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Calcium-mobilizing Agonists Stimulate Anion Fluxes in Cultured Endothelial Cells from Human Umbilical Vein

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Abstract. The goal of the present studies was to determine whether anion fluxes are involved in thrombin- and histamine-activated signal transduction pathways in human umbilical vein endothelial cells (HUVECs). ^{125}Iodine (^{125}I) efflux techniques were used to test the sensitivity of anion fluxes to increases in $[Ca^{2+}]_i$ and activation of protein kinase C. HUVECs exhibited constant ^{125}I efflux rates under basal conditions. Administration of thrombin or histamine stimulated an increase in ^{125}I efflux rates which returned to control values after approximately 1–2 min. Since both agonists stimulate increases in $[Ca^{2+}]_i$, we tested the hypothesis that ^{125}I efflux was sensitive to changes in $[Ca^{2+}]_i$. When HUVECs were exposed to ionomycin or thapsigargin, the ^{125}I efflux rate increased and remained elevated for several minutes. In subsequent experiments, HUVECs were incubated with the cell permanent Ca^{2+} chelator, 1,2-bis-(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid-AM, to buffer changes in $[Ca^{2+}]_i$. This treatment reduced both basal and thrombin-stimulated ^{125}I efflux. However, when Ca^{2+} was removed from the efflux buffer and replaced with EGTA, peak thrombin-stimulated ^{125}I efflux remained unchanged. This anion efflux was also sensitive to activation of protein kinase C since phorbol 12-myristate 13-acetate and phorbol, 12,13-dibutyrate blunted thrombin-mediated increases in ^{125}I efflux. Preincubation of HUVECs with protein kinase C inhibitor peptide [19–36] antagonized the phorbol ester-mediated decrease in thrombin-stimulated ^{125}I efflux. We suggest that ^{125}I efflux in HUVECs represents a Ca^{2+}-sensitive anion conductance and that intracellular Ca^{2+} release, but not extracellular Ca^{2+} influx, is sufficient to initiate channel activity.

Key words: ^{125}I efflux — Cl^- current — Thrombin — PKC — Human umbilical vein endothelial cells

Introduction

In recent years, considerable attention has focused on the role of the endothelium in the regulation of vascular function. In addition to serving as a permeability barrier between the blood and underlying smooth muscle cells, it has been shown that the endothelium serves as the site for the synthesis and release of a number of paracrine substances which affect blood vessel contractility in both normal and disease states [17]. The endothelial cell (EC) agonists, thrombin and histamine, are known to participate in physiological responses to vascular injury and inflammation [4, 11], and also stimulate the release of endothelium-derived relaxing factor (EDRF). The constitutive synthesis of EDRF in response to various agonists requires both calcium (Ca^{2+}) and calmodulin [27]. Mechanisms underlying EC Ca^{2+} mobilization include the release of Ca^{2+} from intracellular storage cites and a membrane potential-sensitive, Ca^{2+} influx pathway [3, 25].

The electrophysiological study of EC ion channels has focused primarily on the characterization of potassium channels and nonspecific cation channels and their role in the regulation of intracellular calcium concentration ($[Ca^{2+}]_i$). In numerous cell types, there is substantial evidence that Cl^- channels play an important role in membrane excitation processes [2, 13, 21, 28]. Ligand- and voltage-gated Cl^- channels have been characterized extensively in excitable cells, including neurons [14, 23], cardiac myocytes [2], and skeletal muscle [6]. Ca^{2+}-

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sensitive Cl^- channels have also been described in various cell types, including parotid acinar cells [19], lacrimal gland cells [24], epithelial cells [10], mast cells [34], platelets [26], neutrophils [28], and vascular smooth muscle cells [13, 21]. In contrast, little information is available concerning the role of Cl^- channels in ECs. At least four cellular mechanisms of Cl^- transport have been identified in recent years. These include: (1) Na^+-dependent chloride/bicarbonate (Cl^-/HCO_3^-) exchange [1, 40]; (2) Na^+-independent Cl^-/HCO_3^- exchange [22]; (3) $Na^+/K^+/Cl^-$ cotransport [7, 20, 32]; and (4) conductive Cl^- channels [16, 33, 35, 39]. The Cl^-/HCO_3^- exchangers provide mechanisms of intracellular pH regulation which are common to most cell types [37]. $Na^+/K^+/Cl^-$ cotransport is thought to be important in cell volume regulation and is also sensitive to changes in $[Ca^{2+}]_i$ in ECs [31]. Patch clamp techniques have been used to identify conductive Cl^- channels in ECs [16, 33, 35]; however, their physiological significance is still poorly understood. The goal of the present studies was to test the hypothesis that anion fluxes are involved in thrombin- and histamine-activated signal transduction pathways in human umbilical vein endothelial cells (HUVECs). In particular, we utilized radioisotope flux techniques to test the sensitivity of anion fluxes to $[Ca^{2+}]_i$ and activation of protein kinase C (PKC).

Materials and Methods

Cell Culture

Primary cultures of HUVECs were established using enzymatic dissociation techniques and propagated as described previously [8]. Primary and passaged cells were grown (passages 1–5) in medium 199 (GIBCO) supplemented with 15% fetal bovine serum, 50 μg/ml endothelial cell mitogen (Biomedical Technologies), 100 μg/ml heparin, 100 μg/ml penicillin and 100 μg/ml streptomycin. HUVECs were detached from culture flasks with trypsin-EDTA and were seeded onto 35 mm culture dishes, previously coated with 0.1% gelatin. Cultured HUVECs were grown to 90% confluence prior to study.

