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Tyrosine kinase inhibitors suppress prostaglandin $F_{2\alpha}$-induced phosphoinositide hydrolysis, Ca^{2+} elevation and contraction in iris sphincter smooth muscle

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Abstract

We investigated the effects of the protein tyrosine kinase inhibitors, genistein, tyrphostin 47, and herbimycin on prostaglandin $F_{2\alpha}$- and carbachol-induced inositol-1,4,5-trisphosphate (IP_3) production, $[Ca^{2+}]_i$ mobilization and contraction in cat iris sphincter smooth muscle. Prostaglandin $F_{2\alpha}$ and carbachol induced contraction in a concentration-dependent manner with EC_{50} values of 0.92×10^{-9} and 1.75×10^{-8} M, respectively. The protein tyrosine kinase inhibitors blocked the stimulatory effects of prostaglandin $F_{2\alpha}$, but not those evoked by carbachol, on IP_3 accumulation, $[Ca^{2+}]_i$ mobilization and contraction, suggesting involvement of protein tyrosine kinase activity in the physiological actions of the prostaglandin. Daidzein and tyrphostin A, inactive negative control compounds for genistein and tyrphostin 47, respectively, were without effect. Latanoprost, a prostaglandin $F_{2\alpha}$ analog used as an antiglaucoma drug, induced contraction and this effect was blocked by genistein. Genistein (10 μM) markedly reduced (by 67%) prostaglandin $F_{2\alpha}$-stimulated increase in $[Ca^{2+}]_i$ but had little effect on that of carbachol in cat iris sphincter smooth muscle cells. Vanadate, a potent inhibitor of protein tyrosine phosphatase, induced a slow gradual muscle contraction in a concentration-dependent manner with an EC_{50} of 82 μM and increased IP_3 generation in a concentration-dependent manner with an EC_{50} of 90 μM. The effects of vanadate were abolished by genistein (10 μM). Wortmannin, a myosin light chain kinase inhibitor, reduced prostaglandin $F_{2\alpha}$- and carbachol-induced contraction, suggesting that the involvement of protein tyrosine kinase activity may lie upstream of the increases in $[Ca^{2+}]_i$ evoked by prostaglandin $F_{2\alpha}$. Further studies aimed at elucidating the role of protein tyrosine kinase activity in the coupling mechanism between prostaglandin $F_{2\alpha}$ receptor activation and increases in intracellular Ca^{2+} mobilization and identifying the tyrosine-phosphorylated substrates will provide important information about the role of protein tyrosine kinase in the mechanism of smooth muscle contraction, as well as about the mechanism of the intraocular pressure lowering effect of the prostaglandin in glaucoma patients.

Keywords: Nonvascular smooth muscle, nonvascular; Tyrosine phosphorylation; 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, 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

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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).

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, 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 smooth muscle, 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 (PIP_2), and to muscle contraction (Yousufzai et al., 1988; Abdel-Latif, 1991). Diacylglycerol activates protein kinase C and IP_3 stimulates the release of intracellular Ca^{2+} (Berridge, 1993; Abdel-Latif, 1996). At least three classes of phospholipase C isozymes (phospholipase C-β, phospholipase C-γ, phospholipase C-δ) induce the hydrolysis of PIP_2 (Rhee and Bae, 1997). Phospholipase C-β isoforms are activated through G proteins, whereas phospholipase C-γ isoforms are activated as a result of phosphorylation by protein tyrosine kinases. In the latter, phosphorylation is catalyzed by either growth factor receptors that possess ligand-activated protein tyrosine kinase domains or by nonreceptor protein tyrosine kinases (Rhee and Bae, 1997). 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 tyrosine kinase in osteoblastic MC3T3-E1 cells (Hakeda et al., 1997).

To our knowledge, the effects of prostaglandin $F_{2\alpha}$ on protein tyrosine kinase activity in contractile tissue have not yet been investigated, although tyrosine dependent signaling pathways are known to be operative in smooth muscle cells for other Ca^{2+}-mobilizing agonists (Di Salvo et al., 1993a,b; Yang et al., 1993; Molloy et al., 1993). There is accumulating evidence which indicates that in addition to the myosin light chain kinase pathway, tyrosine kinase pathways play an important role in the regulation of smooth muscle contraction (Di Salvo et al., 1993a,b, 1997; Hollenburg, 1994). 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 tyrosine phosphorylation is involved in prostaglandin $F_{2\alpha}$- and carbachol-induced contraction in the cat iris sphincter smooth muscle. We found that 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 in this nonvascular smooth muscle.

