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Serotonin-stimulated increase in cytosolic Ca^{2+} in cultured rat heart endothelial cells

Hong-Zin Lee, Chun-Hsiung Wu *

School of Pharmacy, China Medical College, 91, Hsueh-Shih Road, Taichung, 404, Taiwan

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Abstract

This study was designed to investigate the effects of serotonin on changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in cultured rat heart endothelial cells. Serotonin stimulated a biphasic change in cytosolic Ca^{2+} of rat heart endothelial cells: an initial transient increase, which primarily reflects the release of Ca^{2+} from internal stores, followed by a slow rise in $[Ca^{2+}]_i$ during the incubation with serotonin. Our study also demonstrated that the pattern of the serotonin-induced increase in $[Ca^{2+}]_i$ was different from that induced by thrombin in rat heart endothelial cells. In this study, the role of $[Ca^{2+}]_i$ on endothelial paracellular barrier function was also investigated. Serotonin induced an increase in endothelial permeability which paralleled the rise in $[Ca^{2+}]_i$ and was blocked by the 5-HT_2 receptor antagonist cyproheptadine. Therefore, the serotonin-stimulated increase in cytosolic Ca^{2+} and macromolecular permeability was receptor-mediated in rat heart endothelial cells. Further experiments demonstrated that the serotonin-induced increase in $[Ca^{2+}]_i$ was inhibited by the phospholipase C inhibitors, neomycin and [6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U73122). Experiments involving the rapid depletion of intracellular Ca^{2+} stores and Ca^{2+}-free medium demonstrated that the biphasic response of endothelial Ca^{2+} to serotonin was related to the release of Ca^{2+} from intracellular stores and to the influx of extracellular Ca^{2+}. We also suggest that serotonin-induced changes in $[Ca^{2+}]_i$ are related to Ca^{2+} channels sensitive to voltage-operated and inorganic Ca^{2+} channel blockers.

Keywords: 5-Hydroxytryptamine (5-HT), serotonin; Endothelial cell, rat; Permeability; Ca^{2+} concentration, intracellular; Phospholipase C inhibitor; Ca^{2+} channel blocker

1. Introduction

The vascular endothelium is the interface between the blood and the interstitium and it fulfils the essential function of regulating the exchange of fluid, solutes and cells between these two compartments. This barrier function is subject to dynamic regulation and is modulated by many factors in vivo (Majno et al., 1961; Svensjo and Grega, 1986). Important new insights into the mechanisms regulating inflammatory edema have been gained by the development of endothelial cell culture systems. Many vasoactive agents such as thrombin, bradykinin, and histamine have been shown to increase macromolecular transfer across endothelial monolayers obtained from human umbilical vein, bovine pulmonary artery and bovine aorta (Buchan and Martin, 1992; Schaeffer et al., 1993; Garcia et al., 1995). Our previous study showed that serotonin caused a significant increase in the permeability of rat heart endothelial cells and the separation of adjacent cells with the resultant formation of large paracellular openings (Lee, 1997).

An increased cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) is generally thought to activate the contractile apparatus of cell and thereby causes endothelial retraction and increased intercellular passage of water, solutes, macromolecules, and cells. Furthermore, numerous investigators have demonstrated that a rise in endothelial $[Ca^{2+}]_i$ in response to various inflammatory stimuli initiates fluid leakage (Buchan and Martin, 1992; Curry, 1992; Lum et al., 1992; Garcia et al., 1993; Himmel et al., 1993). Brauneis et al. (1992) also reported that Ca^{2+} influx, via a non-specific cation channel, played a major role in serotonin-induced contraction in hepatic endothelial cells. The enhancement of endothelial permeability brought about by many vasoactive agents is link to phospholipase C, the formation of inositol 1,4,5-triphosphate and diacylglycerol, and raised intracellular Ca^{2+} and protein kinase C activity (Stasek et

* Corresponding author. Tel.: +886-4-2058436; fax: +886-4-2039203.

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al., 1992; Patterson et al., 1994). Our previous study reported that the activation of endothelial protein kinase C by serotonin resulted in significant changes in the localization and translocation of protein kinase C isozyme (Lee et al., 1998). Therefore, this study was designed to investigate whether serotonin, by increasing cellular Ca^{2+} concentration, induced the activation of protein kinase C and barrier dysfunction in cultured rat heart endothelial cells. Our study demonstrates that serotonin stimulates an increase in cytosolic Ca^{2+} and that the $[Ca^{2+}]_i$ changes are correlated with serotonin-induced macromolecular permeability in rat heart endothelial cells. The mechanisms by which serotonin changed intracellular Ca^{2+} in rat heart endothelial cells were also investigated.

