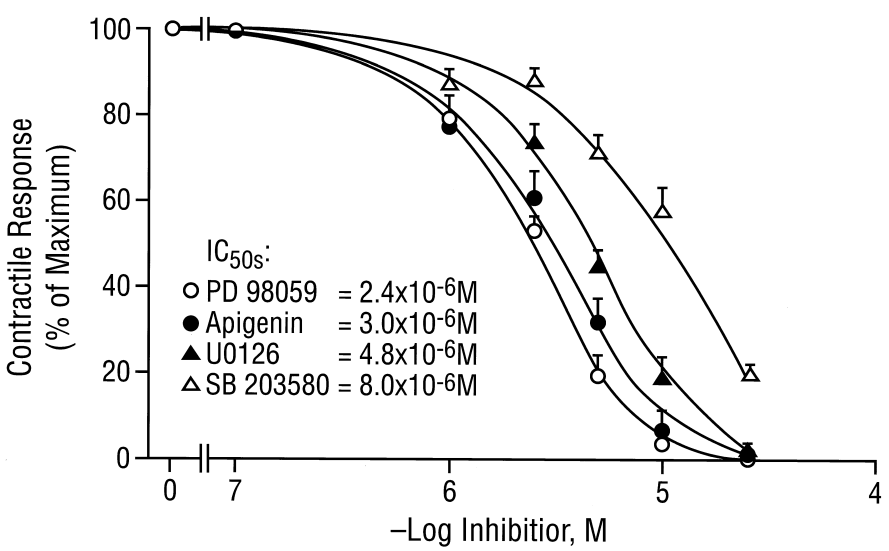

Fig. 2. Concentration–contraction response curves of the effects of MAP kinase inhibitors on prostaglandin $F_{2\alpha}$-induced contraction in cat iris sphincter. The pre-equilibrated muscles were first precontracted with prostaglandin $F_{2\alpha}$ (5 nM) for 3 min, then different concentrations of the inhibitors were added as indicated and changes in tension responses were monitored as described in Section 2.

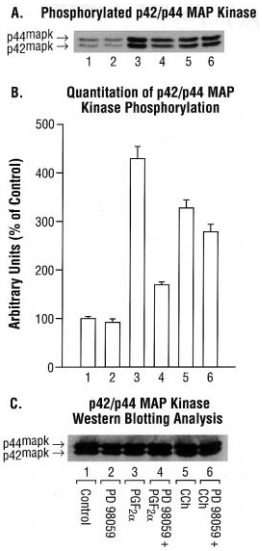

Fig. 3. Effect of PD98059 on prostaglandin $F_{2\alpha}$- and carbachol-induced MAP kinase phosphorylation in iris sphincter. After 90 min of equilibration in Krebs–Ringer bicarbonate buffer (pH 7.4), iris sphincter muscles were treated for 30 min with PD98059 (10 μM) prior to stimulation with prostaglandin $F_{2\alpha}$ (1 μM) or carbachol (10 μM) for 2 min. Phosphorylated MAP kinase was determined by using Western blot and immunodetection as described in Section 2. (A) Phosphorylated p42/p44 MAP kinase. (B) Quantitation of p42/p44 MAP kinase phosphorylation. Phosphorylation of MAP kinases in the iris sphincter was quantitated by densitometry. The autoradiographs were analyzed by densitometry, and the values are expressed in arbitrary optical density units as mean ± S.E.M. of three independent experiments performed in duplicate. (C) Western blot analysis of the immunoprecipitated MAP kinase.

contraction in a concentration-dependent manner with IC_{50} values of 2.4, 3.0, 4.8 and 8 μM, respectively. Under the same experimental conditions, these MAP kinase inhibitors had little effect on carbachol-induced contraction in the smooth muscle (data not shown). PD98059, which was most potent, was used throughout the following studies.