^{125}Iodine Efflux Studies

^{125}Iodine (^{125}I) was chosen as a marker for Cl^- channel activity in these studies for several reasons: (i) it has a higher specific activity than ^{36}Cl; (ii) it is transported poorly by the $Na^+/K^+/Cl^-$ cotransporter and by anion exchangers [12, 32]; and (iii) its permeability through anion-specific channels is greater than that of Cl^- [36]. HUVECs were exposed to 6 μCi/ml ^{125}I (carrier-free, DuPont NEN) in complete growth media for 12–18 hr prior to study. In these experiments, we utilized a standard efflux buffer of the following composition (in mM): 140 NaCl, 10 HEPES, 3.33 KH_2PO_4, 0.83 K_2HPO_4 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose (pH 7.4). In each experiment, the culture dish was placed on a platform in a heated water bath so that the bottom portion of the dish was submerged in water warmed to 37°C. All solutions used in these experiments were maintained at 37°C. Radioactive incubation media were aspirated at the beginning of each experiment. HUVECs were then rinsed with 10 ml of efflux buffer accompanied by simultaneous aspiration to remove excess isotope in the extracellular space. One milliliter of efflux buffer was then added to the dish for a 5 min time period. After this control period, the buffer was aspirated again and replaced with 1 ml of fresh buffer. At this point (time 0), sample collection was initiated. At the end of the first 20 sec sample period, the 1 ml volume of efflux media was removed, decanted into a collection tube and replaced with 1 ml of fresh buffer. Using this buffer replacement protocol, ^{125}I efflux samples in standard buffer were obtained for a 3 min control period. HUVECs were then exposed to agonist (dissolved to the appropriate concentration in standard efflux buffer), and sample collection continued every 20 sec for an additional 5 min. Cell-associated ^{125}I radioactivity remaining at the end of the experiment was extracted with 1.0% sodium dodecyl sulfate/0.1% NaOH and quantified using a Searle gamma counter (model 1285). Total intracellular counts at the beginning of the experiment were estimated by back-adding counts collected during each sample period. ^{125}I efflux rates (% counts effluxed/min) were determined for each 20 sec period by dividing the number of counts effluxed by the total intracellular counts present at the beginning of that sample period.

Measurement of Ca^{2+}-sensitive Fura-2 Fluorescence

Fura-2, a Ca^{2+}-sensitive fluorescent dye (Molecular Probes), was used to monitor changes in $[Ca^{2+}]_i$ in HUVEC suspensions as described by Brock and Capasso [8]. Cells were loaded with Fura-2 (2 μM) for 30 min prior to study. Cytosolic Ca^{2+} concentrations were estimated using the formula:

$$[Ca^{2+}]_i = K_d(R - R_{min})/(R_{max} - R) * (EGTA_{380}/DIG_{380})$$

where R was the fluorescence ratio within the cell, K_d was 224 nM, $EGTA_{380}$ was the fluorescence signal (380 nm) in the presence of 10 mM EGTA (Ph = 10) and DIG_{380} was the fluorescence signal following digitonin addition in the presence of 1.5 mM $CaCl_2$. Fluorescence tracings depicting $[Ca^{2+}]_i$ recordings are typical tracings and representative of at least four independent experiments.

Statistical Analysis

Descriptive statistics reported include mean and standard error of the mean (SEM). Results were analyzed by analysis of variance (ANOVA). The confidence level for comparison was $P < 0.05$.

Results

Cultured HUVECs exhibited constant ^{125}I efflux rates under basal conditions (Fig. 1*A* and Table 1). As illustrated in Fig. 1*B*, thrombin (3 U/ml) caused a rapid, transient increase in ^{125}I efflux above basal values (9.07-fold increase, $n = 33$) which returned to control levels after approximately 1–2 min. Similar results were obtained when HUVECs were challenged with histamine (100 μM) (3.83-fold increase, $n = 6$; Fig. 1*C*). Since both agonists stimulate increases in $[Ca^{2+}]_i$, we tested the hypothesis that ^{125}I efflux was sensitive to changes in $[Ca^{2+}]_i$. When HUVECs were exposed to the Ca^{2+} ionophore, ionomycin (5 μM), the ^{125}I efflux rate rapidly increased (4.88-fold increase) and remained elevated for several minutes (Fig. 2*A*). Thapsigargin (1 μM), which promotes Ca^{2+} release from intracellular storage sites,