2. Materials and methods

2.1. Materials

Reagent sources were as follows: prostaglandin $F_{2\alpha}$ and latanoprost from Cayman, Ann Arbor, MI; genistein and herbimycin from Biomol Research Laboratory, Plymouth Meeting, PA; tyrphostin, Wortmannin and Carbachol from Sigma, St. Louis, MO; Fura-2 acetoxymethyl ester (Fura-2/AM) from Molecular Probes, Eugene, OR; *myo*-[^{3}H]inositol (18 Ci/mmol) from Amersham, Arlington Heights, IL; and Dowex AG 1-X8 anion-exchange resin (200 to 400 mesh, formate form) from BioRad, Melville, NY.

The tyrosine 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 IP_3 formation and muscle 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 (KRB; pH 7.4) containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM $KHPO_4$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 10 mM D-glucose and 1.25 mM $CaCl_2$. The KRB 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. We routinely test the contractility of the muscle with carbachol before the effects of prostaglandin $F_{2\alpha}$ on the pharmacological responses are investigated.

2.3. Immortalized cell culture

Immortalized cat iris sphincter smooth muscle (SV-CISM-2) cells were maintained in Dulbecco's-modified

Eagle medium (DMEM) supplemented with 10% fetal calf serum and 50 μg/ml gentamicin (culture medium) as described previously (Ocklind et al., 1995). To initiate subculture, the confluent SV-CISM-2 cells were washed with Ca^{2+}–Mg^{2+}-free Dulbecco's phosphate buffered salt solution (PBS) and treated with 0.05% trypsin in 0.5 mM EDTA for 3 min at 37°C. This was followed by addition of culture medium, and the cell suspension was centrifuged at $200 \times g$ for 5 min. The pelleted cells were suspended in culture medium and seeded on coverslips in 60 mm dishes for the studies on $[Ca^{2+}]_i$ mobilization, at a split ratio of 1:4. The cultures were maintained by changing the medium every other day until the cells became confluent.

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 KRB 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. LiCl, to a final concentration of 10 mM, was added to each incubation. Ten minutes later, carbachol or prostaglandin $F_{2\alpha}$ was added as indicated, and incubation continued for another 5 min. When used, the tyrosine 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. Measurement of cytosolic Ca^{2+} concentration

SV-CISM-2 cells were subcultured on glass coverslips in 60 mm dishes until they became confluent. $[Ca^{2+}]_i$ was measured in confluent monolayers with the calcium-sensitive dye Fura-2-acetoxymethyl ester (fura-2/AM) as described previously (Ding et al., 1997). Briefly, the cells were washed once with 2 ml KRB buffer, then loaded with 5 μM fura-2/AM in KRB buffer at room temperature for 45 min. At the end of the loading period, the coverslips were washed twice with the buffer and then incubated in buffer without fura-2/AM for another 30 min. The coverslip was inserted into a quartz cuvette in a dual-wavelength spectrofluorometer (Photon Technologies International, South Brunswick, NJ). Fluorescence of Ca^{2+}-bound and unbound fura-2 was measured at room temperature by rapidly alternating the dual excitation wavelengths between 340 and 380 nm and electronically separating the resultant fluorescence signals at an emission wavelength of 510 nm. Autofluorescence was measured in unloaded cells and this value was subtracted from all the measurements. Minimum emission (R_{min}) was measured upon addition of EGTA (8 mM) containing Tris buffer (pH 8.1) followed by addition of ionomycin (10 μM). Maximum emission (R_{max}) was measured by adding 20 mM $CaCl_2$ to the cuvette. Free $[Ca^{2+}]_i$ concentration was calculated using the equation: $[Ca^{2+}]_i = K_d \times (F_{380}\ \text{free}/F_{380}\ \text{sat}) \times (R - R_{min})/(R_{max} - R)$. The K_d of fura-2 for Ca^{2+} was assumed to be 224 nM (Grynkiewicz et al., 1985).

2.6. 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 KRB buffer. A mixture of 97% O_2–3% CO_2 was bubbled continuously through the buffer,

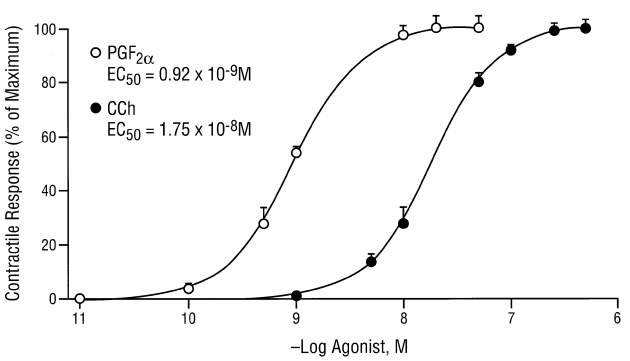

Fig. 1. Concentration–response effects of prostaglandin $F_{2\alpha}$ and carbachol on muscle contraction in cat iris sphincter. The increase in isometric tension after exposure of the muscle to prostaglandin $F_{2\alpha}$ and carbachol was measured as described in Section 2. A supramaximal dose of prostaglandin $F_{2\alpha}$ (50 nM) and carbachol (0.5 μM) elicited contractile responses of 36 ± 1.9 and 29 ± 1.8 mg tension per mg tissue wet weight, respectively, and these values were taken as 100% response. Each point represents data obtained from 3–4 muscle strips.

