Endothelin (ET_A) receptor number and calcium signalling are up-regulated by protein kinase C- β 1 overexpression

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To evaluate the role of protein kinase C (PKC) in regulation of cellular responsiveness to mitogens, we used rat 6 (R6) fibroblasts that stably overexpress the β 1 isoenzyme of protein kinase C (PKC- β 1). The potent vasoconstrictor and mitogen endothelin-1 (ET-1; 100 nM) was substantially more effective in stimulating InsP₃ accumulation in PKC- β 1-overexpressing fibroblasts (PKC3 cells) than in control fibroblasts lacking the PKC- β 1 cDNA insert. PKC3 cells were found to express a 7-fold greater number of endothelin receptors than did control cells, whereas both cell lines showed equivalent K_d values. These receptors were of the ET_A subtype, as defined by a 1000-fold greater affinity for ET-1 than for ET-3. Changes in intracellular free Ca²⁺ levels ([Ca²⁺]_i) in response to ET-1 measured with the fluorescent Ca²⁺ indicator

fura-2 showed that ET-1 was more potent and efficacious in stimulating [Ca²⁺]_i in PKC3 cells than in control fibroblasts. The ET-1-induced Ca²⁺ rise was completely blocked by the selective ET_A antagonist BQ123, but only slightly diminished by extracellular application of 2 mM EGTA. In contrast with the effects of PKC- β 1 overexpression on responsiveness to ET-1, α -thrombin, which was previously found to have a weaker effect on InsP₃ accumulation in PKC- β 1-overexpressing cells, was also a less effective stimulator of [Ca²⁺]_i in PKC3 cells than in control cells. These results demonstrate that, although the Ca²⁺ response to α -thrombin is diminished by PKC- β 1 overexpression, ET_A receptor number and cellular responsiveness to ET-1 are increased in PKC- β 1-overexpressing cells.

INTRODUCTION

The protein kinase C (PKC) family consists of at least eight different isoenzymes that possess phospholipid-dependent protein serine/threonine kinase activity [1,2]. The isoenzymes are non-identical in their tissue expression, subcellular localization and sensitivity to Ca²⁺, suggesting that individual PKC isoenzymes may serve different functional roles [1]. PKC activity is stimulated in response to many growth factors, hormones and neurotransmitters. Upon interaction of these stimuli with their extracellular receptors, PtdIns(4,5)P₂ is hydrolysed to yield the second messengers diacylglycerol (DAG) and InsP₃ [3]. InsP₃ mobilizes Ca²⁺ from intracellular stores [4], and the elevations in DAG, alone or in combination with the elevated cytosolic free Ca²⁺ concentration, directly activate PKC [5]. Once activated, PKC catalyses the phosphorylation of various protein substrates, which in turn regulate many cellular functions, including cell growth [1,6,7].

Since PKC activity is selectively stimulated by tumour-promoting phorbol esters [7,8], PKC has been suggested to play a role in carcinogenesis [7,9]. Furthermore, fibroblasts in which PKC is stably overexpressed display enhanced growth properties, such as decreased doubling time, ability to grow in an anchorage-independent manner, decreased dependence on serum for growth, and enhanced tumorigenicity when injected into nude mice [10,11]. In addition, it has been shown that some tumour cell types naturally overexpress specific isoenzymes of PKC [12,13].

To explore the mechanisms by which PKC might affect cell growth characteristics and cellular responsiveness to growth-inducing mitogens, we have previously used rat 6 (R6) fibroblasts

that stably overexpress cDNA for the β 1 isoform of PKC [10] to measure effects of PKC expression on cellular signal-transduction pathways [14–16]. PKC- β 1 overexpression was found to up-regulate phorbol-ester- or α -thrombin-stimulated phospholipase D hydrolysis of phosphatidylcholine [14,15], which yields the putative second messenger phosphatidic acid [17]. In direct contrast, PKC- β 1 overexpression down-regulates the stimulation of InsP₃ and DAG production by α -thrombin [15]. Thus two different pathways for second-messenger generation that may mediate the mitogenic effects of α -thrombin are oppositely regulated by PKC- β 1 overexpression in fibroblasts.

