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European Journal of Pharmacology 403 (2000) 195–202

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Serotonin-induced protein kinase C activation in cultured rat heart endothelial cells

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Received 6 March 2000; received in revised form 6 July 2000; accepted 11 July 2000

Abstract

This study examined whether serotonin can activate protein kinase C in rat heart endothelial cells. Protein kinase C isozyme translocation was examined by Western blot analysis with isozyme-specific anti-protein kinase C antibody. In this study, only α protein kinase C isozyme was found to be translocated from the cytosolic to the particulate fractions after serotonin stimulation. The effect of serotonin on the incorporation of ^{32}P from [γ-^{32}P]ATP into peptide substrate was studied as another indicator of protein kinase C activation. The experiments in this study demonstrated that the Ca^{2+}-phospholipid-dependent protein kinase, protein kinase C, was activated by serotonin. By investigating [^{3}H]phorbol 12,13-dibutyrate binding to protein kinase C and trypsin-treated protein kinase C activity, we demonstrated that the site of action of serotonin is probably the regulatory domain of protein kinase C. Finally, we also demonstrated that serotonin had no effect on the intracellular concentration of cyclic nucleotides (cAMP, cGMP). These findings support the hypothesis that protein kinase C may be an important participant in serotonin-induced endothelial cell contraction and barrier dysfunction.

Keywords: 5-HT (5-Hydroxytryptamine, serotonin); Endothelial cell, rat; Protein kinase C; [^{3}H]Phorbol 12,13-dibutyrate binding; Trypsin-treated protein kinase C activity; cAMP; cGMP

1. Introduction

Serotonin is a naturally occurring amine with major effects on a variety of bodily functions. To date, the studies concerning serotonin have been focused on vascular and inflammatory responses. Important early studies on the mechanisms of acute inflammation were performed with rats and mice and 5-HT receptor antagonists, and demonstrated the importance of serotonin to the development of inflammation in these models (Spector and Willoughby, 1958a,b, 1959a,b, 1964). Owen (1977) suggested that administration of serotonin to the plantar surface of the rat paw caused edema with striking extravasation of albumin. Serotonin was also reported to induce plasma extravasation as a result of edema formation in vivo (Pierce et al., 1995; Wang et al., 1996).

The vascular endothelium functions as a critical and selective barrier to macromolecular permeability, protecting the underlying tissues from edema. This barrier function is modulated by many vasoactive agents in vivo (Majno and Palade, 1961; Svensjo and Grega, 1986). These vasoactive agents, such as thrombin, bradykinin, and histamine, have been also shown to increase macromolecular transfer across endothelial monolayers obtained from human umbilical vein, bovine pulmonary artery and bovine aorta in vitro (Buchan and Martin, 1992; Schaeffer et al., 1993; Garcia et al., 1986). Endothelial cell contraction, with subsequent formation of intercellular gaps, has been hypothesized as the mechanism by which increased paracellular macromolecular transport and increased endothelial cell permeability occur (Majno and Palade, 1961; Boswell et al., 1992; Garcia et al., 1995; Lee, 1997). Nowadays, the molecules involved in the regulation of this contraction are recognized. They include intracellular Ca^{2+}, protein kinase C, and actin.

Many investigators suggest that protein kinase C activation is an important signal transduction pathway by which extracellular mediators increase endothelial macromolecular transport (Lynch et al., 1990; Stasek et al., 1992; Krizbai et al., 1995; Nagpala et al., 1996). The activation of protein kinase C, which can occur as a result of the

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0014-2999/00/$ - see front matter
PII: S0014-2999(00)00495-7

generation of 1,2-diacylglycerol (Nishizuka, 1984), is associated with the phosphorylation of specific cytoskeletal proteins and decreased cell–cell contact (Stasek et al., 1992). Studies have demonstrated that inflammatory mediators which increase endothelial permeability, such as bradykinin, thrombin, and hydrogen peroxide, also activate protein kinase C (Lynch et al., 1990; Siflinger-Birnboim et al., 1992; Aschner et al., 1993). Protein kinase C inhibitors, such as staurosporine or H-7, reduce vasoactive agent-induced increases in endothelial permeability (Lynch et al., 1990; Garcia et al., 1991; Siflinger-Birnboim et al., 1992).