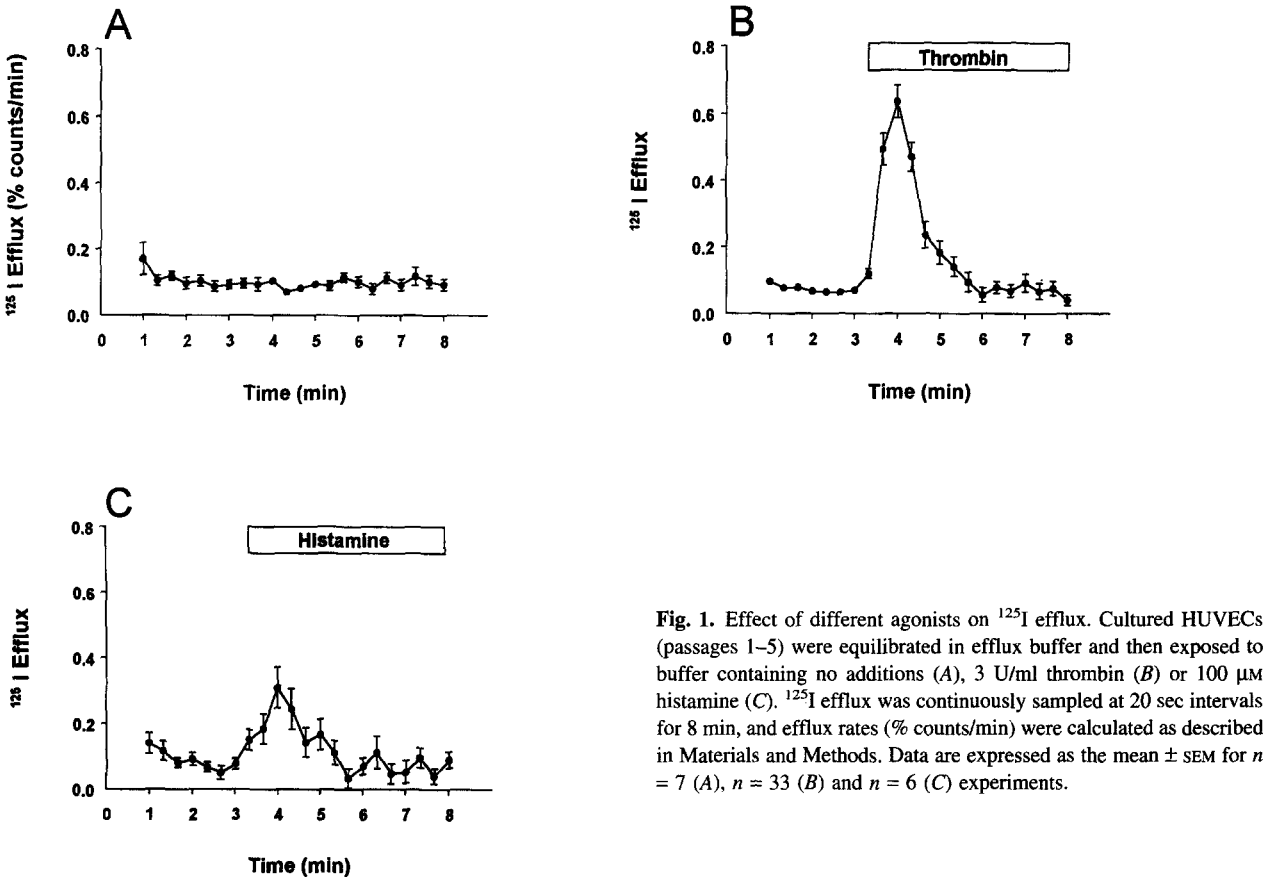

Fig. 1. Effect of different agonists on ^{125}I efflux. Cultured HUVECs (passages 1–5) were equilibrated in efflux buffer and then exposed to buffer containing no additions (*A*), 3 U/ml thrombin (*B*) or 100 μM histamine (*C*). ^{125}I efflux was continuously sampled at 20 sec intervals for 8 min, and efflux rates (% counts/min) were calculated as described in Materials and Methods. Data are expressed as the mean ± SEM for $n = 7$ (*A*), $n = 33$ (*B*) and $n = 6$ (*C*) experiments.

also stimulated a sustained increase (2.55-fold) in ^{125}I efflux (Fig. 2*B*). In subsequent experiments, 1,2-bis-(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA) was used as a cell Ca^{2+} buffer to dampen agonist-stimulated increases in $[Ca^{2+}]_i$. Incubation of HUVECs with the acetoxymethyl ester of BAPTA (BAPTA-AM/20 μM, 20 min) completed inhibited thrombin-stimulated ^{125}I efflux ($n = 8$, Fig. 2*C*, Table 1). However, when Ca^{2+} was removed from the efflux buffer and replaced with the chelating agent, EGTA (2 mM), the peak thrombin-stimulated ^{125}I efflux remained unchanged (Fig. 2*D*). Both BAPTA-AM treatment and extracellular Ca^{2+} removal caused basal ^{125}I efflux rates to decrease in the absence of thrombin (Table 1).

Fluorescence techniques were used to monitor changes in $[Ca^{2+}]_i$ under similar experimental conditions. In Fura-2-located HUVECs, resting $[Ca^{2+}]$ was 110 ± 10 nM ($n = 13$). Thrombin (3 U/ml) induced a rapid increase in $[Ca^{2+}]_i$ with a peak concentration reaching 533 ± 24 nM (Fig. 3). In cells pretreated with BAPTA-AM (20 μM), a significant reduction in resting $[Ca^{2+}]$ was noted (63 ± 5 nM/$n = 9$). Additionally, the thrombin-mediated increase in $[Ca^{2+}]_i$ was completely blocked by BAPTA-AM treatment (peak increase = 79 ± 15 nM/$n = 4$; Fig. 3). Replacement of extracellular Ca^{2+} with 2 mM EGTA did not prevent the thrombin-stimulated increase in $[Ca^{2+}]_i$ (*data not shown*). Thapsigargin (1 μM) induced a slowly developing but sustained increase in $[Ca^{2+}]_i$ (Fig. 3) which was temporally related to thapsigargin-stimulated ^{125}I efflux. These results support the hypothesis that in-

Table 1. Effect of experimental treatments on mean ^{125}I efflux rates

Treatment	Mean ^{125}I Efflux (% counts effluxed/min)	SEM	*P*	*n*
Control	0.097	0.003		7
BAPTA-AM	0.048	0.007	**	6
EGTA	0.054	0.010	**	7
Forskolin	0.069	0.005	**	6
KCl	0.062	0.013	*	4
PMA	0.067	0.005	**	7
PKCI [19–36]	0.099	0.007	NS	4
Thrombin + BAPTA-AM	0.091	0.012	NS	8
Thrombin + $ZnCl_2$	0.061	0.006	**	4

Means represent the average ^{125}I efflux calculated over the last 5 min of each experiment (sample intervals nos. 10–24). Mean ^{125}I efflux rates under the conditions noted were compared to control efflux rates (*$P < 0.05$; **$P < 0.01$). Studies were performed using low-passage HUVECs (subcultures 1–5). The concentrations of agents were: thrombin (3 U/ml); BAPTA-AM (20 μM); EGTA (2 mM); forskolin (2.5 μM); KCl (140 mM); PMA (0.1 μM); PKCI [19–36] (1 μM); $ZnCl_2$ (1 mM).