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 (BSA), cyproheptadine, endothelial cell growth supplement, ethyleneglycol-bis-β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), Evans blue, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (IgG), neomycin, serotonin, thrombin, and verapamil were purchased from Sigma (St. Louis, MO); Transwells (diameter, 0.65 cm; pore size, 3 μm) were from Corning Costar (Cambrige, MA); human fibronectin, lactate dehydrogenase (LDH) Detection Kit, and mouse anti-human factor VIII were from Boehringer Mannheim (Germany); [6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U73122) was from Biomolecules (Plymouth Meeting, PA); 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid pentaacetoxymethyl ester (fura-2 AM) was from Molecular Probes (Eugene, OR); nickel chloride was from Merck (Taiwan). Serotonin, thrombin, and nickel chloride were dissolved in Hank's Balanced Salt Solution (HBSS) and other substances were dissolved in less than 0.5% dimethylsulfoxide (DMSO).

2.2. Culture of rat heart endothelial cells

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 $1000 \times g$ and allowed to adhere to a 24-well tissue culture cluster plate 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 the barrier function of rat heart endothelial cell monolayers

Rat heart endothelial cells cultured on filters were used 3 days after seeding (1×10^5 cells/well). Exchange of macromolecules through the endothelial cell monolayers was investigated by assaying the transfer of Evans blue dye bound-BSA (4% final concentration) as described previously (Lee, 1997). Briefly, endothelial cell monolayers were cultured on 48 Transwell polycarbonate membrane assemblies. The membrane assemblies were then placed in 0.6 ml of DMEM containing 20% fetal bovine serum in 24-well plates and the cells were allowed to grow to form a tight monolayer. For experimentation, membrane assemblies with attached cells were washed twice by immersion in HBSS and transferred to 24-well plates. Thereafter, 600 μl of HBSS was placed in each of the wells, which formed the lower chamber, and 100 μl of HBSS was placed above the endothelial monolayers. These volumes were chosen so as to avoid creating a hydrostatic gradient across the monolayers. Drugs were then added to the top and bottom chambers, and the lower chamber was stirred for rapid mixing. The entire system was kept at 37°C by a thermostatically regulated water bath. At the end of the experiment, a 200-μl aliquot was removed from each of the lower chambers and the transfer of Evans blue dye bound-BSA across the monolayers was quantified by measuring optical density at 600 nm.

2.4. Measurement of cytosolic free Ca^{2+} concentration

Cytosolic free Ca^{2+} concentration was measured with the fluorescent indicator, fura-2. Rat heart endothelial cells were grown to confluency in 100-mm dishes. Cells were dispersed from the bottom of the culture dish by incubation with 0.25% trypsin for 3–4 min. The action of trypsin was immediately terminated by the addition of an equal volume of medium containing 20% serum. The cells were centrifuged, washed, and resuspended in 2 ml of HEPES-buffered saline (HBS) (118 mM NaCl, 4.6 mM KCl, 24.9 mM $NaHCO_3$, 1 mM KH_2PO_4, 1.1 mM $MgSO_4$, 11.1 mM D-glucose, 1 mM $CaCl_2$, 0.1% BSA, 5 mM HEPES, pH

7.4) containing 20 μM fura-2/AM for 30 min at 37°C. The concentration of cells during the incubation with fura-2/AM was $2–3 \times 10^7$ cells/ml. The cell suspensions were diluted with HBS to a final volume of 20 ml, incubated for an additional 30 min, centrifuged, washed with 20 ml of HBS, and resuspended in 16 ml of HBS and maintained on ice in the dark. Aliquots (2 ml) were subsequently removed, centrifuged, and resuspended immediately prior to fluorescence measurement. The cells ($3–4 \times 10^6$ /ml) were kept in suspension in the cuvette with a magnetic stirrer. Fluorescence was monitored at 37°C in a Fluorescence spectrophotometer (Hitachi F-4010) at excitation and emission wavelengths of 342 and 498 nm, respectively. Intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ was determined by using the equation $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$ where the dissociation constant (K_d) for the Ca-fura-2 complex at 37°C is 224 nM (Lin and Rui, 1994). F is the fluorescence intensity emitted from intact cells, F_{max} is the maximum fluorescence obtained on disruption of the cells with 0.1% Triton X-100, and F_{min} is the minimum fluorescence obtained by the subsequent addition of EGTA, resulting in a final concentration of 15 mM. The results shown are representative of at least 3–5 experiments performed under similar conditions.