Serotonin is known to act in peripheral tissues to produce pain and inflammation, yet the mechanisms underlying serotonin-induced inflammation have not been well studied. Our previous study indicated that serotonin caused an increase in rat heart endothelial cell monolayer paracellular permeability (Lee, 1997). In our other studies, serotonin caused a significant increase in the concentration of cytosolic Ca^{2+} in rat heart endothelial cells (Lee and Wu, 1999). The activation of protein kinase C by vasoactive agents appears to be the result of phospholipase C activation and the production of diacylglycerol and inositol 1,4,5-triphosophate, which can directly or indirectly activate protein kinase C by the mobilization of Ca^{2+} (Rhee et al., 1989; Nishizuka, 1992). Therefore, this study examined whether serotonin can directly activate protein kinase C in rat heart endothelial cells. In the present study, we demonstrated that serotonin stimulated the translocation of cytosolic α protein kinase C to the particulate fraction and the incorporation of ^{32}P from [γ-^{32}P]ATP into peptide substrate. We also demonstrated that serotonin probably acted on the regulatory domain of protein kinase C by measuring [^{3}H]phorbol 12,13-dibutyrate binding to protein kinase C and trypsin-treated protein kinase C activity.

2. Materials and methods

2.1. Materials

All culture media and fetal bovine serum were obtained from Gibco BRL (Grand island, NY). Bovine serum albumin, endothelial cell growth supplement, serotonin, forskolin, staurosporine, 3-isobutyl-1-methylxanthine (IBMX), phenylmethylsulfonyl fluoride, antipain, leupeptin, pepstatin, ethylenediaminetetraacetic acid (EDTA), ethyleneglycol-bis-(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), tris (hydroxymethyl) aminomethane (Tris), dithiothreitol, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (IgG), phorbol 12,13-dibutyrate, and phosphatidylserine were purchased from Sigma (St. Louis, MO); 1-(5-isoquinoline sulfonyl)-2-methylpiperazine (H-7) was from Research Biochemicals International (Natick, MA); anti-Von Willebrand factor, human fibronectin, and lactate dehydrogenase (LDH) detection kit were from Boehringer Mannheim (Germany); Immobilon-P transfer membrane was from Millipore (Bedford, MA); the monoclonal antibodies against specific protein kinase C isozymes-α, -β, -δ, -ε, -μ, -θ, -ζ and -λ were purchased from Transduction Laboratory (Lexington, KY); anti-γ protein kinase C antibody was from Gibco BRL; anti-mouse IgG peroxidase-conjugated secondary antibody, cyclic nucleotide enzyme immunoassay kit, enhanced chemiluminescent (ECL) detection reagents, [γ-^{32}P]ATP, and protein kinase C [^{32}P] enzyme assay system were purchased from Amersham (Buckinghamshire); [^{3}H]phorbol 12,13-dibutyrate was from Dupont NEN (Boston, MA); Whatman GF/C filter was from Whatman (Singapore); sodium nitroprusside was from Merck (Taiwan). Serotonin was dissolved in Hank's Balanced Salt Solution (HBSS) and other substances were dissolved in less than 0.5% dimethylsulfoxide (DMSO). In the trypsin-treated protein kinase C activity experiment, serotonin was dissolved in DMSO.

2.2. Rat heart endothelial cell culture preparation

Rat heart endothelial cells were isolated as described previously (Lee, 1997). Ventricles from three hearts were removed from 4-day-old donor rats. The tissue was then subjected to four successive trypsinization steps. After each trypsinization, free cells were removed and 2 ml of endothelial cell culture medium consisting of Dulbecco's modified essential medium (DMEM) and 20% fetal bovine serum was added. The cells were recovered by centrifugation at 1000 g and allowed to adhere to a 24-well tissue culture cluster plate (Falcon) for 90 min. The culture medium was then removed and the culture wells were washed twice with HBSS to remove non-adherent cells. Thereafter, culture medium supplemented with 150 μg of endothelial cell growth supplement and 1000 U penicillin-1000 μg streptomycin per milliliter as well as 20% fetal bovine serum was added to the adherent cells. All cultures had a typical contact-inhibited cobblestone appearance. Factor VIII-related antigen was confirmed by indirect immunofluorescence using human factor VIII antiserum and FITC-conjugated goat anti-mouse IgG as previously described (Jaffe et al., 1973). All experimental data were obtained from rat heart endothelial cells in their second and third passage.