To determine whether the negative effect of PKC- β 1 overexpression on α -thrombin-stimulated PtdInsP₂ hydrolysis is also observable with other mitogens, we conducted the present study on the effect of PKC- β 1 overexpression on endothelin-1 (ET-1)-stimulated InsP₃ production. ET-1 is a member of a family of 21-amino-acid peptides [18], and was originally isolated from cultured aortic endothelial cells as a potent vasoconstrictor [19]. Subsequently, endothelins and their receptors have been found to be widely distributed in peripheral tissues and the central nervous system [20–24], and ET-1 has been shown to be a potent mitogen for vascular smooth-muscle cells [25,26] and fibroblasts [27–29]. The present paper shows that, in direct contrast with the down-regulation of α -thrombin-stimulated InsP₃ formation by PKC- β 1 overexpression [15], PKC- β 1 overexpression greatly enhances the stimulation of inositol polyphosphate levels by ET-1 in rat 6 fibroblasts. This enhancement is accompanied by increased effectiveness of ET-1 to induce mobilization of intracellular Ca²⁺ stores in PKC- β 1-overexpressing fibroblasts. Finally, we demonstrate that the increased responsiveness of the PKC- β 1-overexpressing fibroblasts results, at least in part, from

Abbreviations used: PKC- β 1, the β 1 isoenzyme of protein kinase C; ET-1, endothelin-1; ET-3, endothelin-3; [Ca²⁺]_i, intracellular free Ca²⁺ concentration; DAG, 1,2-diacylglycerol; DMEM, Dulbecco's modified Eagle's medium.

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an increase in the number of endothelin receptors of the ET_A subtype.

EXPERIMENTAL

Materials

myo-[2-³H]inositol was from NEN (Boston, MA, U.S.A.) and ¹²⁵I-ET-1 was from Amersham (Arlington Heights, IL, U.S.A.). AG1-X8 resin (formate form) was from Bio-Rad (Richmond, CA, U.S.A.). Cell-culture reagents were from GIBCO (Grand Island, NY, U.S.A.). Fura-2 acetoxymethyl ester was obtained from Molecular Probes (Eugene, OR, U.S.A.). Endothelin peptides were from Peninsula Laboratories (Belmont, CA, U.S.A.). α -Thrombin and all other reagents were from Sigma (St. Louis, MO, U.S.A.).

Cell culture

Control (R6 C1) and PKC- β 1-overexpressing cells (R6 PKC3) were obtained from Dr. I. B. Weinstein (Columbia University) and grown in monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal-calf serum, 1% penicillin/streptomycin, 1% L-glutamine and 50 μ g/ml G418 at 37 °C in a humidified atmosphere of CO₂/air (1:19). For fura-2 and ¹²⁵I-ET-1-binding experiments, cells were then serum deprived for 24 h in DMEM supplemented as above, except that G418 and penicillin/streptomycin were omitted and serum content was lowered to 0.5%. For inositol phosphate measurements, cells were prelabelled for 48 h with 10 μ Ci of [³H]inositol/dish in inositol-free DMEM with 0.5% serum.

Measurement of inositol polyphosphates

[³H]inositol-prelabelled cells in 1 ml of serum-free DMEM received 20 μ l of DMEM or concentrated drug. Reactions were terminated and inositol phosphates separated by anion-exchange chromatography as previously described [30]. Data are expressed as percentage of total chloroform-extractable [³H]inositol-labelled lipid [31].