2.5. Cytotoxicity assay

LDH released from rat heart endothelial cell monolayers was used as a marker of overt cytotoxicity. Endothelial cells grown on plastic tissue culture plates were exposed to various concentrations of reagent in DMSO in HBSS with 0.1% BSA for 30 min. The supernatant was removed and centrifuged at 1000 g for 10 min. LDH activity in the supernatant was determined by the LDH Detection Kit.

2.6. Statistical analysis

Statistically significant differences from the control group were identified by Student's *t* test for paired data. A *P* value less than 0.05 was considered significant for all tests.

3. Results

3.1. Effects of serotonin on $[Ca^{2+}]_i$ in cultured rat heart endothelial cells

The first series of experiments was designed to determine the effect of serotonin on Ca^{2+} changes in rat heart endothelial cells. In this study, endothelial cells suspended in buffer had a resting $[Ca^{2+}]_i$ of 95 ± 8 nM (mean ± S.E.M., $n = 5$). 1 mM serotonin stimulates a biphasic change in cytosolic Ca^{2+} of rat heart endothelial cells at 37°C: an initial increase, which then decreased slightly, followed by a further slow rise in $[Ca^{2+}]_i$ (Fig. 1A). The

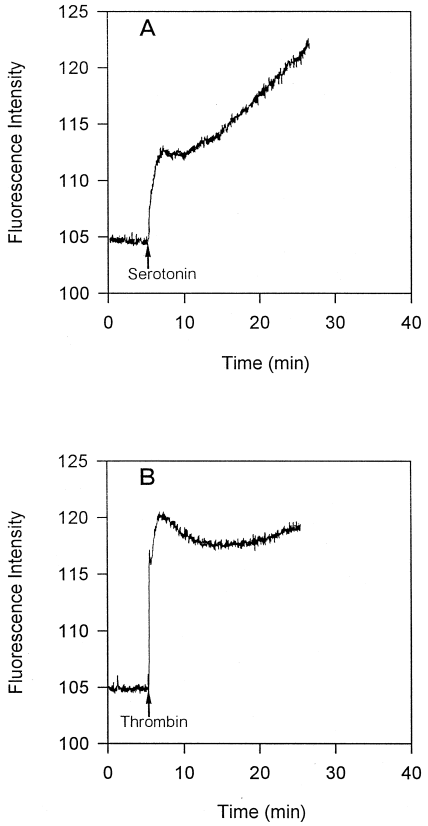

Fig. 1. The effects of serotonin and thrombin on $[Ca^{2+}]_i$ in rat heart endothelial cells. Endothelial cells were preloaded with fura-2 AM and the effects of various compounds on $[Ca^{2+}]_i$ were determined fluorometrically, as described in Section 2. (A) Cells stimulated with 1 mM serotonin. (B) Cells treated with 1 U/ml thrombin. The results shown are representative of the results obtained for four to five separate cell preparations.

serotonin- and thrombin-mediated changes in rat heart endothelial cells cytosolic free Ca^{2+} were different. The response of the rat heart endothelial cells to thrombin also occurred in two phases. The initial phase of the thrombin response exhibited no detectable latency, peaked 1–10 s after the addition of 1 U/ml stimulus, and gradually decayed. The second phase followed as a sustained elevation of cytosolic Ca^{2+} to 3–4-fold above resting levels (Fig. 1B).

3.2. The relationship between the change in cytosolic Ca^{2+} and the increase in endothelial permeability mediated by serotonin

Incubation of rat heart endothelial cell monolayers with serotonin induced a concentration-dependent increase in the passage of Evans blue dye bound-BSA, as shown in Fig. 2. The mean increase in the passage of Evans blue dye bound-BSA induced by a 30-min incubation with 1 mM serotonin was about 3-fold of control. Therefore, 1 mM serotonin was used in our study to cause a rapid change in endothelial cell permeability and to mimic acute inflamma-