2.3. Measurement of intracellular distribution of protein kinase C activity

After treatment, endothelial cells were washed twice with HBSS and scraped, on ice, into ice-cold lysis buffer containing 20 mM Tris–HCl, pH 8.0, 0.5 mM EDTA, 0.5 mM EGTA, 25 μg/ml leupeptin, 10 μg/ml antipain, 10 μg/ml pepstatin, 1 mM dithiothreitol, and 2.5 mM phenylmethylsulfonyl fluoride. The cells were collected and sonicated for 10 pulses. The sonicated samples were centrifuged at $100{,}000 \times g$ for 30 min at 4°C and the resulting supernatant was collected as the "cytosolic" frac-

tion. The pellet was resuspended in lysis buffer plus 0.5% Triton X-100, sonicated, and centrifuged as before. The supernatant was collected as the "particulate" fraction. Protein kinase C activity in both fractions was determined by measuring the incorporation of ^{32}P into peptide substrates using a protein kinase C [^{32}P] enzyme assay system. Briefly, 50 μl reaction mixture contained 50 mM Tris–HCl buffer, pH 7.5, 3 mM calcium acetate, 18 mM magnesium chloride, 7.5 mM Hepes, 7.5 mM dithiothreitol, 75 μg/ml phosphatidylserine, 6 μg/ml of phorbol myristate acetate (PMA), 0.3 mM ATP (0.2 μCi [γ-^{32}P]ATP per tube), 225 μM protein kinase C substrate, and 30 μg protein kinase C sample. After addition of stop reagent, aliquots (35 μl) of the reaction mixtures were spotted on phosphocellulose discs and washed with three changes of 75 mM orthophosphoric acid. The protein kinase C-dependent phosphorylated peptide substrate bound to the filter was quantified by scintillation counting.

2.4. [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDB) binding assay

[^{3}H]PDB binding was determined as described previously (Goodwin and Weinberg, 1982; Wang et al., 1997) with some modifications. Confluent endothelial cells were washed twice with HBSS and scraped, on ice, into ice-cold lysis buffer containing 20 mM Tris–HCl, pH 8.0, 0.5 mM EDTA, 0.5 mM EGTA, 25 μg/ml leupeptin, 10 μg/ml antipain, 10 μg/ml pepstatin, 1 mM dithiothreitol, 2.5 mM phenylmethylsulfonyl fluoride, and 0.5% Triton X-100. The cells were collected and sonicated for 10 pulses. The sonicated samples were centrifuged at 100,000 × g for 30 min at 4°C. The supernatant was fractionated into aliquots (30 μg/tube) and stored at −80°C until assayed. The binding assay was done in 1.5-ml polypropylene tubes containing a final volume of 50 μl. The reaction mixture contained 20 mM Tris–HCl, pH 7.2, 100 mM KCl, 50 μg/ml of phosphatidylserine, 0.5 mM calcium chloride, 30 nM [^{3}H]PDB, and protein kinase C sample. Before the addition of [^{3}H]PDB to the reaction mixture, cell protein was preincubated with various agents at 37°C for the indicated time period. Nonspecific binding was determined in the presence of excess unlabeled PDB (30 μM). All results are presented as specific binding (that is, the difference between [^{3}H]PDB bound in the presence and absence of excess unlabeled PDB). After incubation with [^{3}H]PDB at 30°C for 30 min, the reaction was terminated by the addition of 0.5% DMSO solution at 4°C. Aliquots (35 μl) of the reaction mixtures were spotted on Whatman GF/C filter. The filter was then washed and radioactivity was quantified by scintillation counting.

2.5. Trypsin-treated protein kinase C activity

Trypsin-treated protein kinase activity was determined as previously described (Lee and Bell, 1986) with some modifications. Confluent endothelial cells were washed twice with HBSS and scraped, on ice, into ice-cold lysis buffer containing 20 mM Tris–HCl, pH 8.0, 0.5 mM EDTA, 0.5 mM EGTA, 25 μg/ml leupeptin, 10 μg/ml antipain, 10 μg/ml pepstatin, 1 mM dithiothreitol, 2.5 mM phenylmethylsulfonyl fluoride, and 0.5% Triton X-100. The cells were collected and sonicated for 10 pulses. The sonicated samples were centrifuged at 100,000 × *g* for 30 min at 4°C. The supernatant was fractionated into aliquots (30 μg/tube) and stored at −80°C until assayed. Cell protein (30 μg) was first preincubated for 10 min at 30°C with trypsin (0.0025% final concentration) in a solution which contained 10 mM Tris–HCl at pH 8.0 and 5 mM 2-mercaptoethanol. The trypsin-treated protein kinase C was then incubated with DMSO and various agents for 10 min at 37°C before the addition of [γ-^{32}P]ATP and protein kinase C substrate to start the reaction. Trypsin-treated protein kinase C activity was determined by measuring the incorporation of ^{32}P into peptide substrates using a protein kinase C [^{32}P] enzyme assay system as described above, except that calcium chloride, phosphatidylserine and PMA were left out of the reaction mixture.