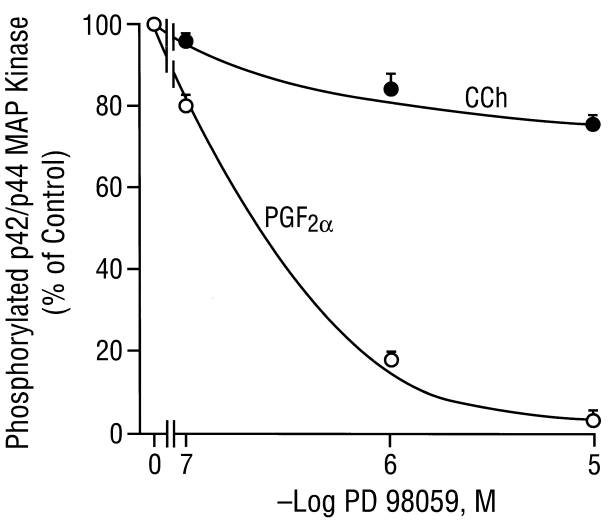

Fig. 4. Concentration-dependence of PD98059-induced inhibition of prostaglandin $F_{2\alpha}$- and carbachol-stimulated MAP kinase phosphorylation in cat iris sphincter. Incubation conditions and methods of analysis were the same as described in Fig. 3 except that various concentrations of PD98059 were added as indicated. Iris sphincter muscles were incubated with different concentrations of PD98059, stimulated with either prostaglandin $F_{2\alpha}$ (1 μM) or carbachol (10 μM) for 2 min, and then analyzed for phosphorylated p42/p44 MAP kinase. Values shown are means ± S.E.M. for two experiments each run in triplicate.

3.4. Effects of PD98059 on prostaglandin $F_{2\alpha}$- and on carbachol-induced MAP kinase phosphorylation

MAP kinase can be activated by either tyrosine kinase- or G-protein-linked receptors. In previous communications from this laboratory we demonstrated the presence of p42/p44 MAP kinase (Husain and Abdel-Latif, 1998) and p38 MAP kinase (Husain and Abdel-Latif, 1999) in the cat iris sphincter. The effects of PD98059 on prostaglandin $F_{2\alpha}$- and on carbachol-induced MAP kinase phosphorylation were determined. Fig. 3A and C shows representative immunoblots of phosphorylated (active) and total MAP kinase, respectively, from iris sphincter treated with the agonists in the absence and presence of PD98059. Fig. 3B is a quantitation of p42/p44 kinase phosphorylation. As can be seen from Fig. 3, stimulation of prostaglandin $F_{2\alpha}$

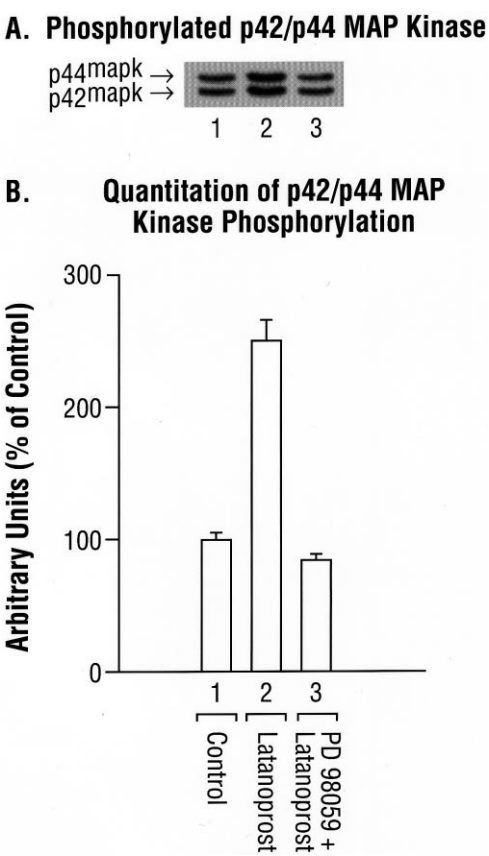

Fig. 5. Effect of PD98059 on latanoprost-induced MAP kinase phosphorylation in cat iris sphincter. Incubation conditions and methods of analysis were the same as described under Fig. 3 except that latanoprost was added instead of prostaglandin $F_{2\alpha}$. (A) Phosphorylated p42/p44 MAP kinase. (B) Quantitation of p42/p44 MAP kinase phosphorylation. The results are means ± S.E.M. obtained from two to three experiments each run in triplicate.