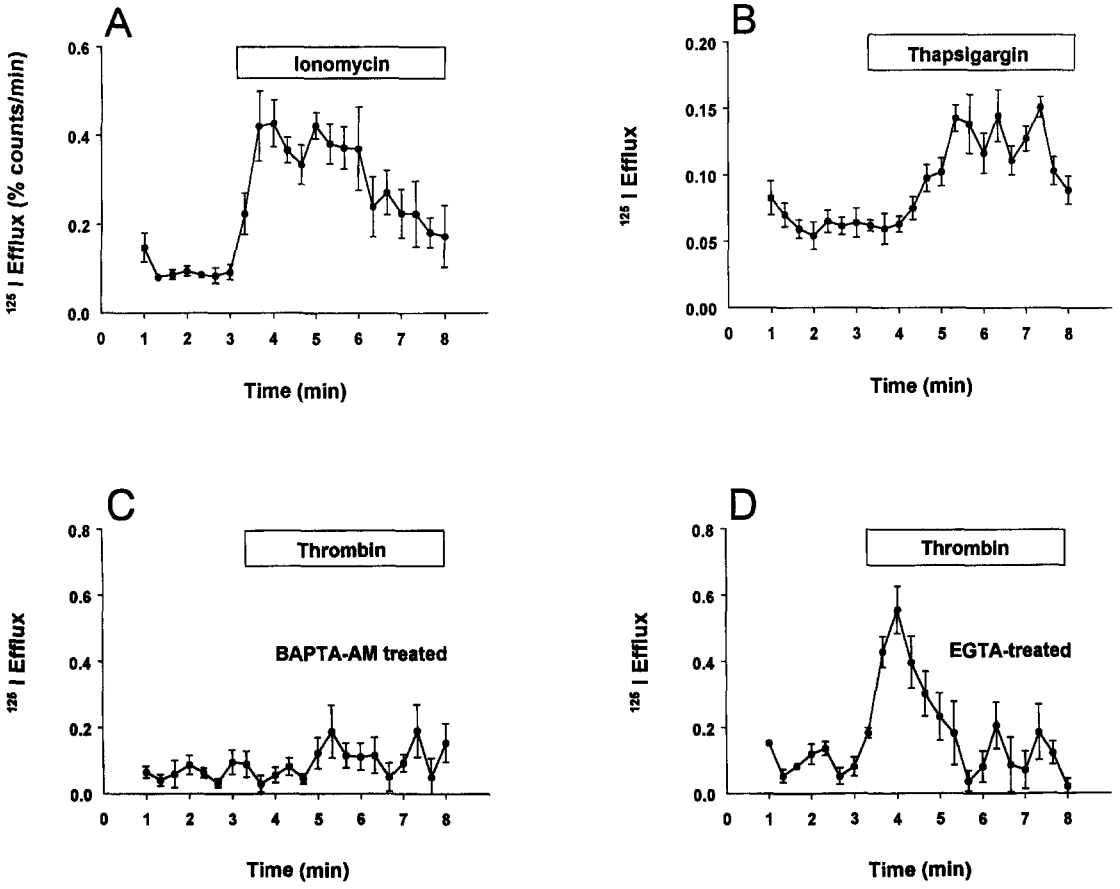

Fig. 2. Effects of cellular Ca^{2+} modulators on ^{125}I efflux in HUVECs. (*A*) Ionomycin (5 μM) stimulated a prolonged increase in ^{125}I efflux compared to thrombin treatment in Fig. 1. (*B*) Effect of thapsigargin (1 μM) on ^{125}I efflux. (*C*) Pretreatment of HUVECs with BAPTA-AM (20 μM/20 min) inhibited thrombin-stimulated ^{125}I efflux. (*D*) Extracellular Ca^{2+}-chelation (2 mM EGTA was substituted for Ca^{2+} in the efflux buffer) had no effect on thrombin-stimulated ^{125}I efflux. ^{125}I efflux rates were calculated as described in Materials and Methods. Data are expressed as the mean ± SEM for $n = 11$ (*A*), $n = 5$ (*B*), $n = 8$ (*C*) and $n = 8$ (*D*) experiments.

tracellular Ca^{2+} release, but not extracellular Ca^{2+} influx, is sufficient to initiate agonist-stimulated ^{125}I efflux.

In other experiments, we tested the effects of cyclic AMP (cAMP) and of membrane depolarization on ^{125}I efflux. Forskolin has been shown to activate epithelial cell Cl^- channels by stimulating the production of cAMP [10]. Similarly, cAMP stimulates ^{36}Cl efflux in bovine pulmonary artery endothelial cells [38]. In HUVECs, however, we found that forskolin (2.5 μM) reduced ^{125}I efflux (Table 1). When HUVECs were depolarized by exposing monolayers to efflux buffer containing 140 mM KCl, ^{125}I efflux was reduced below control values (Table 1). If the equilibrium potential for Cl^- (E_{Cl}) is near the resting membrane potential, KCl-induced membrane depolarization would be expected to produce a slight decrease in ^{125}I efflux, since inward currents would be predicted at membrane potentials more depolarized than the E_{Cl}. Alternatively, isotonic KCl may induce cellular swelling leading to changes in anion transport mechanisms. For example, this treatment has been shown to inhibit $Na^+/K^+/Cl^-$ cotransport in bovine aortic ECs [20].

Since Na^+-dependent and -independent Cl^-/HCO_3^- transport mechanisms are inactive in HCO_3^--free efflux media [37], our data indicate that these particular transporters do not mediate agonist-stimulated ^{125}I efflux. To test the potential role of other Cl^- transport mechanisms in mediating agonist-stimulated ^{125}I efflux, HUVECs were pretreated with either bumetanide (100 μM, 30 min), DIDS (50 μM, 10 min) or $ZnCl_2$ (1 mM, 10 min). In HUVECs pretreated with bumetanide, peak ^{125}I efflux in response to thrombin was not significantly elevated compared to thrombin treatment alone (0.824 ± 0.113 *vs.* 0.636 ± 0.048) (Fig. 4*A*). In contrast, when HUVECs were pretreated with DIDS, a Cl^- channel blocker, thrombin-stimulated ^{125}I efflux was inhibited by approximately 48% (Fig. 4*B*). Pretreatment of HUVECs with 1 mM $ZnCl_2$, a reversible blocker of Cl^- channels, completely abolished thrombin-stimulated increases in ^{125}I efflux (Fig. 4*C*) and significantly reduced baseline efflux to values below control (Table 1).