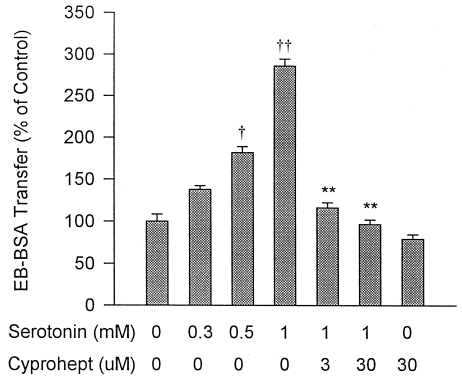

Fig. 2. Effects of serotonin on the transfer of macromolecules across rat heart endothelial cell monolayers grown on filters and of cyproheptadine (Cyprohept) on serotonin-induced endothelial cell monolayer barrier dysfunction ($n = 23–27$). Cells were stimulated with the indicated concentrations of serotonin for 30 min or were preincubated with the 5-HT_2 receptor antagonist, cyproheptadine, for 10 min before the addition of 1 mM of serotonin, and Evans blue dye bound-bovine serum albumin (EB-BSA) transfer was measured after 30 min. EB-BSA was determined spectrophotometrically, as described in Section 2. Cyproheptadine alone had no effect on the passage of macromolecules across the monolayers. The results are expressed as the mean % control ± S.E.M. †$P < 0.05$ compared to the control values. ‡$P < 0.01$ compared to the control values. **$P < 0.01$ compared to the serotonin-treated monolayers.

tion in vivo. The 5-HT_2 receptor antagonist, cyproheptadine, was used to prevent serotonin-induced macromolecular monolayer permeability (Fig. 2) Cyproheptadine, 3 and 30 μM, caused a significant dose-dependent inhibition of serotonin-induced permeability. Fig. 2 also shows that cyproheptadine (30 μM) alone had no effect on the passage of macromolecules across the monolayers in 30 min. To confirm that the increase in $[Ca^{2+}]_i$ was correlated with endothelial permeability, rat heart endothelial cells were pretreated with cyproheptadine (10 μM) for 5 min. As shown in Fig. 3, cyproheptadine attenuated the serotonin-induced increase in $[Ca^{2+}]_i$. The result affirmed that serotonin-induced macromolecular permeability in rat heart endothelial cells was receptor-mediated and mediated by $[Ca^{2+}]_i$.

3.3. Inhibition of serotonin response by phospholipase C inhibitors

To investigate whether the serotonin-induced $[Ca^{2+}]_i$ increase was mediated by phospholipase C, endothelial cells were pretreated with neomycin (1.5, 3 mM; Fig. 4A) and U73122 (1, 2.5 μM; Fig. 4B) for 5 min prior to stimulation with 1 mM serotonin. Neomycin and U73122 are inhibitors of phospholipase C and phospholipid turnover with a less potent effect on phospholipase A_2. As shown in Fig. 4A and B, both neomycin (3 mM) and U73122 (2.5 μM) abolished the serotonin-induced $[Ca^{2+}]_i$ increase in this study.

3.4. Response of endothelial cells to serotonin in the absence of extracellular Ca^{2+}

The next set of experiments was performed to investigate the effect of serotonin on intracellular Ca^{2+} release in the absence of extracellular Ca^{2+}. To this end, cells were

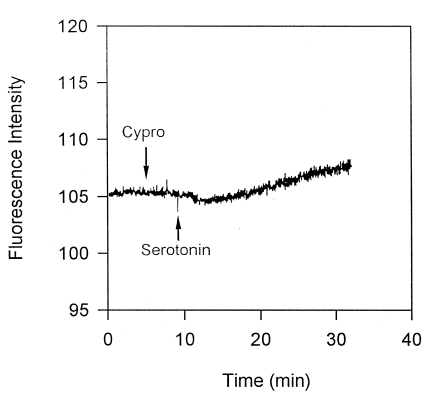

Fig. 3. The effect of cyproheptadine (Cypro) on the serotonin-elicited $[Ca^{2+}]_i$ response in rat heart endothelial cells. Fura-2-loaded endothelial cells suspended in HBS were stimulated with serotonin (1 mM) after addition of 10 μM cyproheptadine (5 min).