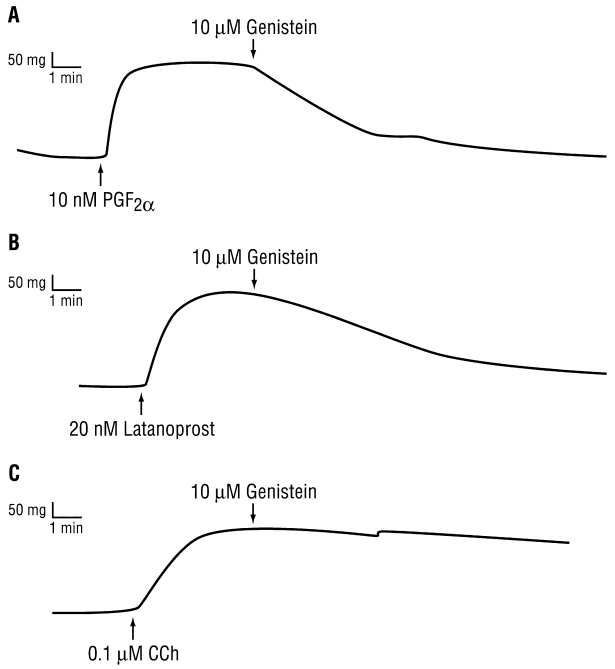

Fig. 2. Representative recordings of mechanical responses of cat iris sphincter muscle to prostaglandin $F_{2\alpha}$ (A), latanoprost (B) and carbachol (C) in the absence and presence of genistein. The muscles were pre-equilibrated in KRB buffer for 90 min, prostaglandin $F_{2\alpha}$ (10 nM), latanoprost (20 nM) and carbachol (0.1 μM) were then added as indicated for 3 min followed by addition of genistein (10 μM).

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).

3. Results

3.1. Concentration–response effects of prostaglandin $F_{2\alpha}$ and carbachol on muscle contraction in cat iris sphincter

Previously, we reported that prostaglandin $F_{2\alpha}$-1-isopropyl ester induces contraction in isolated cat iris sphincter with an EC_{50} of 1.3×10^{-9} M (Yousufzai et al., 1988). As can be seen from Fig. 1, 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.

3.2. Representative recordings of the effects of genistein on prostaglandin $F_{2\alpha}$-, latanoprost-, and carbachol-induced contraction in cat iris sphincter

The purpose of this experiment was to demonstrate whether tyrosine phosphorylation is involved in the mechanism of agonist-induced contraction in the cat iris sphincter. Fig. 2 shows typical recordings of mechanical responses of the sphincter muscle to prostaglandin $F_{2\alpha}$, latanoprost and carbachol in the absence and presence of the specific tyrosine kinase inhibitor genistein. At 10 μM, genistein completely blocked (relaxed) prostaglandin $F_{2\alpha}$- and latanoprost-induced contraction but had little effect on that of carbachol. The inhibition was rapid, it began within 1 min and stabilized at 10–15 min. This time course of inhibition is in agreement with the data reported by Yang et al. (1992) who showed that genistein inhibited prostaglandin $F_{2\alpha}$- and epidermal growth factor-induced contraction in gastric smooth muscle within 10–15 min. The inhibitory action of genistein was readily reversible upon washing the preparation free from the enzyme inhibitor. These results demonstrate that in cat iris sphincter, protein tyrosine kinase activity is involved in the signal transduc-

Table 1
Effects of various protein tyrosine 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 $F_{2\alpha}$ (10 nM)	Percent of control	Carbachol (0.1 μM)	Percent of control
Genistein	1	9.0 ± 1.0	45	16.0 ± 1.2	100
Genistein	10	2.8 ± 0.12	14	13.7 ± 1.2	85
Tyrphostin 47	1	13 ± 1.0	61	16.0 ± 1.0	101
Tyrphostin 47	10	4.0 ± 0.6	19	13.8 ± 1.0	87
Herbimycin	1	14.5 ± 1.0	59	15.6 ± 1.0	100
Herbimycin	10	6.4 ± 0.5	26	12.9 ± 1.2	86
Daidzein	25	–	no effect	no effect	–
Tyrphostin A	25	–	no effect	no effect	–
Wortmannin	1	9.2 ± 1.0	50	6.3 ± 0.3	48
	10	3.4 ± 0.4	18	2.0 ± 0.4	20

Sphincter muscles were contracted by prostaglandin $F_{2\alpha}$ or carbachol for 3 min followed by addition of the inhibitors as indicated for 10 min. The data are the mean ± S.E.M. of 2–3 different experiments.