Since reports suggest that EC Cl^- channels may be regulated by PKC-dependent phosphorylation [16, 29],

experiments were performed to test the effects of phorbol esters (activators of PKC) on ^{125}I efflux. Ten minute preincubation of HUVEC monolayers with the phorbol ester, phorbol 12-myristate 13-acetate (PMA: 0.1 μM), caused a significant reduction in basal ^{125}I efflux below control values (Table 1). PMA and the phorbol ester, phorbol, 12,13-dibutyrate (PDBu: 0.1 μM) attenuated the peak ^{125}I efflux response to thrombin by 71 and 41%, respectively (Fig. 5). Control experiments were performed to determine the specificity of these responses. HUVECs were pretreated (10 min) with 4α-phorbol, 12, 13-didecanoate, an inactive phorbol ester, prior to study. As seen in Fig. 6*A*, preincubation with 4α-phorbol, 12, 13-didecanoate (0.1 μM) had no effect on thrombin-mediated ^{125}I efflux. In other experiments, ^{125}I efflux responses were studied in HUVECs pretreated with the PKC inhibitor, protein kinase C inhibitor peptide [19–36] (PKCI [19–36]). PKCI [19–36] acts as a substrate antagonist and is believed to inhibit PKC via its interaction with the active site of the enzyme [18]. HUVECs were incubated with the peptide (1 μM) for 18 hr prior to study in serum-free media containing 6 μCi/ml ^{125}I. No change in basal (Table 1) or thrombin-stimulated ^{125}I efflux (Table 2) was noted following PKCI [19–36] exposure. The ability of PKCI [19–36] to reverse the PMA-mediated inhibition of thrombin-stimulated ^{125}I efflux was tested in subsequent experiments. PMA was added to PKCI [19–36]-incubated HUVECs for 10 min prior to initiation of the efflux assay. Under these conditions, PKCI [19–36] prevented the inhibition of thrombin-stimulated ^{125}I efflux by PMA (Fig. 6*B*, Table 2). While the peak increase in thrombin-stimulated ^{125}I efflux occurred more rapidly under these conditions, the maximum response was not statistically different from that seen to thrombin in untreated HUVECs. In PMA-treated cells, a significant difference, however, was noted between peak responses to thrombin in the presence and absence of PKCI [19–36] ($P < 0.05$). These results suggest that PKC may be involved in the regulation of the agonist-sensitive ^{125}I efflux pathway.

To determine potential effects of PKC on $[Ca^{2+}]_i$ mobilization processes, Fura-2-loaded HUVECs were studied. Basal and thrombin-stimulated increases in $[Ca^{2+}]_i$ are depicted in Fig. 7. PMA pretreatment (0.1 μM, 10 min) significantly reduced thrombin-stimulated increases in $[Ca^{2+}]_i$ (peak $[Ca^{2+}] = 258 \pm 17$ nM). Overnight incubation with PKCI [19–36] did not affect the peak response to thrombin in the absence of PMA. However, PKCI [19–36] partially blocked the PMA-mediated inhibition of thrombin-stimulated Ca^{2+} mobilization (Fig. 7).

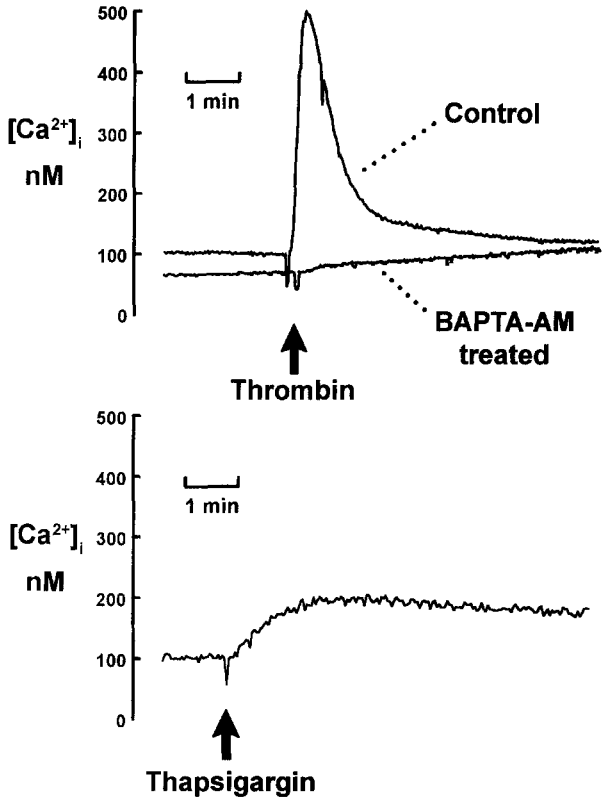

Fig. 3. Intracellular calcium transients in suspensions of Fura-2 loaded HUVECs. (Top panel) Fluorescence emission spectra for HUVECs exposed to thrombin (3 U/ml) in the presence and absence of BAPTA-AM. HUVECs were loaded with Fura-2 (2 μM) for 30 min prior to study. BAPTA-AM (20 μM) was added to the cell suspension 10 min into the Fura-2 incubation period. The rapid increase in $[Ca^{2+}]_i$ in response to thrombin under control conditions was totally abolished in HUVECs incubated with BAPTA-AM. (Bottom panel) Effect of thapsigargin (1 μM), a receptor-independent Ca^{2+} agonist, on $[Ca^{2+}]_i$ in a HUVEC suspension. $[Ca^{2+}]_i$ rose slowly in response to thapsigargin and remained elevated for several minutes. Sample tracings are representative of at least four individual experiments.

Discussion

The results of the present studies show that ^{125}I efflux in cultured HUVECs is activated in response to agonists such as thrombin and histamine. Furthermore, ^{125}I efflux is mediated by changes in $[Ca^{2+}]_i$, as shown by our observations that agonist-stimulated increases in ^{125}I efflux from HUVECs were mimicked using ionomycin and thapsigargin, and were inhibited using BAPTA-AM to blunt agonist-stimulated $[Ca^{2+}]_i$ increases. We suggest that the ^{125}I efflux pathway described in these studies represents the activation of conductive Cl^- channels. This assumption is based on the following observations. Cl^- channels are relatively nonselective for anions [35] and have a greater permeability for I^- than for Cl^- [9]. In the present studies, efflux media were buffered with HEPES to inhibit Cl^-/HCO_3^- exchange, since this transporter is inactive in HCO_3^--free media [37]. Additionally, ^{125}I has little affinity for anion exchange transporters or for $Na^+/K^+/Cl^-$ cotransport mechanisms [9]. In contrast, ^{36}Cl can be transported by multiple types of anion carriers, i.e., $Na^+/K^+/Cl^-$ cotransporter, Na^+-