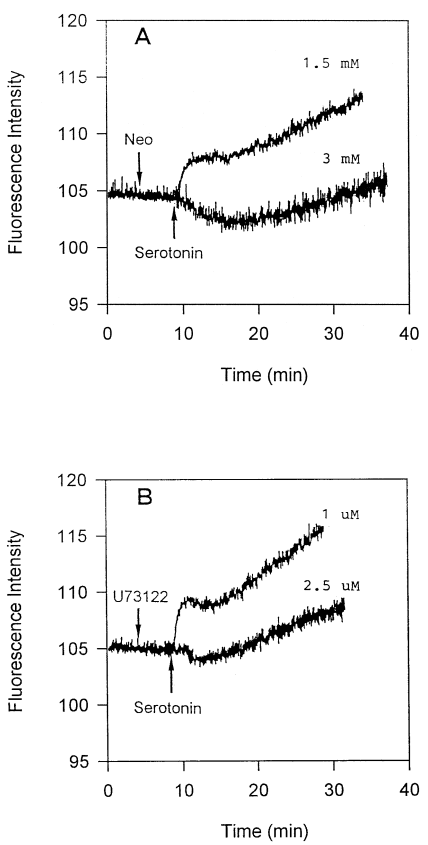

Fig. 4. The effects of the phospholipase C inhibitors, neomycin and U73122, on the serotonin-elicited $[Ca^{2+}]_i$ response in rat heart endothelial cells. Endothelial cells were pretreated with (A) 1.5 or 3 mM neomycin (Neo), (B) 1 or 2.5 μM U 73122 for 5 min prior to stimulation with 1 mM serotonin.

treated with nominally Ca^{2+}-free medium that was further supplemented with 5 mM EGTA for 15 s or 7 min. Treatment of cells with EGTA-containing medium resulted in an early decrease of the resting Ca^{2+} levels, as depicted in Fig. 5. When extracellular Ca^{2+} was less than cytosolic Ca^{2+} concentrations as a result of addition of 5 mM EGTA 15 s before the addition of 1 mM serotonin, the initial and second responses to serotonin were partially affected (Fig. 5A). However, both phases of the serotonin response were inhibited completely by exposing the cells to 5 mM EGTA for 7 min before the addition of 1 mM serotonin (Fig. 5B).

3.5. Distribution of sequestered intracellular Ca^{2+} on serotonin-elicited increase of cytosolic free Ca^{2+}

This study investigated whether Ca^{2+} was slowly released from intracellular stores by serotonin in rat heart endothelial cells. Extracellular Ca^{2+} was rapidly chelated with 5 mM EGTA for 15 s after the cells were stimulated with 1 mM serotonin for 30 min and then the cells were immediately exposed to 4 μM ionomycin (a Ca^{2+} ionophore). As shown in Fig. 6, treatment with ionomycin caused a transient increase of cytosolic Ca^{2+}, which was demonstrated by a transient increase in fluorescence. It was presumably due to the release of residual Ca^{2+} from intracellular Ca^{2+} stores into the cytosolic compartment.

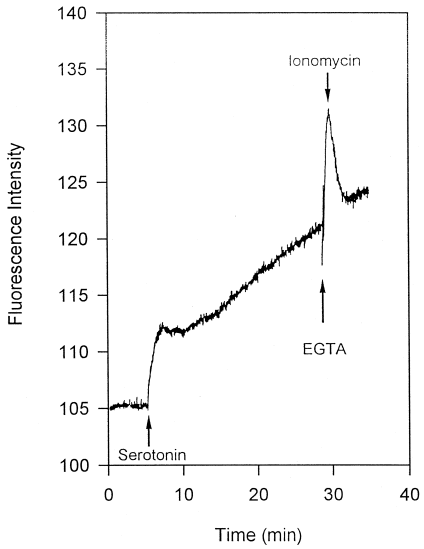

Fig. 6. Depletion of intracellular Ca^{2+} stores by serotonin. Representative tracings of fura-2-loaded endothelial cells suspended in HBS and exposed to serotonin followed by 5 mM EGTA 15 s before addition of 4 μM ionomycin. The release of residual Ca^{2+} from intracellular stores is demonstrated by an increase in fluorescence after the 30-min exposure to serotonin.