2.6. Western blot analysis

After treatment, endothelial cells were harvested and sonicated in ice-cold buffer containing 20 mM Tris–HCl, pH 8.0, 0.5 mM EDTA, 0.5 mM EGTA, 25 μg/ml leupeptin, 10 μg/ml antipain, 10 μg/ml pepstatin, 1 mM dithiothreitol, and 2.5 mM phenylmethylsulfonyl fluoride, and were fractionated into cytosolic and particulate fractions according to the method described above. Equal amounts of protein (30 μg/lane) were separated by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-separated proteins were equilibrated in transfer buffer (50 mM Tris, pH 9.0–9.4, 40 mM glycine, 0.375% SDS, 20% methanol) and electrotransferred to Immobilon-P transfer membranes. The blot was blocked with a solution containing 5% nonfat dry milk in Tris-buffered saline (10 mM Tris, 150 mM NaCl) with 0.05% Tween 20 (TBST) for 1 h, washed, and incubated with the protein kinase C isozyme-specific antibodies in TBST with 0.5% nonfat dry milk for 1 h. The blot was washed and incubated with an anti-mouse IgG peroxidase-conjugated second antibody. The ECL detection system was used for immunoblot protein detection.

2.7. Measurement of cyclic nucleotides

Cyclic nucleotides were measured by enzyme immunoassay as previously described (Lee, 1997). Cells were plated onto 24-well Falcon plates at 2×10^5 cells/well and grown to confluence in DMEM with 20% fetal bovine serum for 3 days prior to cyclic nucleotide determination. Before cyclic nucleotides were measured, the cells were washed twice in HBSS and incubated in HBSS alone or in

Table 1
Effect of serotonin on protein kinase C translocation and protein kinase C inhibitors on serotonin-induced protein kinase C translocation in rat heart endothelial cells

Reagents	Time (min)	Cytosolic[a]	Particulate[a]
DMSO	10	2.18 ± 0.18	1.49 ± 0.11
+ HBSS	10		
DMSO	10	0.96 ± 0.10[b]	3.46 ± 0.13[b]
+ Serotonin (1 mM)	10		
Staurosporine (3×10^{-9} M)	10	1.61 ± 0.11[c]	2.77 ± 0.15[c]
+ Serotonin (1 mM)	10		
H-7 (4×10^{-5} M)	10	1.23 ± 0.09[c]	2.02 ± 0.12[c]
+ Serotonin (1 mM)	10		

[a] Rat heart endothelial cells were cultured to confluence in 100-mm petri dishes. After various treatments, endothelial cells were fractionated into cytosolic and particulate fractions according to the method described above. Protein kinase C activity in both fractions was determined by measuring the incorporation of ^{32}P into peptide substrates using a protein kinase C [^{32}P] enzyme assay system. Specific protein kinase C activity is expressed as pmol [γ-^{32}P]ATP incorporated/μg/min. Data are expressed as the means ± S.E.M. of five independent experiments.

[b] $P < 0.01$ compared to the corresponding control.

[c] $P < 0.01$ compared to the serotonin-treated cells.

HBSS containing the agents at 37°C in a 5% CO_2-humidified atmosphere. After incubation for the indicated time, the medium was removed completely and the cells were washed twice with HBSS containing 0.5 mM isobutylmethylxanthine (IBMX) to inhibit phosphodiesterase and to prevent subsequent breakdown of the cyclic nucleotide during cell solubilization, sample collection, and processing. The HBSS containing IBMX was decanted and the cells were immediately solubilized in 250 μl of ice-cold 0.1 N HCl, and the plates were stored at −70°C. Extracts were removed from the plates and centrifuged at $1000 \times g$ for 10 min. A 200-μl aliquot of the supernatant was acetylated, and the amount of cyclic nucleotide was determined by enzyme immunoassay.

2.8. Assay of LDH release

LDH release from rat heart endothelial cell monolayers was used as a marker of overt cytotoxicity. The rat heart endothelial cells grown on the plastic tissue culture plates were exposed to various concentrations of reagent in DMSO in HBSS with 0.1% bovine serum albumin for 30 min. The supernatant was removed and centrifuged at $1000 \times g$ for 10 min. LDH activity in the supernatant was determined with the LDH detection kit.

2.9. Statistical analysis

Results were analyzed for statistical significance by analysis of variance with repeated measures and a Newman–Keuls test. A *P*-value of less than 0.05 was considered significant for all tests.