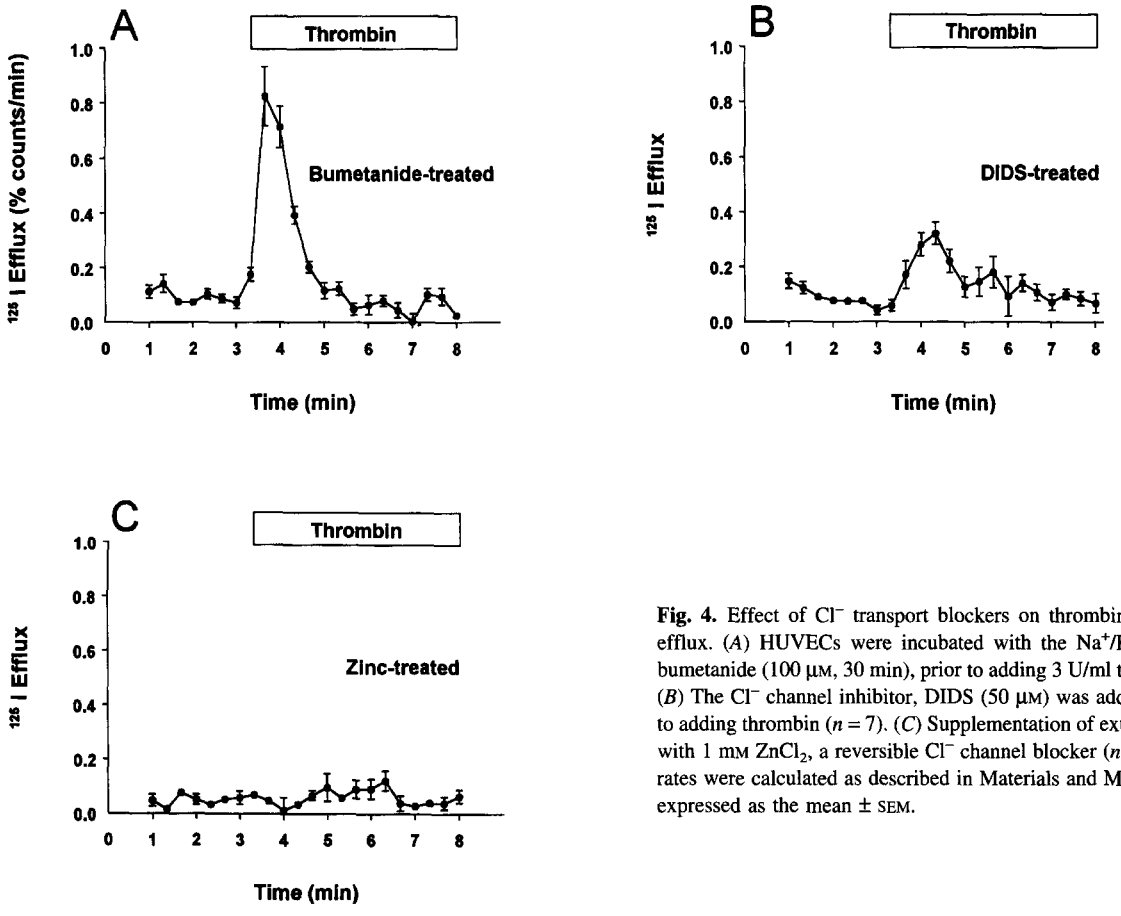

Fig. 4. Effect of Cl^- transport blockers on thrombin-stimulated ^{125}I efflux. (*A*) HUVECs were incubated with the $Na^+/K^+/Cl^-$ inhibitor, bumetanide (100 μM, 30 min), prior to adding 3 U/ml thrombin ($n = 9$). (*B*) The Cl^- channel inhibitor, DIDS (50 μM) was added 10 min prior to adding thrombin ($n = 7$). (*C*) Supplementation of extracellular buffer with 1 mM $ZnCl_2$, a reversible Cl^- channel blocker ($n = 4$). ^{125}I efflux rates were calculated as described in Materials and Methods. Data are expressed as the mean ± SEM.

dependent Cl^-/HCO_3^- exchanger, and Na^+-independent Cl^-/HCO_3^- exchanger.

Previous studies have used radioisotope flux techniques to track Cl^- movements in endothelial cells. Ueda and co-workers [38] have reported that bovine pulmonary artery endothelial cells (BPAECs) readily accumulate ^{36}Cl under basal conditions. Concurrent administration of isoproterenol (ISO) and isobutylmethylxanthine (IBMX), agents which stimulate increases in cellular cAMP, inhibited this ^{36}Cl uptake mechanism. Additionally, ISO/IBMX stimulated ^{36}Cl efflux in BPAECs when cells were pretreated with ouabain and the $Na^+/K^+/Cl^-$ cotransport inhibitor bumetanide [38]. In our studies, forskolin, which also increases intracellular cAMP concentration, caused a slight decrease in ^{125}I efflux. We did not test ^{125}I efflux responses to forskolin in the presence of bumetanide, however, and do not know whether this treatment would reveal changes in ^{125}I efflux in HUVECs. These observations suggest that anion fluxes in ECs may be under the regulation of different cellular signaling mechanisms. Alternatively, these differential responses to agents which stimulate cAMP formation may represent variable responses of ECs isolated from different vascular regions. Other studies have shown that Ca^{2+}-mobilizing agonists stimulate Cl^- transport mechanisms in ECs. In bovine aortic endothelial cells, angiotensin II, vasopressin and bradykinin stimulate $Na^+/K^+/Cl^-$ cotransport as measured by ^{86}Rb uptake (K^+ influx) [31]. $Na^+/K^+/Cl^-$ cotransport was inhibited by removal of extracellular Ca^{2+} and by treatment of cells with BAPTA and the calmodulin antagonist W-7, suggesting an important role for Ca^{2+} in the activation of the cotransporter [31].