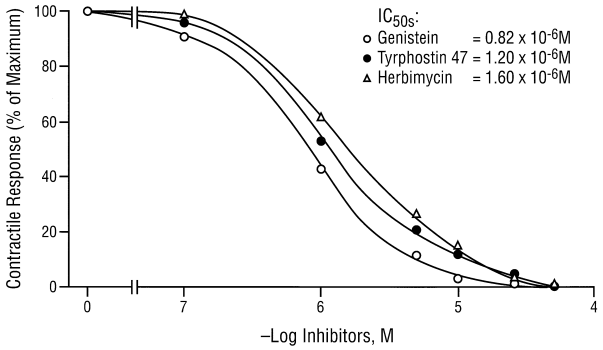

Fig. 3. Concentration–contraction response curves of the effects of protein tyrosine kinase inhibitors on prostaglandin $F_{2\alpha}$-induced contraction in cat iris sphincter. The pre-equilibrated muscles were first precontracted with prostaglandin $F_{2\alpha}$ (10 nM) for 3 min, then different concentrations of the inhibitors were added as indicated and changes in tension responses were monitored as described in Section 2.

tion pathway by which muscle contraction is induced by prostaglandin $F_{2\alpha}$ and its analog latanoprost, but not by carbachol.

3.3. Effects of various protein tyrosine kinase inhibitors on prostaglandin $F_{2\alpha}$- and on carbachol-induced contraction in cat iris sphincter

To further investigate the role of tyrosine phosphorylation in prostaglandin $F_{2\alpha}$-induced contraction in the iris sphincter we investigated the effects of three structurally unrelated tyrosine kinase inhibitors, genistein (Akiyama et al., 1987), tyrphostin (Gazit et al., 1989) and herbimycin (Fukazawa et al., 1991). Table 1 shows the effects of the tyrosine kinase inhibitors genistein, a natural tyrosine kinase inhibitor, tyrphostin 47, a synthetic tyrosine kinase inhibitor, herbimycin, a natural tyrosine kinase inhibitor, and daidzein and tyrphostin A, inactive negative control compounds for genistein and tyrphostin 47, respectively. At 1 μM concentrations, genistein, tyrphostin 47 and herbimycin inhibited prostaglandin $F_{2\alpha}$-induced contraction by 55, 39 and 41%, respectively, and at 10 μM they inhibited the contractile response by 86, 81 and 74%, respectively. At the same concentrations, these inhibitors had little effect on carbachol-induced contraction. Both daidzein and tyrphostin A had no effect neither on prostaglandin $F_{2\alpha}$- nor on carbachol-induced contractions (Table 1). At 1 μM, Wortmannin, which inhibits myosin light-chain kinase at concentrations 100-fold higher than that required for inhibition of PI 3-kinase (Nakanishi et al., 1992), inhibited prostaglandin $F_{2\alpha}$- and carbachol-induced contractions by 50 and 52%, respectively, and at 10 μM, the inhibition rose to 82 and 80%, respectively (Table 1). These data demonstrate that in cat iris sphincter protein tyrosine kinase activity is involved in prostaglandin $F_{2\alpha}$-induced contraction, but not in carbachol-induced contraction.

3.4. Concentration–contraction response curves of the effects of tyrosine kinase inhibitors on prostaglandin $F_{2\alpha}$-induced contraction in cat iris sphincter

Results of concentration–response studies on the inhibitory effects of genistein, tyrphostin 47 and herbimycin, which are known to inhibit protein tyrosine kinase activity through different mechanisms, on prostaglandin $F_{2\alpha}$-induced contraction are given in Fig. 3. Genistein, tyrphostin 47 and herbimycin inhibited prostaglandin $F_{2\alpha}$-induced contraction in a concentration-dependent manner with IC_{50} values of 0.82, 1.2 and 1.6 μM, respectively. These results indicate that in spite of the differences in their mechanism of action the three inhibitors markedly inhibited prostaglandin $F_{2\alpha}$-induced contraction and that their potency was in the following order: genistein > tyrphostin 47 > herbimycin. Genistein was used throughout the following experiments.