3. Results

3.1. Characterization of serotonin-induced protein kinase C activation

The activity of protein kinase C in confluent rat heart endothelial cells was studied after exposure to 1 mM serotonin (Table 1). Quiescent monolayers exhibited protein kinase C activity predominantly in the cytosolic fraction (Table 1). Treatment of rat heart endothelial cells with 1 mM serotonin for 10 min resulted in translocation of protein kinase C activity from the cytosolic to the particulate fraction, as assessed by [γ-^{32}P]ATP incorporation in peptide substrate (Table 1). As shown in Table 1, serotonin-induced protein kinase C activity was suppressed by the protein kinase C inhibitors staurosporine (3×10^{-9} M) and H-7 (4×10^{-5} M). In this study, staurosporine and H-7 prevented the evolution of morphologic changes elicited by serotonin (data not shown).

3.2. Binding of [^{3}H]PDB to protein kinase C

In order to determine which region of protein kinase C is the site of action of serotonin, PDB binding to rat heart endothelial cell protein kinase C was examined. In the assay of PDB binding, serotonin, like PMA, significantly diminished the binding of [^{3}H]PDB to endothelial protein kinase C (Table 2). Binding to protein kinase C was specific; it was blocked by the addition of a 100-fold excess unlabeled phorbol dibutyrate (data not shown).

3.3. Trypsin-treated protein kinase C activity

To further investigate whether the activation of protein kinase C by serotonin could be linked to binding of the

Table 2
Effect of serotonin on [^{3}H]PDB binding to protein kinase C in rat heart endothelial cells

Reagents	Time (min)	[^{3}H]PDB bound (c.p.m.)[a]
DMSO	10	1337 ± 125
+ HBSS	10	
DMSO	10	771 ± 80[b]
+ Serotonin (1 mM)	10	
HBSS	10	689 ± 85[b]
+ PMA (10^{-5} M)	10	

[a] Rat heart endothelial cells were cultured to confluence in 100-mm petri dishes. The cells were collected, sonicated, and fractionated into aliquots (30 μg/tube). Aliquots of cell protein were pretreated with various reagents at 37°C for the specified time period before the addition of [^{3}H]PDB to the reaction mixture. After termination of the reaction, protein was harvested on a filter, and the radioactivity on the filter was counted as described in Materials and Methods. Data are expressed as the means ± S.E.M. of five independent experiments.

[b] $P < 0.01$ compared to the control values.

regulatory domain of the protein kinase C, we used trypsin to remove the regulatory region of protein kinase C. The remaining protein kinase C activity was measured by detecting the incorporation of ^{32}P from [γ-^{32}P]ATP into peptide substrate in the absence of calcium, phosphatidylserine, and PMA. After partial digestion of protein kinase C with trypsin, the remaining catalytic region of protein kinase C was not activated by serotonin or PMA (Table 3). However, H-7, a potent inhibitor of protein kinase C acting at the catalytic domain, effectively attenuated the trypsin-treated protein kinase C activity (Table 3).

3.4. Effects of serotonin on the expression and translocation of protein kinase C isozymes in rat heart endothelial cells

In this experiment, we examined the effect of serotonin on the translocation of the various protein kinase C isozymes from the cytosolic to the particulate fraction by Western blot analysis as another indicator of protein kinase C activation. The expression of protein kinase C isozymes in rat heart endothelial cells was examined using isozyme-specific anti-protein kinase C antibodies. β, γ, and μ protein kinase C isozymes were not found in rat heart endothelial cell extracts even with a variety of primary and secondary antibody dilutions (data not shown). In contrast to these three isozymes, immunoreactive bands of four protein kinase C isozymes, α, δ, ε, and ζ, were clearly detected in the cytosolic or particulate fractions, while very faint immunoreactive bands of θ and λ protein kinase C were observed (Fig. 1). Isozymes δ, ε, and θ mainly appeared in the particulate protein fraction, whereas ζ protein kinase C was detected in the cytosolic fraction. Isozymes α, δ, ε, ζ, θ, and λ had apparent molecular masses of 82, 78, 90, 72, 79 and 74 kDa, respectively.