It has been suggested that increases in EC Cl^- channel activity may be responsible for changes in the permeability of the endothelium [38]. Stimulation of Cl^- channel activity, as measured by ^{36}Cl efflux, resulted in configurational changes in cultured BPAECs associated with cytoplasmic retraction and the formation of dendritic processes. This response could be inhibited by Cl^- channel blockers [38]. Of related interest is an earlier report that thrombin stimulates the formation of spindle-like processes in human ECs [15]. Presumably, these configurational changes in cultured cells correlate with an increase in diffusion across the endothelium *in situ*. The mechanism(s) by which Cl^- channels alter the permeability of the endothelium are poorly understood, though it has been suggested that changes in cell volume may underlie this phenomenon [39]. It is uncertain, under our experimental conditions, how much cell volume

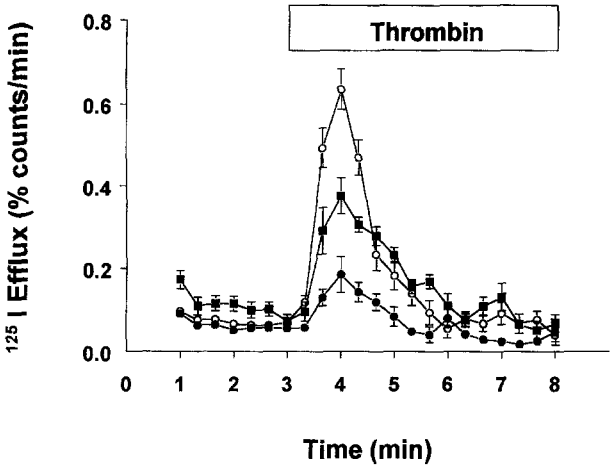

Fig. 5. Effect of phorbol esters on ^{125}I efflux. HUVECs were preincubated with PDBu (0.1 μM) or PMA (0.1 μM) for 10 min prior to study. ^{125}I efflux responses to thrombin (3 U/ml) were subsequently tested. PDBu (■, n = 6) and PMA (●, n = 5) blunted peak efflux responses to thrombin by 41 and 71%, respectively. Open circles (○, n = 33) represent the control ^{125}I efflux profile for thrombin. Data are expressed as the mean ± SEM.

may be altered by thrombin or histamine treatment of HUVECs.

It is well documented that agonists such as thrombin and histamine stimulate phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) in HUVECs yielding 1,4,5-inositol trisphosphate (IP_3) and 1,2 diacylglycerol (DAG) [5, 8, 17]. IP_3 stimulates Ca^{2+} release from the endoplasmic reticulum [5], while DAG is believed to exert its physiological actions by activating the Ca^{2+}- and phospholipid-dependent enzyme, protein kinase C [30]. We observed that the relative magnitude and time course of thrombin-activated ^{125}I efflux are closely related to thrombin-stimulated increases in $[Ca^{2+}]_i$. A similar correlation was found between histamine-stimulated ^{125}I efflux in the current studies and increases in $[Ca^{2+}]_i$ in HUVECs [8]. Collectively, these results suggest that ^{125}I efflux in HUVECs is closely coupled with $[Ca^{2+}]_i$ mobilization processes.

Results of the current studies suggest that ^{125}I efflux in HUVECs may be regulated by PKC as well as by $[Ca^{2+}]_i$. We found that the phorbol esters PMA and PDBu attenuated thrombin-stimulated increases in ^{125}I efflux and that the PKC antagonist PKCI [19–36] reversed the inhibitory effect of PMA. These results indicate that the activation of PKC inhibits ^{125}I efflux mechanisms. In the current studies, short-term incubation of HUVECs with PMA blunted thrombin-stimulated increases in $[Ca^{2+}]_i$. This response was partially blocked by PKCI [19–36]. We have previously reported that the inhibitory effect of PMA on $[Ca^{2+}]_i$ mobilization is related to a reduction in agonist-stimulated IP_3 formation, suggesting that stimulation of PKC inhibits phospholipase C activity [8]. It has been suggested that this inhibitory effect of PKC modulates the responsiveness of ECs to vasoactive agents [8, 16].