Preparation of membranes for ¹²⁵I-ET-1 binding

Cells were grown in 100 mm tissue-culture dishes as above. With dishes on ice, cells were harvested by replacing media with 100 mM Tris/HCl, pH 7.4, and scraping cells with a rubber policeman. After a second wash, pooled cell suspensions were centrifuged at low speed for 5 min at 4 °C. Cell pellets were rapidly frozen and stored at -80 °C. Pellets were later thawed and resuspended in 10 vol. of ice-cold hypotonic buffer (10 mM Tris/HCl, pH 7.4, 2 mM EDTA). After 15 min on ice, the suspension was homogenized with a motor-driven Teflon/glass homogenizer. Sucrose was added to obtain a final concentration of 0.25 M, and the homogenate was spun at 1500 g for 20 min at 4 °C. The resulting pellet was resuspended in hypotonic buffer, homogenized, supplemented with sucrose, and the mixture was spun at 1500 g for 20 min. Supernatant fractions were pooled and centrifuged for 1 h at 100000 g. The resulting pellet was suspended in buffer containing 10 mM Tris/HCl, pH 7.4, and 0.125 M sucrose. Samples were rapidly frozen in a solid-CO₂/methanol bath and stored at -80 °C.

Protein concentration was determined by the Amido Black binding assay [32] with BSA as a standard.

Endothelin binding

Samples of membrane preparations obtained from R6 C1 and R6 PKC3 cells, containing 10 and 1.5 μ g of protein respectively, were incubated with various concentrations (15–300 pM) of ¹²⁵I-ET-1 (4000–5000 d.p.m./fmol) in a 500 μ l reaction mixture containing 50 mM Tris/HCl, pH 7.4, 150 mM NaCl and 5 mg/ml BSA. Reactions were carried out for 90 min at 37 °C, and were terminated by rapid filtration over Whatman GF/B glass micro-fibre filters presoaked in buffer containing 50 mM Tris/HCl, pH 7.4, 10 mg/ml BSA and 0.1% NaN₃ for 30 min. Test tubes and filters were rinsed with 5 $\times$ 5 ml of ice-cold buffer containing 10 mM Tris/HCl, pH 7.4, and 100 mM NaCl. Radioactivity retained on filters was counted for 1 min in an LKB-Wallac γ -radiation counter (1272 Clinigamma). Non-specific binding was determined in the presence of 1 μ M ET-1. Total and non-specific binding assays were carried out in duplicates. Data were analysed as described by Scatchard [33] to obtain K_d and maximal number of binding sites (B_{max}).

To determine selectivity of ET receptors for ET-1 or ET-3, assays were carried out in the presence of 30 pM ¹²⁵I-ET-1 and various concentrations of unlabelled ET-1 or ET-3 (10 pM–1 μ M). Reactions were initiated by addition of sample containing membrane, and were carried out as described above.

Measurement of intracellular free Ca²⁺ concentration ([Ca²⁺]_i)

This was done by fura-2 fluorescence essentially as described by Takuwa et al. [34]. Cells were harvested by exposure to 0.25% trypsin/1 mM EDTA, washed three times in buffer A (140 mM NaCl, 4 mM KCl, 1 mM Na₂HPO₄, 1 mM MgCl₂, 1.25 mM CaCl₂, 11 mM glucose, 0.2% BSA and 10 mM Hepes, pH 7.4), and loaded with dye by 45 min incubation of cells in buffer A containing 4 μ M fura-2 acetoxymethyl ester at room temperature. Cells were then washed three times with buffer A and resuspended in buffer A to 1 $\times$ 10⁶ cells/ml. Fluorescence of the cell suspension was measured in a constantly stirred quartz cuvette (Hellma Cells, Jamaica, NY, U.S.A.) at room temperature with a DM3000 spectrofluorimeter (SPEX Industries, Edison, NJ, U.S.A.) with excitation at 340 and 380 nm and emission at 505 nm. Maximum and minimum fluorescence ratios were measured by addition of 0.1% Triton X-100 and addition of 10 mM EGTA/30 mM Tris base, respectively. After subtraction of cellular autofluorescence from all data, free Ca²⁺ concentrations were calculated in accordance with Grynkiewicz et al. [35], by using a K_d value for fura-2 at 22 °C of 138 nM.