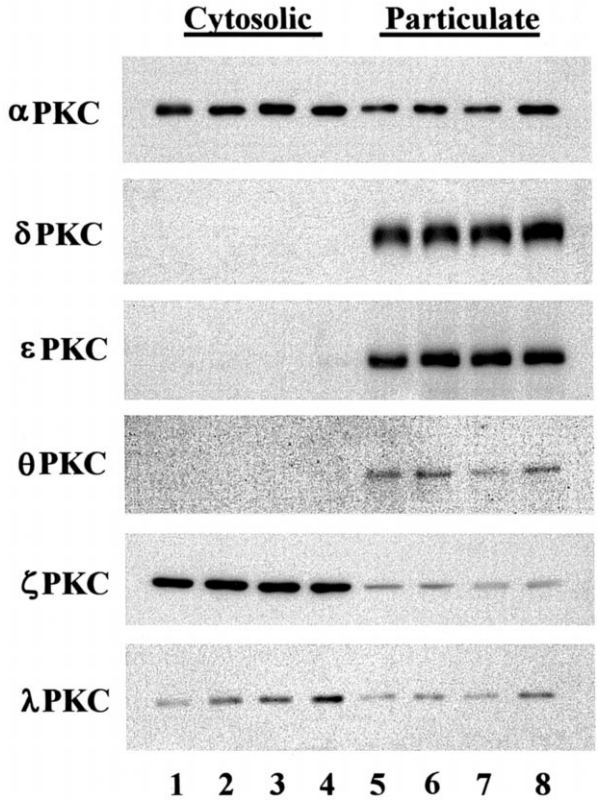

Fig. 1. Western blot showing the effects of 1 mM serotonin for 5 and 10 min on protein kinase C isozyme translocation in rat heart endothelial cells. Cytosolic and particulate fractions were analyzed by 7.5% SDS-PAGE and probed with antibodies against peptides specific for protein kinase C isozymes. Western blot analysis using a mAb to detect protein kinase C isozymes was as described in Section 2. Lane 1 and 5, control cells (HBSS, 5 min); lane 2 and 6, serotonin-treated cells (1 mM serotonin, 5 min); lane 3 and 7, control cells (HBSS, 10 min); lane 4 and 8, serotonin-treated cells (1 mM serotonin, 10 min). Results are representative of three independent experiments.

When endothelial cells were treated with 1 mM serotonin for 5 and 10 min, there was a translocation of cytosolic α protein kinase C to the particulate fraction (Fig. 1). In

Table 3
Effect of serotonin on trypsin-treated protein kinase C activity in rat heart endothelial cells

Reagents	Time (min)	pmol $^{32}P/\mu g/min$[a]
Control	10	2.32 ± 0.18
Serotonin (1 mM)	10	2.22 ± 0.21
PMA (10^{-5} M)	10	2.32 ± 0.15
H-7 (4×10^{-5} M)	10	1.13 ± 0.11[b]

[a]Rat heart endothelial cells were cultured to confluence in 100-mm petri dishes. The cells were collected, sonicated, and fractionated into aliquots (30 μg/tube). A 30-μg aliquot of cell protein was first preincubated for 10 min at 30°C with trypsin (0.0025% final concentration). The trypsin-treated protein kinase C was then incubated with DMSO and various agents for 10 min at 37°C before the addition of [γ-^{32}P]ATP and protein kinase C substrate to start the reaction. Trypsin-treated protein kinase C activity was determined by measuring the incorporation of ^{32}P into peptide substrates using a protein kinase C [^{32}P] enzyme assay system as described above except that calcium, phosphatidylserine, and PMA were left out of the reaction mixture. Data are expressed as the means $\pm$ S.E.M. of five independent experiments.

[b]$P < 0.01$ compared to the control values.

Table 4
Effect of serotonin on intracellular cAMP and cGMP levels in rat heart endothelial cells

Reagents	cAMP concentration[a] (% of control)	cGMP concentration[a] (% of control)
Control	100	100
Forskolin (10^{-6} M)	333 ± 12[b]	–[c]
Sodium nitroprusside (10^{-6} M)	–[c]	177 ± 5[b]
Serotonin (1 mM)	90 ± 8	85 ± 5

[a]Rat heart endothelial cells were cultured to confluence in 24-well plates. After a 10-min incubation, the intracellular cyclic nucleotides were measured by enzyme immunoassay as described under Section 2. The results are expressed as the mean % control $\pm$ S.E.M. ($n = 12$–15).

[b]$P < 0.01$, compared to the relative control values.

[c]Not determined.