3.5. Concentration–response effects of genistein on prostaglandin $F_{2\alpha}$- and carbachol-induced IP_3 production in cat iris sphincter

Activation of prostaglandin $F_{2\alpha}$ receptors in the iris sphincter leads to the hydrolysis of PIP_2 and the generation of the two second messengers IP_3 and diacylglycerol (Yousufzai et al., 1988). Genistein (10^{-7} to 2.5×10^{-5} M) inhibited prostaglandin $F_{2\alpha}$-induced IP_3 production in a concentration-dependent manner with IC_{50} value of 1 μM (Fig. 4). Under the same experimental conditions the tyrosine kinase inhibitor had little effect on carbachol-

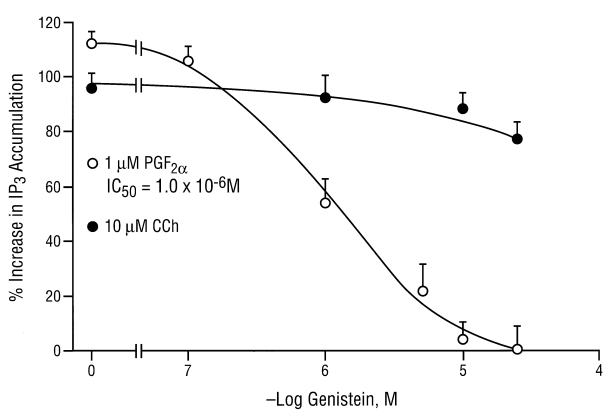

Fig. 4. Concentration–response effects of genistein on prostaglandin $F_{2\alpha}$- and on carbachol-induced IP_3 formation in cat iris sphincter. Muscles were labeled with [^{3}H]inositol (10 μCi/ml) in KRB buffer for 90 min at 37°C. The labeled muscles were washed with non-radioactive buffer, and different concentrations of genistein were added for 10 min. Prostaglandin $F_{2\alpha}$ (1 μM) or carbachol (10 μM) were then added for 5 min and [^{3}H]inositol phosphates were analyzed by anion-exchange chromatography. The basal level of IP_3 production was 5631 ± 469 dpm/mg protein. The results are means ± S.E.M. obtained from three different experiments.

induced IP_3 production in this smooth muscle (Fig. 4). These results demonstrate the involvement of protein tyrosine kinase activity in prostaglandin $F_{2\alpha}$-stimulated IP_3 formation in this tissue.

3.6. Effects of genistein on prostaglandin $F_{2\alpha}$- and carbachol-induced $[Ca^{2+}]_i$ mobilization in cat iris sphincter smooth muscle cells

As can be seen from Fig. 5A, prostaglandin $F_{2\alpha}$ induced an 86% increase in $[Ca^{2+}]_i$ in the iris sphincter smooth muscle cells. Genistein (10 μM) reduced the prostaglandin $F_{2\alpha}$-stimulated increase in $[Ca^{2+}]_i$ by 67% but had little effect on that of carbachol (Fig. 5B). Daidzein, the inactive negative control compound for genistein, had little effect on the prostaglandin $F_{2\alpha}$-stimulated Ca^{2+} mobilization. These results demonstrate the involvement of protein tyrosine kinase activity in prostaglandin $F_{2\alpha}$-induced increase in $[Ca^{2+}]_i$ in this tissue.