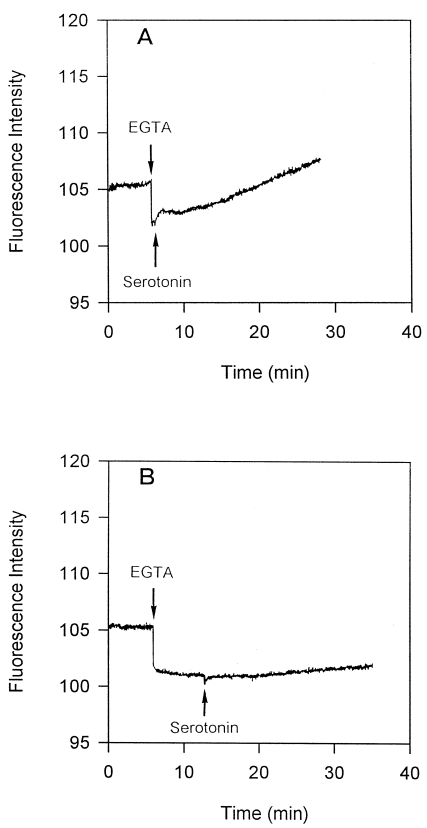

Fig. 5. The effects of prolonged treatment of rat heart endothelial cells with a Ca^{2+}-depleted medium on the serotonin-induced increase of cytosolic Ca^{2+} levels. (A) 15-s pretreatment in Ca^{2+}-deficient medium; (B) 7-min pretreatment in Ca^{2+}-deficient medium (5 mM EGTA).

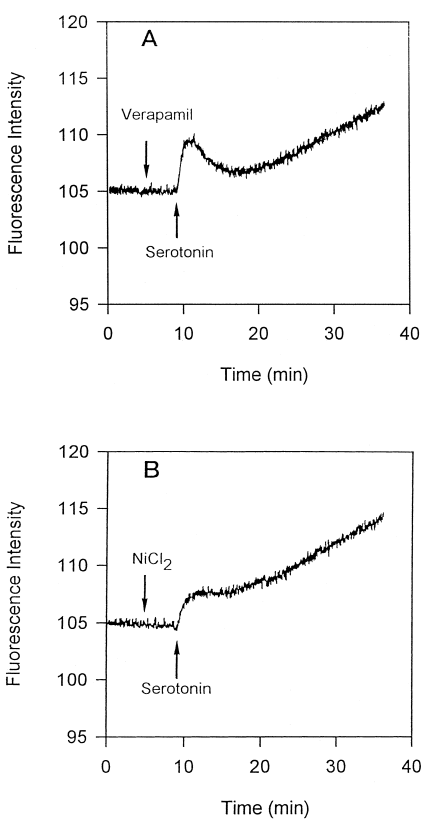

Fig. 7. The effects of Ca^{2+} channel blockers on the serotonin-elicited $[Ca^{2+}]_i$ response in rat heart endothelial cells. Fura-2-loaded cells were incubated for 5 min with (A) verapamil (20 μM), or (B) nickel chloride (Ni^{2+}) (1 mM) before the addition of 1 mM serotonin.

Table 1
LDH release from rat heart endothelial cells

Reagents	LDH release (% of control)[a]
Control (vehicle)	100
Serotonin (1 mM)	99.9 ± 0.1
Cyproheptadine (30 μM)	99.9 ± 0.2
Neomycin (3 mM)	100.0 ± 0.2
U73122 (10 μM)	100.0 ± 0.2
Verapamil (20 μM)	99.8 ± 0.3
Nickel chloride (1 mM)	99.7 ± 0.2

[a]Rat heart endothelial cells were cultured in 24-well plates and then incubated with various concentrations of reagent in HBSS with 0.1% BSA for 30 min. LDH activity in the supernatant was determined by the LDH Detection Kit. The results are expressed as the mean % control ± S.E.M. ($n = 20$).

The result indicated that the ionomycin-sensitive Ca^{2+} stores were still demonstrable at 30 min after stimulation with 1 mM serotonin.

3.6. Inhibition of serotonin response by selective ion channel blockers

Pretreatment of the cells with the non-specific voltage-sensitive Ca^{2+} channel blocker, verapamil (20 μM, 5 min), or the inorganic Ca^{2+} channel blocker, nickel chloride (1 mM, 5 min), had different effects on the Ca^{2+} release elicited by serotonin. In the presence of verapamil, the early response to serotonin was decreased and the initial stage of the second phase of the serotonin response was inhibited (Fig. 7A). However, the biphasic response of serotonin-induced $[Ca^{2+}]_i$ was inhibited partially by nickel chloride (Fig. 7B). These results indicate that the response of endothelial Ca^{2+} to serotonin was related to the influx of extracellular Ca^{2+}.

3.7. Cytotoxicity assay

Treatment of rat heart endothelial cells with various reagents for 30 min did not increase LDH release (Table 1). These results indicate that the increase in endothelial permeability and cytosolic Ca^{2+} concentration was not due to a cytolytic effect of these interventions.