Table 5
LDH release from rat heart endothelial cells

Reagents	LDH release (% of control)[a]
Control	100
Serotonin (2×10^{-3} M)	99.9 ± 0.1
Staurosporine (10^{-6} M)	99.7 ± 0.1
IBMX (10^{-5} M)	100.0 ± 0.1
Forskolin (10^{-5} M)	100.0 ± 0.1
Sodium nitroprusside (10^{-6} M)	100.0 ± 0.1
H-7 (4×10^{-5} M)	99.9 ± 0.2

[a]Endothelial cells were cultured in 24-well plates and then incubated with various concentrations of reagent in HBSS with 0.1% bovine serum albumin for 30 min. LDH activity in the supernatant was determined by using an LDH detection kit. The results are expressed as the mean % of control ± S.E.M. ($n = 20$).

addition, no significant changes in protein content of other protein kinase C isozymes were noted in either cytosolic or particulate fractions after serotonin stimulation (Fig. 1).

3.5. Effects of serotonin on the cAMP and cGMP concentration in rat heart endothelial cell monolayers

To further investigate whether the effect of serotonin on endothelial barrier dysfunction could be linked to the attenuation of endothelial cAMP, we tested the effect of serotonin on the intracellular cAMP concentration. Confluent monolayers of endothelial cells were stimulated with forskolin, adenylate cyclase activator, (10^{-6} M, 10 min) and serotonin (1 mM, 10 min). In the presence of IBMX, a phosphodiesterase inhibitor, forskolin induced an increase in the cellular cAMP concentration. Serotonin had no effect on the endothelial cAMP concentration (Table 4). This study also tested the effect of serotonin on the intracellular cGMP concentration in endothelial cell monolayers, on the assumption that the cGMP concentration is related to endothelial cell permeability. Confluent monolayers of endothelial cell were incubated with sodium nitroprusside, guanylate cyclase activator, (10^{-6} M, 10 min) and serotonin (1 mM, 10 min). In the presence of IBMX, sodium nitroprusside induced an increase in the cellular cGMP concentration. However, serotonin had no effect on the endothelial cGMP concentration (Table 4).

3.6. Cytotoxicity assay

Treatment of rat heart endothelial cells with various agents for 30 min did not increase LDH release (Table 5). This finding indicates that the result of each experiment in rat heart endothelial cells was not due to a cytolytic effect of these agents.

4. Discussion

Serotonin is a naturally occurring amine with major effects on a variety of bodily functions. To date, the studies concerning serotonin have been focused on vascular and inflammatory responses. Serotonin was reported to induce plasma extravasation as a result of the edema formation in vivo (Pierce et al., 1995; Wang et al., 1996). However, the mechanisms underlying serotonin-induced edema formation have not been well studied. The vascular endothelium functions as a critical and selective barrier to macromolecular permeability, protecting the underlying tissues from edema. Endothelial cell contraction plays a pivotal role in the increased extravasation of fluid and macromolecules in vascular leakage. In our previous study, serotonin caused significant dose-dependent increases in the passage of Evans blue-bovine serum albumin in rat heart endothelial cells (Lee et al., 1998b) as well as other vasoactive agents in various cell cultures such as human umbilical vein endothelial cells, bovine artery endothelial cell, and porcine pulmonary artery endothelial cells (Buchan and Martin, 1992; Schaeffer et al., 1993; Sheldon et al., 1993; Garcia et al., 1995).

Our study also demonstrated that serotonin-induced endothelial cell contraction and increased permeability to macromolecules are receptor-mediated events (Lee, 1997). Receptor-mediated responses of endothelial cells include activation of phospholipase C, increase in cytosolic Ca^{2+}, and increased permeability to macromolecules. In our other studies, we also demonstrated that serotonin caused a significant increase in the concentration of cytosolic Ca^{2+} in rat heart endothelial cells (Lee and Wu, 1999). The activation of protein kinase C by vasoactive agents appears to be the result of phospholipase C activation and the production of diacylglycerol and inositol 1,4,5-triphosophate, which can directly or indirectly activate protein kinase C by the mobilization of Ca^{2+} (Rhee et al., 1989; Nishizuka, 1992). In addition, many investigators suggest that protein kinase C activation is an important signal transduction pathway by which extracellular mediators increase endothelial macromolecular transport (Lynch et al., 1990; Stasek et al., 1992; Krizbai et al., 1995; Nagpala et al., 1996). Our previous study has suggested that protein kinase C-mediated events are important cellular mechanisms leading to serotonin-induced permeability (Lee et al., 1998a). Therefore, the aim of this study was to determine whether serotonin can directly activate protein kinase C in rat heart endothelial cells.