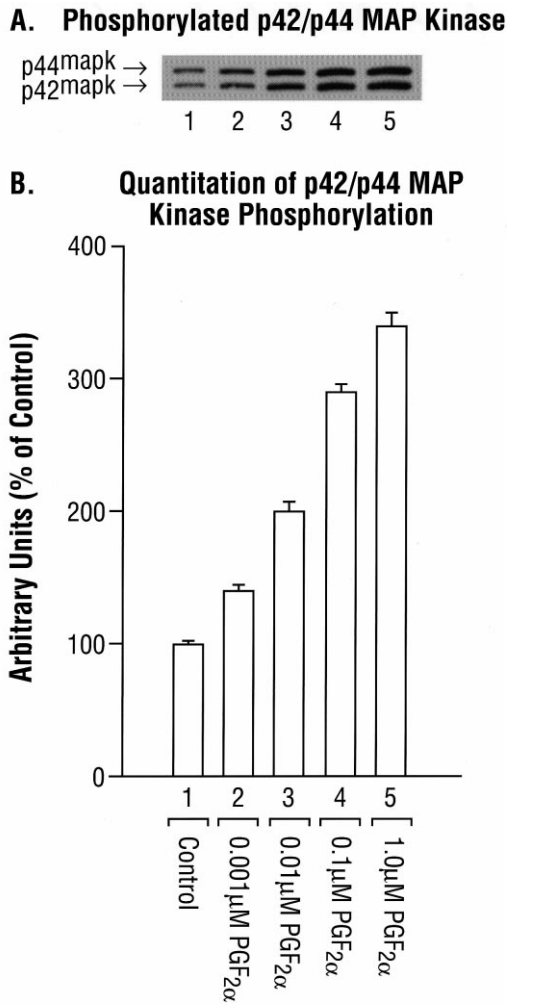

Fig. 6. Concentration–response to prostaglandin $F_{2\alpha}$ for MAP kinase phosphorylation in cat iris sphincter. Incubation conditions and methods of analysis were the same as described in Fig. 3 except that various concentrations of prostaglandin $F_{2\alpha}$ were added as indicated. (A) Phosphorylated p42/p44 MAP kinase. (B) Quantitation of p42/p44 MAP kinase phosphorylation. The results are means ± S.E.M. obtained from two to three experiments each run in triplicate.

receptor, a G-protein-linked receptor, by prostaglandin $F_{2\alpha}$, resulted in a 330% increase in MAP kinase phosphorylation (indicative of MAP kinase activity) and this was blocked by PD98059. In contrast, activation of muscarinic receptors by carbachol resulted in a 230% increase in MAP kinase phosphorylation and this was not significantly inhibited by PD98059. These results demonstrate that both prostaglandin $F_{2\alpha}$ and muscarinic receptors are linked to the activation of MAP kinase but only activation of MAP kinase by prostaglandin $F_{2\alpha}$ is sensitive to PD98059 inhibition.

3.5. Concentration dependence of PD98059-induced inhibition of prostaglandin $F_{2\alpha}$- and carbachol-stimulated MAP kinase phosphorylation

The effects of different concentrations of PD98059 on prostaglandin $F_{2\alpha}$- and carbachol-induced MAP kinase phosphorylation was also determined. In the presence of prostaglandin $F_{2\alpha}$, phosphorylated (active) MAP kinase levels decreased with increasing concentrations of PD98059 (Fig. 4). At 1 μM, PD98059 inhibited prostaglandin $F_{2\alpha}$-induced MAP kinase phosphorylation by 86%, but it had little effect on that of carbachol.

3.6. Effect of PD98059 on latanoprost-induced MAP kinase phosphorylation

Latanoprost, the prostaglandin $F_{2\alpha}$ analog, increased MAP kinase phosphorylation by 150%, and this was completely blocked by PD98059 (Fig. 5). These results indicate that stimulation of prostaglandin $F_{2\alpha}$ receptors by latanoprost is linked to the activation of MAP kinase.