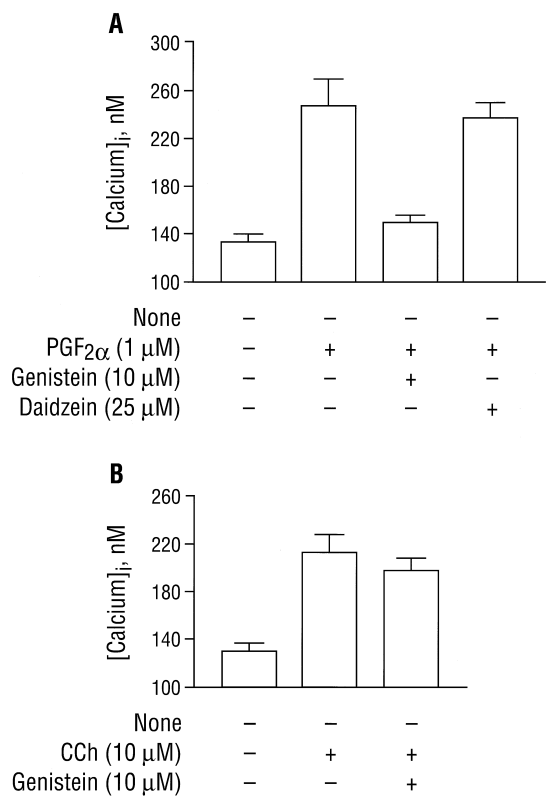

Fig. 5. Effects of genistein on prostaglandin $F_{2\alpha}$- and on carbachol-induced $[Ca^{2+}]_i$ mobilization in cat iris sphincter smooth muscle cells. The smooth muscle cells were first loaded with Fura-2/AM then stimulated with either 1 μM $PGF_{2\alpha}$ (A) or 10 μM carbachol (B) for 3 min, followed by the addition of genistein (10 μM) or daidzen (25 μM) as indicated. The fluorescence of the Ca^{2+} bound and unbound Fura-2 was measured using a dual-wavelength spectrofluorimeter as described in Section 2. The results are means ± S.E.M. obtained from 4–5 experiments.

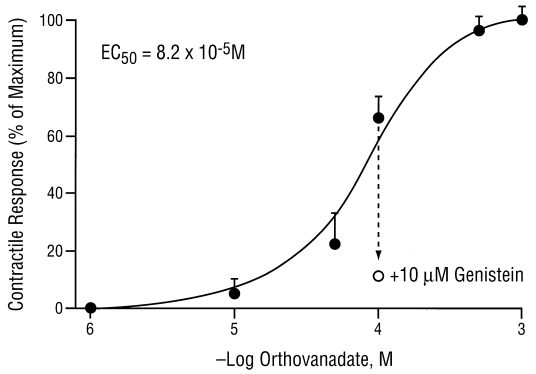

Fig. 6. Concentration–response to orthovanadate for contraction in cat iris sphincter. Incubation conditions were the same as described under Fig. 1 except that various concentrations of orthovanadate were added as indicated. Genistein (10 μM) was added 10 min before the addition of orthovanadate (100 μM). The results are means ± S.E.M. obtained from 2–3 experiments.

3.7. Concentration–response to sodium orthovanadate for contraction and IP_3 production in cat iris sphincter

Sodium orthovanadate, a potent inhibitor of protein tyrosine phosphatases (Tonks et al., 1988), has been reported to induce contraction in guinea pig taenia coli (Di Salvo et al., 1993a,b) and increase generation of inositol phosphates and tension in rat myometrium (Palmier et al., 1996). In the cat iris sphincter, vanadate induced a slow gradual muscle contraction, $t_{1/2}$ value = 7.3 min as compared to 0.8 min for prostaglandin $F_{2\alpha}$ (data not shown), in a concentration-dependent manner with an EC_{50} of 82 μM (Fig. 6) and increased IP_3 generation in a concentration-dependent manner with an EC_{50} of 90 μM (Fig. 7). Genistein (10 μM) completely blocked both vanadate-induced contraction and IP_3 generation (Figs. 6 and 7), thus demonstrating that in cat iris sphincter protein tyrosine

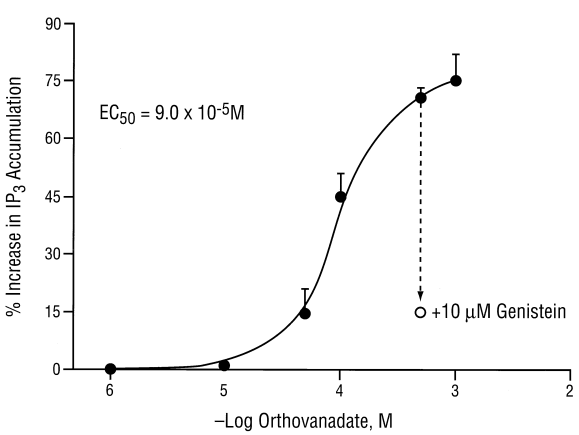

Fig. 7. Concentration–response to orthovanadate for IP_3 accumulation in cat iris sphincter. Incubation conditions were the same as described under Fig. 4 except that various concentrations of orthovanadate were added as indicated for 10 min. Genistein (10 μM) was added 10 min before the addition of orthovanadate (0.5 mM). The average basal release of IP_3 after 10 min of incubation was 4361 ± 381 dpm/mg protein. The results are means ± S.E.M. obtained from three experiments.

kinase/phosphatase activities are involved in the generation of IP_3 and contraction.