Many protein kinase C isozymes have been described in vascular endothelial cells such as α, β, γ, δ, ε, ζ, θ, ι, λ and μ (Nishizuka, 1988, 1989). In our studies, protein kinase C isozyme translocation in endothelial cells was examined by Western blot analysis using isozyme-specific anti-protein kinase C antibodies. This study showed that rat heart endothelial cells contain various amount of protein kinase C isozymes α, δ, ε, ζ, θ and λ but not β, γ, and μ. It is generally considered that the enzymes, when quiescent, are located in the cytoplasm and upon activation are translocated to the plasma membrane (Nishizuka, 1984; Lynch et al., 1990; Braiman et al., 1999). In the present

study, it is interesting to notice that only α protein kinase C isozyme was found to be translocated from the cytosolic to the particulate fraction after serotonin stimulation of rat heart endothelial cells. No significant changes in protein content of other protein kinase C isozymes were noted. This is consistent with the notion that not all protein kinase C isozymes in endothelial cells and other cell types are translocated by an agonist. Differences in tissue distribution, subcellular localization, and translocation following activation have also been demonstrated (Mochly-Rosen et al., 1990; Mochly-Rosen, 1995; Disatnik et al., 1994; Braiman et al., 1999). The explanation for the difference among the various protein kinase C isozymes is not clear but could be multiple. The variety of expression patterns of protein kinase C family members in endothelial cells of different types may reflect differences in the functional responsiveness to environmental stimuli. In this study, it is interesting to speculate that the predominant activation of α protein kinase C isozyme could be the result of serotonin inducing an increase in Ca^{2+} flux which could stimulate, in concert, α protein kinase C isozyme to translocate since this isozyme contains a Ca^{2+} binding site.

In the present study, the effect of serotonin on the incorporation of ^{32}P from [γ-^{32}P]ATP into peptide substrate was studied as another indicator of protein kinase C activation. The receptor-mediated agonist serotonin increased the incorporation of ^{32}P from [γ-^{32}P]ATP into peptide substrate in the particulate fraction. The structure of protein kinase C consists of a single polypeptide with an amino-terminal regulatory domain and a carboxy-terminal catalytic domain (Kikkawa et al., 1987; Ono et al., 1988). In order to determine which region is the site of action of serotonin, PDB binding to protein kinase C and trypsinized protein kinase C activity was examined. Lee and Bell (1986) indicated that the lipid binding, regulatory domain, of protein kinase C is the [^{3}H]PDB binding fragment. Newton (1995) has also reported that phorbol esters bind to the C_1 (diacylglycerol/phorbol ester binding site) domain of the protein kinase C regulatory region and can substitute for diacylglycerol in activating protein kinase C. In the assay of PDB binding, serotonin, like PMA, diminished the binding of [^{3}H]PDB to endothelial protein kinase C, which suggests that the serotonin-induced activation of protein kinase C could be linked to binding at the C_1 domain of the protein kinase C regulatory region. Wang et al. (1997) suggested that the regulatory domain of protein kinase C was removed after partial digestion of protein kinase C with trypsin. In our study, the remaining catalytic region of protein kinase C was not activated by serotonin or PMA. These results lead us to suggest that the site of action of serotonin is probably the regulatory domain of protein kinase C.

Finally, the study investigated whether serotonin, by changing the cell concentration of cyclic nucleotides, increased endothelial permeability to macromolecules. Recent studies have demonstrated that elevation of the intracellular concentrations of cyclic nucleotides can improve endothelial barrier function (Stelzner et al., 1989; Loffon et al., 1990; Langeler and Van Hinsbergh, 1991; Westendrop et al., 1994). In this study, serotonin had no effect on the intracellular cAMP and cGMP concentrations. These results appear to suggest that the serotonin regulation of endothelial permeability is not mediated by a decrease in intracellular cyclic nucleotide levels.

In conclusion, our results suggest that protein kinase C may be an important participant in serotonin-induced endothelial cell contraction and barrier dysfunction. Serotonin-mediated responses, such as the change in cell shape and increase in monolayer permeability, can be significantly attenuated by protein kinase C inhibition. This study has demonstrated that the Ca^{2+}-phospholipid-dependent protein kinase, protein kinase C, is activated by serotonin. The site of action of serotonin is probably the regulatory domain of protein kinase C. The serotonin-mediated activation of protein kinase C may be due to the translocation of α protein kinase C from cytosolic to particulate fractions in rat heart endothelial cells. We also demonstrated that serotonin enhancement of endothelial permeability is not correlated with intracellular levels of cyclic nucleotides. Therefore, these results suggest that serotonin increases endothelial macromolecular transport by activating the phospholipase C–protein kinase C signal transduction pathways.

Acknowledgements

The authors thank Hsu, S.L. PhD for her generous support throughout these studies. This work was supported by China Medical College of the Republic of China.

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