3.7. Concentration–response to prostaglandin $F_{2\alpha}$ for MAP kinase phosphorylation

Exposure of iris sphincter muscle to increasing concentrations of prostaglandin $F_{2\alpha}$ for 2 min increased MAP kinase phosphorylation in a concentration-dependent manner (Fig. 6). A significant increase (40%) was already observed with 0.001 μM, and at 1 μM the increase in MAP kinase phosphorylation due to prostaglandin $F_{2\alpha}$ was 240%.

3.8. Effects of MAP kinase inhibitors on prostaglandin $F_{2\alpha}$- and carbachol-induced IP_3 production

Activation of prostaglandin $F_{2\alpha}$ receptors in the iris sphincter leads to the hydrolysis of polyphosphoinositides and the generation of the two second messengers IP_3 and

Table 2
Effects of MAP kinase inhibitors on prostaglandin $F_{2\alpha}$- and carbachol-induced IP_3 production in cat iris sphincter

Additions	[^{3}H]IP_3 production (dpm/mg protein)	% of Control
None	18,596 ± 1651	100
1 μM $PGF_{2\alpha}$	49,864 ± 3850[a]	268
1 μM $PGF_{2\alpha}$ + 10 μM PD98059	46,118 ± 2516[a]	248
10 μM Carbachol	44,816 ± 3921[a]	241
10 μM Carbachol + 10 μM PD98059	45,932 ± 4196[a]	247

Muscles were labeled with *myo*-[^{3}H]inositol (10 μCi/ml) in Krebs–Ringer bicarbonate buffer for 90 min at 37°C. The labeled muscles were washed with non-radioactive buffer and the tissues were incubated in absence or presence of different MAP kinase inhibitors for 30 min. Prostaglandin $F_{2\alpha}$ (1 μM) or carbachol (10 μM) were then added as indicated for 5 min and *myo*-[^{3}H]inositol phosphates analyzed by anion-exchange chromatography. The results are means ± S.E.M. obtained from three to four different experiments, each run in triplicate.

[a] Significant differences from control, $P < 0.001$; no significant differences between the effects of the agonists on [^{3}H]IP_3 production in the absence and presence of the MAP kinase inhibitors.

Table 3
Effect of PD98059 on prostaglandin $F_{2\alpha}$- and carbachol-induced myosin-light chain phosphorylation (MLC) in cat iris sphincter

Additions	^{32}P-Radioactivity in MLC (cpm/mg protein)	% of Control
None	76,671 ± 7503	100
PD98059 (10 μM)	76,742 ± 7346	100
Prostaglandin $F_{2\alpha}$ (1 μM)	129,036 ± 10,946	168
Prostaglandin $F_{2\alpha}$ (1 μM) + PD98059 (10 μM)	85,541 ± 7701	112
Carbachol (10 μM)	116,540 ± 8891	152
Carbachol (10 μM) + PD98059 (10 μM)	110,073 ± 9473	144

Time of incubation with the MAP kinase inhibitor was 30 min prior to addition of the agonists. Prostaglandin $F_{2\alpha}$ and carbachol were added for 2 min. Incubation conditions and analysis of MLC phosphorylation were the same as described under Methods. The results are means ± S.E.M. obtained from five to seven experiments, each run in triplicate.

diacylglycerol (Yousufzai et al., 1988). Addition of either prostaglandin $F_{2\alpha}$ or carbachol to iris sphincter muscle prelabeled with *myo*-[^{3}H]inositol increased [^{3}H]IP_3 production by about 150% (Table 2). However, pre-incubation of the labeled tissue with the MAP kinase inhibitor, PD98059, had no inhibitory effect on the stimulation of IP_3 production by prostaglandin $F_{2\alpha}$ or by carbachol. These results indicate that MAP kinase is not associated with the production of IP_3.