4. Discussion

In the present study, we employed three structurally unrelated protein tyrosine kinase inhibitors, genistein, tyrphostin 47 and herbimycin which are known to inhibit the kinase activity through different mechanisms, and orthovanadate, a potent protein tyrosine phosphatase inhibitor, to demonstrate that in cat iris sphincter smooth muscle tyrosine phosphorylation is involved in the mechanism of prostaglandin $F_{2\alpha}$-induced IP_3 production, $[Ca^{2+}]_i$ mobilization and contraction. In contrast, we found that enhanced tyrosine phosphorylation has little effect on muscarinic stimulation of these responses in this tissue (Table 1, Figs. 2 and 4). Thus, while prostaglandin $F_{2\alpha}$ and carbachol induced contraction in a concentration-dependent manner with EC_{50} values of 0.92×10^{-9} and 1.75×10^{-8} M, respectively (Fig. 1), genistein, tyrphostin 47 and herbimycin (1 μM each) inhibited the contractile response to prostaglandin $F_{2\alpha}$ by 55, 39 and 41% respectively, but had little effect on the pharmacological response to carbachol (Table 1). Higher concentrations of the inhibitors (10 μM) abolished the prostaglandin $F_{2\alpha}$ response, and inhibited that of carbachol by about 10–15%. In contrast, Wortmannin, a myosin light chain kinase inhibitor, inhibited both prostaglandin $F_{2\alpha}$- and carbachol-induced contraction (Table 1). These findings could suggest different mechanisms for the actions of prostaglandin $F_{2\alpha}$ and carbachol on the contractile response in this smooth muscle. Thus, in the carbachol-stimulated smooth muscle the resultant phosphorylation of myosin light chain (MLC_{20}) by myosin light chain leads to the interaction of actin with myosin, triggering a contractile response (Kamm and Stull, 1985; Abdel-Latif, 1996). Wortmannin, a fungal extract produced by *Talaromyces wortmannii* KY-12420, which has been shown to be a highly specific inhibitor of the smooth muscle myosin light chain kinase by irreversibly binding at, or close to, the ATP-binding site (Nakanishi et al., 1992) inhibited both prostaglandin $F_{2\alpha}$- and carbachol-induced contraction (Table 1). By blocking ATP binding, Wortmannin prevents phosphorylation of the myosin light chain, binding of myosin to actin, and the subsequent generation of force. It has been shown in vascular smooth muscle that inhibition of myosin light chain kinase by Wortmannin suppresses contractions (Takayama et al., 1994). However, Wortmannin inhibits myosin light chain kinase at concentrations 100-fold higher than that required for inhibition of PI 3-kinase, a protein tyrosine kinase enzyme (Nakanishi et al., 1992). These results suggest that Wortmannin inhibits downstream from the stimulated $[Ca^{2+}]_i$ mobilization. In contrast, in the prostaglandin $F_{2\alpha}$-stimulated sphincter protein tyrosine kinase/phosphatase activities are involved in the coupling mechanism between receptor activation and contraction. This conclusion is based on the following findings in the present work: (1) Genistein, tyrphostin 47 and herbimycin inhibited prostaglandin $F_{2\alpha}$-induced contraction in a concentration-dependent manner with IC_{50} values of 0.82, 1.20 and 1.60 μM, respectively (Fig. 3). The protein tyrosine kinase inhibitors also blocked the action of latanoprost on muscle contraction (Fig. 2). While protein tyrosine kinase inhibitors have been reported to block agonist-induced contraction in smooth muscle (for review, Di Salvo et al., 1997), this is the first report to show that these inhibitors completely blocked prostaglandin $F_{2\alpha}$-induced contraction in a smooth muscle. Yang et al. (1992), working with guinea pig gastric smooth muscle, reported that the contractile action of prostaglandin $F_{2\alpha}$ (1 μM) was partially inhibited by genistein (7.4 μM), up to $10 \pm 5\%$ in the longitudinal preparation and $35 \pm 3\%$ in the circular preparation. The finding that in the iris sphincter the protein tyrosine kinase inhibitors blocked prostaglandin $F_{2\alpha}$- but not carbachol-induced contraction is in accord with the observations of Yang et al. (1993) who reported that in guinea pig gastric longitudinal smooth muscle genistein and tyrphostin selectively and completely blocked angiotensin-II-mediated contraction, without affecting contractions caused by carbachol and bradykinin. However, in the gastric longitudinal muscle indomethacin blocked the angiotensin-II-mediated contraction (Yang et al., 1993), implying the involvement of prostaglandins. More recently, Wiederholt et al. (1998) reported that epidermal growth factor (EGF) induced a relaxation in trabecular meshwork and a contraction in ciliary muscle precontracted with carbachol, and when trabecular meshwork and ciliary muscle were activated by the epidermal growth factor, inhibition of protein tyrosine kinase activity by genistein relaxed the cell preparations. (2) Genistein inhibited prostaglandin $F_{2\alpha}$-induced IP_3 production in a concentration-dependent manner with IC_{50} of 1.0×10^{-6} M, without affecting contraction caused by carbachol (Fig. 4, Table 1). In general, activation of phospholipase C-β isoforms in the iris sphincter, as well as in other smooth muscles, is linked to stimulation of G-protein coupled receptors (Abdel-Latif, 1996), whereas activation of phospholipase Cγ isoforms is largely mediated by its direct tyrosine phosphorylation (Rhee and Bae, 1997). In the present work we found that incubation of the sphincter muscle with 1 μM prostaglandin $F_{2\alpha}$ resulted in an increase in tyrosine phosphorylation of several protein bands, with the most prominent phosphoproteins migrating at relative molecular weights of approximately 170, 125, 115, 95 and 73 kDa; however, there was no increase in tyrosine phosphorylation of phospholipase C-γ_1 by the prostaglandin (N. Ali, S.Y.K. Yousufzai and A.A. Abdel-Latif, unpublished work). Palmier et al. (1996), working with rat myometrium, reported that orthovanadate, a potent protein tyrosine phosphatase inhibitor, mediated an increased generation of inositol phosphates and tension in this tissue