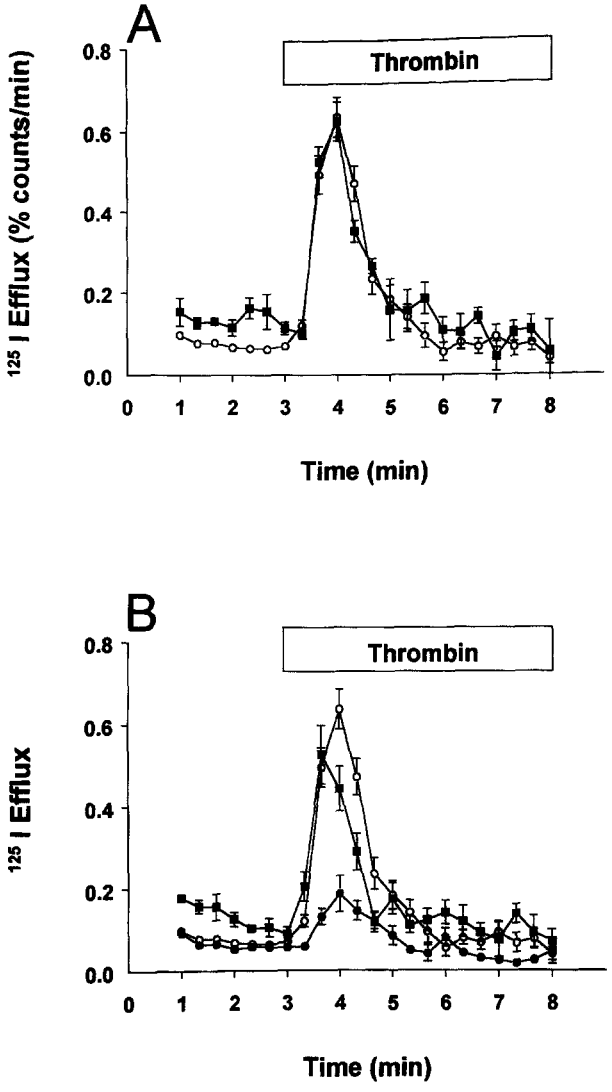

Fig. 6. (*A*) Effect of the inactive phorbol ester, 4α-phorbol, 12, 13, didecanoate (0.1 μM), on thrombin-stimulated ^{125}I efflux. HUVECs were treated with 4α-phorbol, 12, 13, didecanoate (●, n = 6) 20 min prior to study. Open circles (○, n = 33) represent the control ^{125}I efflux profile for thrombin. (*B*) Effect of PKCI [19–36] on thrombin-stimulated ^{125}I efflux in PMA-treated HUVECs. Cells were incubated with PKCI [19–36] for 18 hr prior to study. Ten minutes prior to the efflux assay, PMA (0.1 μM) was added to the incubation media. PKCI [19–36] (■, n = 10) blocked the inhibitory effect of PMA on thrombin-stimulated ^{125}I efflux. Open circles (○, n = 33) represent the control ^{125}I efflux profile for thrombin. Filled circles denote the inhibition of thrombin-stimulated ^{125}I efflux by PMA (●, n = 5). ^{125}I efflux rates were calculated as described in Materials and Methods. Data are expressed as the mean ± SEM.

Reports suggest that PKC may modulate anion transport mechanisms in ECs and other cell types. An inhibitory effect of PKC on $Na^+/K^+/Cl^-$ cotransport [31] and on Cl^- currents [16, 29] has been described in ECs.

Table 2. Effect of experimental treatments on peak, thrombin-simulated ^{125}I efflux rates

Treatment	Peak ^{125}I Efflux (% counts effluxed/min)	SEM	P	n
Control	0.636	0.048		33
DIDS	0.321	0.041	**	7
Bumetanide	0.824	0.113	NS	9
EGTA	0.555	0.071	NS	8
PMA	0.186	0.043	**	5
PDBu	0.376	0.044	*	6
4α-phorbol, 12,13-didecanoate	0.626	0.047	NS	6
PKCI [19–36]	0.560	0.032	NS	5
PKCI [19–36] + PMA	0.526	0.071	NS	10

Peak ^{125}I efflux represents the mean, maximum response to thrombin calculated for each experiment. Peak thrombin responses for a given treatment were compared to thrombin responses under control conditions (*$P < 0.05$; **$P < 0.01$). The concentrations of agents were: thrombin (3 U/ml); DIDS (50 μM); bumetanide (100 μM); EGTA (2 mM); PMA (0.1 μM); PDBu (0.1 μM); 4α-phorbol; 12,13-didecanoate (0.1 μM); PKCI [19–36] (1 μM).

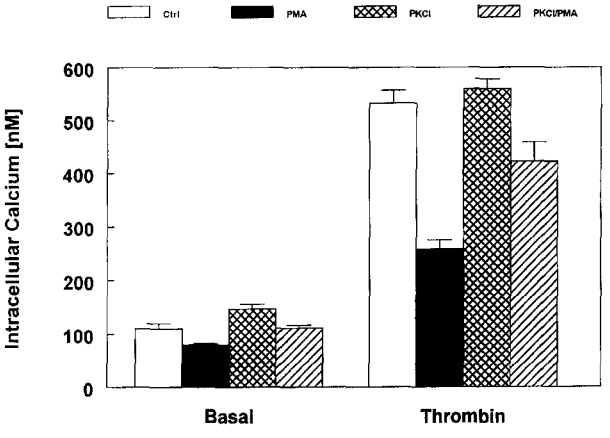

Fig. 7. Effect of PMA and PKCI [19–36] on intracellular Ca^{2+} transients in HUVEC suspensions. Basal and thrombin-stimulated increases in $[Ca^{2+}]_i$ were recorded in control (open bar, $n = 13$), PMA-treated (filled bar, $n = 6$), PKCI [19–36]-treated (cross-hatched bar, $n = 7$) and PKCI [19–36] + PMA-treated (diagonal-hatched bar, $n = 8$) HUVECs. In experiments with PKCI [19–36], cells were incubated for 18 hr with the peptide (1 μM). In other experiments, PMA (0.1 μM) was added to cell suspensions 10 min prior to study. Data are expressed as the mean ± SEM.

In the current studies, PMA reduced basal ^{125}I efflux. At this time, we do not know whether PKC exerts a direct inhibitory effect on Cl^- channels. In porcine aortic ECs, the PKC inhibitor, polymyxin B, induces Cl^- channel activity with a frequency similar to that seen with induction by a Ca^{2+} ionophore [16]. Such a conductance may underlie the agonist-mediated increases in ^{125}I efflux which we have noted in HUVECs. Studies in oocytes expressing cDNA for a neuronal Cl^- channel suggest that PKC may directly modulate channel activity [23]. PMA-mediated inhibition of Cl^- currents was reversed by PKCI in these oocytes, suggesting that PKC-dependent phosphorylation of the channel protein itself may inhibit current flow [23].

The physiological role of agonist-stimulated ^{125}I efflux is not known at this time; however, we suggest that increases in $[Ca^{2+}]_i$ lead to the activation of an anion conductance in HUVECs. Direct measurements of Cl^- channel activity are required to test this hypothesis further. We postulate that Cl^- channels play a role in EC membrane excitability and Ca^{2+} homeostasis. Agonist-mediated increases in $[Ca^{2+}]_i$ in endothelial cells have been linked to a number of processes including the synthesis of EDRF [27]. Increases in $[Ca^{2+}]_i$ following agonist stimulation are thought to be derived from both the release of Ca^{2+} from intracellular storage sites and from a hyperpolarization-driven entry of Ca^{2+} down its relative steep concentration gradient from the extracellular space into the cytosol. It has been suggested that this second mechanism of Ca^{2+} mobilization is due to the activation of a Ca^{2+}-dependent potassium channel which induces endothelial cell hyperpolarization [25]. Anion transport mechanisms may also be an important determinant of Ca^{2+} mobilization and therefore of HUVEC excitability. Cl^- efflux from the cell would be expected to depolarize the cell membrane and therefore reduce the driving force for Ca^{2+} entry. In this manner, a Cl^- conductance may act to both stabilize EC membrane potential and serve as part of a cellular feedback mechanism to regulate the hyperpolarization-induced influx of Ca^{2+} after agonist stimulation.

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