3.9. Effects of prostaglandin $F_{2\alpha}$ and carbachol, in the absence and presence of PD98059, on MLC phosphorylation in iris sphincter muscle

MLC phosphorylation catalyzed by the Ca^{2+}/calmodulin-dependent MLC kinase is the major regulatory pathway for excitation–contraction coupling in smooth muscle (Kamm and Stull, 1985). Thus, it was of interest in the present work to determine the effects of PD98059 on prostaglandin $F_{2\alpha}$- and on carbachol-induced MLC phosphorylation in this smooth muscle. As can be seen from Table 3, when the muscles were incubated in a medium containing ^{32}P and then stimulated with prostaglandin $F_{2\alpha}$ or carbachol the increases in ^{32}P labeling of MLC were 68% and 52%, respectively. The stimulatory effects of prostaglandin $F_{2\alpha}$, but not that of carbachol, were markedly blocked by PD98059. PD98059 alone had no effect on the labeling of MLC. These results demonstrate that in this smooth muscle, MAP kinase activity is involved in the signal transduction pathway by which muscle MLC phosphorylation is induced by prostaglandin $F_{2\alpha}$.

4. Discussion

We have previously reported that in cat iris sphincter the protein tyrosine kinase inhibitor, genistein, blocked the stimulatory effects of prostaglandin $F_{2\alpha}$, but not those evoked by carbachol, on IP_3 accumulation, intracellular Ca^{2+} mobilization and contraction, suggesting involvement of protein tyrosine kinase activity in the physiological actions of the prostaglandin (Yousufzai and Abdel-Latif, 1998). Furthermore, in cat iris sphincter smooth muscle cells genistein inhibited endothelin-1-stimulated p42/p44 MAP kinase activity (Husain and Abdel-Latif, 1998). Taken together, these studies suggest that prostaglandin $F_{2\alpha}$ may stimulate tyrosine kinase(s) to result in smooth muscle contraction. In the present work, we have demonstrated that stimulation of the iris sphincter with prostaglandin $F_{2\alpha}$ increases p42/p44 MAP kinase activity, MLC phosphorylation and contraction (Figs. 1, 3 and 6, Table 1) and that these effects are inhibited by PD98059. In addition, we observed that enhanced MAP kinase phosphorylation due to carbachol is not involved in the muscarinic stimulation of MLC phosphorylation and contraction (Fig. 1, Table 3) — thus suggesting that the stimulation of the iris sphincter with prostaglandin $F_{2\alpha}$ and carbachol activate two separate pathways. PD98059 alone had no effect on basal MAP kinase phosphorylation, IP_3 production, MLC phosphorylation, or contraction. The conclusion that p42/p44 MAP kinase activity is involved in prostaglandin $F_{2\alpha}$-induced contraction in this smooth muscle is supported by the following findings in the present work: (1) Prostaglandin $F_{2\alpha}$ increased p42/p44 MAP kinase phosphorylation (activation) in a concentration-dependent manner with EC_{50} value of 1.1×10^{-8} M (Fig. 6) and induced contraction in a concentration-dependent manner with EC_{50} value of 0.92×10^{-9} M (Yousufzai and Abdel-Latif, 1998). (2) Latanoprost, a prostaglandin $F_{2\alpha}$ analog, also increased MAP kinase phosphorylation and contraction and these effects were inhibited by PD98059 (Figs. 1 and 5). (3) The MAP kinase inhibitors PD98059, Apigenin and UO126 inhibited prostaglandin $F_{2\alpha}$-induced contraction in a concentration-dependent manner with IC_{50} values of 2.4, 3.0 and 4.8 μM, respectively (Fig. 2). (4) PD98059 inhibited prostaglandin $F_{2\alpha}$-induced MAP kinase phosphorylation with IC_{50} of 2.1×10^{-7} M, without affecting MAP kinase phosphorylation caused by carbachol (Fig. 4). Prostaglandin $F_{2\alpha}$ stimulated the MAP kinase signaling pathway in cultured rat puerperal uterine myometrial cells through Gβγ protein (Ohmichi et al., 1997). Several investigators have shown that MAP kinases are activated during stimulation of vascular smooth muscle (Adam et al., 1995; Katoch and Moreland, 1995; Gerthof-