through phosphorylation and activation of phospholipase C-γ_1. In contrast, Di Salvo and Nelson (1998), working with Al0 aortic smooth muscle cells, reported that tyrosine phosphorylation of phospholipase C-γ_1, is not obligatory for protein tyrosine kinase dependent increases in $[Ca^{2+}]_i$ that occur in response to stimulation of G-protein coupled receptors in these cells. Thus, the mechanism of activation and phosphorylation of phospholipase C-γ_1 in the cat iris sphincter remains to be determined. (3) Genistein suppressed prostaglandin $F_{2\alpha}$-induced intracellular Ca^{2+} mobilization, without affecting carbachol-induced Ca^{2+} mobilization in iris sphincter smooth muscle cells (Fig. 5). IP_3 is a second messenger that controls many cellular processes by causing release of Ca^{2+} from intracellular stores. Thus, it is not unreasonable to assume that since genistein inhibited prostaglandin $F_{2\alpha}$-induced IP_3 production (Fig. 4) it will also block prostaglandin $F_{2\alpha}$-induced Ca^{2+} mobilization and consequently contraction. This result is consonant with results reported from other laboratories. Thus, Di Salvo et al. (1997) reported that in vascular smooth muscle cells both the transient and the sustained components of [arginine^8]-vasopressin receptor-activated increases in $[Ca^{2+}]_i$ are virtually abolished by genistein. Gould et al. (1995) reported that genistein inhibited both contraction and intracellular Ca^{2+} mobilization induced by activation of receptors for histamine in porcine carotid arterial smooth muscle. (4) Vanadate, a potent inhibitor of protein tyrosine phosphatases in smooth muscle (Di Salvo et al., 1993a,b; Palmier et al., 1996) induced a slow gradual muscle contraction in a concentration-dependent manner with EC_{50} value of 82 μM and increased IP_3 production in a concentration-dependent manner with EC_{50} value of 90 μM and these effects were inhibited by 10 μM genistein (Figs. 6 and 7). These results are in agreement with those of Di Salvo et al. (1993a,b) who reported that enhanced protein tyrosine phosphorylation occurs during vanadate-induced contraction of intact taenia coli preparations.

It is of considerable interest that the actions of prostaglandin $F_{2\alpha}$ on IP_3 production, Ca^{2+} mobilization and contraction are inhibited by the protein tyrosine kinase inhibitors in cat iris sphincter smooth muscle. This finding could shed more light on the molecular mechanism underlying the intraocular pressure lowering effects of this prostaglandin and its analog, latanoprost, an antiglaucoma 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 protein tyrosine kinase activity in the contractile action of this prostaglandin in the iris sphincter. Protein tyrosine kinase activity does not seem to be involved in the physiological actions of the muscarinic agonist carbachol; however, it is involved in the increases in $[Ca^{2+}]_i$ evoked by activation of the prostaglandin $F_{2\alpha}$ receptor. This conclusion is supported by the findings on the inhibitory effects of Wortmannin which acts downstream from the stimulated $[Ca^{2+}]_i$ mobilization.

In conclusion, the results obtained with the protein tyrosine kinase inhibitors and the protein tyrosine phosphatase inhibitor vanadate suggest an important role for protein tyrosine kinase activity in the stimulatory action of prostaglandin $F_{2\alpha}$ on IP_3 production, $[Ca^{2+}]_i$ mobilization and contraction in the cat iris sphincter. Further studies aimed at elucidating the role of protein tyrosine kinase activity in the coupling mechanism between the activated prostaglandin $F_{2\alpha}$ receptor and intracellular $[Ca^{2+}]$ mobilization and identifying the tyrosine-phosphorylated substrates will provide important information about the role of this enzyme 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 antiglaucoma drugs.

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