4. Discussion

The vascular endothelial cell is uniquely situated to play an active role in the induction of the inflammatory response. Endothelial cell motility and contractility directly influence the inflammatory response by modulating leukocyte emigration and plasma protein leakage. It has been proposed that Ca^{2+}-dependent contractile mechanisms modulate changes in the permeability of endothelial cells to water and macromolecules (Schilling et al., 1991). Many investigators also reported that the changes in intracellular Ca^{2+} concentration might function as critical signals regulating endothelial barrier function in response to permeability-increasing agents, such as bradykinin and thrombin (Schilling et al., 1988; Goligorsky et al., 1989). Therefore, changes in $[Ca^{2+}]_i$ play a key role in the responses of vascular endothelial cells to vasoactive substances. Our previous studies have demonstrated that serotonin causes a significant change in the morphology of rat heart endothelial cell monolayers and the separation of adjacent cells with the resultant formation of large paracellular openings (Lee, 1997). We also suggested that the activation of endothelial protein kinase C by serotonin resulted in significant changes in the localization and translocation of α protein kinase C isozyme (Lee et al., 1998). Xia et al. (1996) have indicated that diacyglycerol levels and Ca^{2+} flux can stimulate protein kinase C isoforms α and β to translocate since both of these isoforms contain diacyglycerol and Ca^{2+} binding sites. It is interesting to speculate that the predominant activation of α protein kinase C isozyme could be the result of the serotonin-induced increases in Ca^{2+} flux. This study was designed to investigate whether serotonin, by increasing cellular Ca^{2+} concentration, induced the contraction of adjacent endothelial cells. We demonstrated that serotonin caused a significant increase in the concentration of cytosolic Ca^{2+} in rat heart endothelial cells. The 5-HT_2 receptor antagonist, cyproheptadine, prevented the serotonin-induced permeability of monolayers to macromolecules and attenuated the serotonin-induced increase in $[Ca^{2+}]_i$. This indicated that serotonin-induced macromolecular permeability in rat heart endothelial cells was receptor-mediated and associated with $[Ca^{2+}]_i$. Previous observations showed that the thrombin- and histamine-induced increase in cytosolic Ca^{2+} concentration consisted of an initial and a sustained phase (Rotrosen and Gallin, 1986; Goligorsky et al., 1989). In our study, serotonin stimulated a biphasic change in cytosolic Ca^{2+} of endothelial cells: an initial steep increase that the decreased slightly, followed by a slow rise in $[Ca^{2+}]_i$. This result is not consistent with other observations that vasoactive agents elicit a transient increase in cytosolic free Ca^{2+} followed by a progressive decline to a steady state. However, our study demonstrated that the thrombin-mediated pattern of biphasic change in $[Ca^{2+}]_i$, as well as that of other vasoactive agents in various endothelial cell cultures (Schilling et al., 1988; Goligorsky et al., 1989), consisted of an initial phase, in which there was an immediate increase in Ca^{2+} that gradually decayed, and a second phase in which there was a sustained elevation of cytosolic Ca^{2+} to 3–4-fold above resting levels. The pattern of the biphasic change in $[Ca^{2+}]_i$ was different for serotonin and thrombin. The result indicates that serotonin- or thrombin-induced increase in $[Ca^{2+}]_i$ may be mediated by different mechanisms.

Previous studies have suggested that some metabolite of phospholipase C may function to both release Ca^{2+} and

stimulate influx (Carney et al., 1985; Schilling and Elliott, 1992; Stanimirovic et al., 1996). Carney et al. (1985) and Schilling et al. (1991) demonstrated that neomycin (a phospholipase C inhibitor) inhibited thrombin-stimulated phosphoinositide turnover and U73122 (a phospholipase C inhibitor) blocked the bradykinin-stimulated change of $[Ca^{2+}]_i$, respectively. Based on previous studies and the above observation, it seemed reasonable to surmise that thrombin or bradykinin lead to the activation of phospholipase C, stimulation of phosphatidylinositol turnover, and mobilization of Ca^{2+}. Indeed, experiments showed that the inhibitors of phospholipase C, neomycin and U73122, suppressed the serotonin-induced increase in $[Ca^{2+}]_i$, confirming that serotonin mobilized Ca^{2+} from intracellular stores or the extracellular compartment by activating a phosphoinositide-specific phospholipase C.