fer et al., 1996; Watts, 1996). Whether or not MAP kinase activation is important in the actual contractile event is controversial. Watts (1996) and Dessy et al. (1998) have suggested that a small but significant proportion of force development is sensitive to inhibition of MAP kinase. Gerthoffer et al. (1996) demonstrated that the addition of activated MAP kinase to a permeabilized fiber resulted in a contraction. In contrast, Gorenne et al. (1998) reported that inhibition of p42/p44 MAP kinase does not alter smooth muscle contraction in swine carotid artery. PD98059, the p42/p44 MAP kinase inhibitor, had no effect on neither prostaglandin $F_{2\alpha}$ — nor on carbachol-induced production of IP_3 in this smooth muscle (Table 2). In contrast, Genistein inhibited prostaglandin $F_{2\alpha}$-induced IP_3 Production in a concentration-dependent manner with IC_{50} of 1×10^{-6} M, without affecting IP_3 production caused by carbachol (Yousufzai and Abdel-Latif, 1998). Nohara et al. (1996), working with uterine smooth muscle in pregnant rat, reported that PD98059 had no effect on oxytocin-induced intracellular Ca^{2+} mobilization in either pregnant human or pregnant rat uterine cells, however, it partly inhibited oxytocin-induced pregnant rat uterine contraction in a concentration-dependent manner. These data suggest that the mechanism of prostaglandin $F_{2\alpha}$-induced contraction in this smooth muscle involves both the MAP kinase pathway and the Ca^{2+} mobilization pathway. The observation that the MAP kinase inhibitor has little effect on carbachol-induced MLC phosphorylation and contraction suggest that the mechanism of carbachol-induced iris sphincter contraction involves mainly the Ca^{2+} mobilization pathway. The finding that KN-93, a calmodulin kinase II inhibitor, inhibited both prostaglandin $F_{2\alpha}$- and carbachol-induced contraction (Table 1) could suggest that calmodulin kinase II is an upstream activator of MAP kinase. A Ca^{2+}-dependent isoform of protein kinase C and calmodulin kinase II have been suggested as upstream activators of MAP kinase in the swine carotid artery (Katoch et al., 1999).

It is of considerable interest that the stimulatory actions of prostaglandin $F_{2\alpha}$ on MLC phosphorylation and contraction are inhibited by the MAP kinase inhibitors in this smooth muscle. This observation could shed more light on the molecular mechanism underlying the intraocular pressure lowering effects of prostaglandin $F_{2\alpha}$ and its analog, latanoprost, an anti-glaucoma drug routinely used in the treatment of glaucoma patients (Stjernschantz et al., 1995). These effects of prostaglandin $F_{2\alpha}$ are specific and supportive of a role for MAP kinase activity in the contractile action of this prostaglandin in the iris sphincter. MAP kinase activity does not seem to be involved in the physiological actions of the muscarinic agonist carbachol.

In conclusion, the results obtained with the MAP kinase inhibitors suggest an important role for protein MAP kinase activity in the stimulatory action of prostaglandin $F_{2\alpha}$ on MLC phosphorylation and contraction in the cat iris sphincter. In addition, the data presented clearly indicate that the stimulation of the iris sphincter with prostaglandin $F_{2\alpha}$ and carbachol activate two distinct pathways, namely the MAP kinase pathway and the Ca^{2+} mobilization pathway. This is supported by the differences presented on the effects of PD98059 on MLC phosphorylation and contraction between prostaglandin $F_{2\alpha}$ and carbachol stimulation. Further studies aimed at elucidating the role of MAP kinase activity in the coupling mechanism between the activated prostaglandin $F_{2\alpha}$ receptor and MLC phosphorylation and contraction and identifying the tyrosine-phosphorylated substrates will provide important information about the role of MAP kinase in the mechanism of smooth muscle contraction. Furthermore, understanding the mechanism of action of prostaglandin $F_{2\alpha}$ and latanoprost in the smooth muscles of the iris–ciliary body could lead to the development of better anti-glaucoma drugs.

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