The early mobilization of Ca^{2+} from intracellular pools followed by the influx of extracellular Ca^{2+} has been described by previous investigators. In the present study, the ability of serotonin to change intracellular Ca^{2+} concentration in the virtual absence of extracellular Ca^{2+} was also investigated. We used relatively mild chelation (5 mM EGTA, 15 s) of Ca^{2+} in the experiment in order to effectively lower extracellular Ca^{2+} below cytosolic concentrations yet not strip Ca^{2+} from intracellular sites. When extracellular Ca^{2+} was removed by the addition of EGTA immediately before the addition of serotonin, the biphasic response to serotonin was changed. This result indicated that the biphasic response of endothelial Ca^{2+} to serotonin was related to the influx of extracellular Ca^{2+}. It is interesting to note that the second phase of the serotonin-elicited change in $[Ca^{2+}]_i$ was partially attenuated, but the slow increase in $[Ca^{2+}]_i$ during the incubation with serotonin was still seen in Ca^{2+}-free medium. This indicated that the second response of endothelial cell Ca^{2+} to serotonin was also related to the release of Ca^{2+} from internal stores. Our study also demonstrated that increasing the time of pretreatment of the cells with Ca^{2+}-deficient medium from 15 s to 7 min caused a significant decrease in the serotonin-elicited Ca^{2+} response. It suggested that the serotonin-sensitive Ca^{2+} store was depleted following incubation in EGTA buffer for 7 min. Collectively, these data suggested that the biphasic response of rat heart endothelial cell Ca^{2+} to serotonin was related to the mobilization of Ca^{2+} from intracellular pools and to the influx of Ca^{2+} from the extracellular space.

Furthermore, this study examined whether the serotonin-induced increase in $[Ca^{2+}]_i$ in rat heart endothelial cells rapidly depleted intracellular Ca^{2+} stores. The hypothesis suggested by Morgan-Boyd et al. (1987) is that endothelial cell desensitization may be the expression of intracellular Ca^{2+} depletion. This was investigated by treating the cells with a Ca^{2+} ionophore, ionomycin, in the presence of Ca^{2+}-deficient medium. This approach is a well-established way to cause the rapid depletion of intracellular Ca^{2+} stores (Chandler et al., 1983; Morgan-Boyd et al., 1987). Our study demonstrated that intracellular Ca^{2+} pools were not rapidly depleted by serotonin, and the Ca^{2+} stores were still demonstrable at 30 min after serotonin. These results suggested that the serotonin-induced gradual increase in $[Ca^{2+}]_i$ in the second phase was also associated with the release of Ca^{2+} from intracellular stores and that this is probably linked to the serotonin-evoked continuous sensitization of rat heart endothelial cells.

Finally, this study was designed to investigate the role of Ca^{2+} channels in serotonin-evoked modulation of $[Ca^{2+}]_i$ by the use of the Ca^{2+} channel blockers. We demonstrated that the serotonin-induced changes in $[Ca^{2+}]_i$ were related to Ca^{2+} channels sensitive to voltage-operated and inorganic Ca^{2+} channel blockers in rat heart endothelial cells. These results are consistent with those of other studies reporting that voltage-gated Ca^{2+} channels and inorganic Ca^{2+} channels present in endothelial cells may be involved in agonist-stimulated Ca^{2+} influx (Singer and Peach, 1982; Rotrosen and Gallin, 1986).

In conclusion, this study has established the relationship of serotonin-induced intracellular Ca^{2+} levels in regulating the permeability to macromolecules of cultured rat heart endothelial cells. The increase in Ca^{2+} appears to be the result of an interaction of serotonin with 5-HT_2 receptors. Experiments also demonstrated that the activation of phospholipase C was involved in the serotonin-induced increase in $[Ca^{2+}]_i$. In Ca^{2+}-deficient medium, we demonstrated that the biphasic response of rat heart endothelial cell Ca^{2+} to serotonin was related to the mobilization of Ca^{2+} from intracellular pools and to the influx of Ca^{2+} from the extracellular space. Cells appear to continue to be sensitive to serotonin, since ionomycin-sensitive Ca^{2+} stores could still be demonstrated during the incubation with serotonin. Finally, serotonin-induced changes in $[Ca^{2+}]_i$ were also inhibited by the Ca^{2+} channel blockers, verapamil and nickel chloride, in this study.

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