RESULTS

Stimulation of inositol polyphosphates by ET-1

To assess the effect of PKC- β 1 overexpression on endothelin-stimulated PtdInsP₂ hydrolysis, inositol polyphosphate accumulation in the presence of ET-1 was measured in control (R6 C1) and PKC- β 1-overexpressing (R6 PKC3) cells. Figure 1 shows that the levels of InsP₂ and InsP₃ increased within 30 s in R6 C1 cells upon treatment with 100 nM ET-1. The magnitude of the effect of ET-1 on both InsP₂ and InsP₃ accumulation was markedly greater in the R6 PKC3 cells, although the time course of stimulation was unaffected by PKC- β 1 overexpression. Figure 1(c) shows the relationship between ET-1 concentration and accumulation of InsP₃ within 20 s. Greater sensitivity of the R6 PKC3 cells is clearly evident in response to ET-1 concentrations as low as 1 nM. As a control for these experiments, the effect of 20 s treatment with 10 nM α -thrombin was routinely tested along with the ET-1 treatments (results not shown). In agreement

Figure 1 Stimulation of inositol polyphosphates by ET-1: enhancement by PKC- β 1 overexpression

Time courses of $[^3\text{H}]\text{InsP}_2$ (a) and $[^3\text{H}]\text{InsP}_3$ (b) accumulation in response to 100 nM ET-1 were measured in the absence of LiCl in R6 C1 (○) and R6 PKC3 (●) cells. (c) Effects of 20 s treatment with increasing ET-1 concentrations on $[^3\text{H}]\text{InsP}_3$ accumulation in R6 C1 and R6 PKC3 cells. Each point represents the mean of duplicate determinations. Results shown are representative of at least two separate experiments.

Figure 2 Effect of PKC- β 1 overexpression on ET-1 binding in rat 6 fibroblasts

Typical Scatchard plots of ^{125}I -ET-1 binding to membrane preparations from R6 C1 (○) and R6 PKC3 (●) cells. ^{125}I -ET-1 binding was carried out as described in the Experimental section.

with our previous report [15], α -thrombin was always found to be a less effective stimulator of InsP_2 and InsP_3 accumulation in R6 PKC3 cells than in the control cells.

Figure 3 Selectivity of endothelin binding sites in R6 C1 and R6 PKC3 cells for ET-1

Competition for binding between ET-1 (○, ●) and ET-3 (□, ■) to the endothelin binding sites in membrane preparations from R6 C1 (●, ■) and R6 PKC3 (○, □) cells was measured as described in the Experimental section. Higher affinity of the binding sites for ET-1, as shown in this Figure, is a characteristic of the ET_A subtype.

Endothelin receptors

To determine whether the greater inositol polyphosphate response to ET-1 in R6 PKC3 cells resulted from an effect of PKC- β 1 overexpression on endothelin receptors, the binding of ^{125}I -ET-1 to membranes prepared from control and PKC- β 1-overexpressing cells was measured (Figure 2). Single-site Scatchard analysis [33] showed that the average number of endothelin receptors ($n = 5$) was approx. 7-fold greater in the R6 PKC3 cells than in the R6 C1 cells ($B_{\text{max}} \pm \text{S.E.M.} = 2195 \pm 186$ and 318 ± 72 fmol/mg of protein respectively). The equilibrium binding constants were approximately the same for membranes from R6 PKC3 and R6 C1 cells ($K_d \pm \text{S.E.M.} = 45.4 \pm 9.6$ and 45.8 ± 6.2 pM respectively).

To determine which subtype(s) of endothelin receptor are present on R6 C1 cells, and whether PKC- β 1 overexpression affects the endothelin receptor subtypes expressed, competition between ^{125}I -ET-1 and unlabelled ET-1 or ET-3 was quantified for membranes from the two cell types (Figure 3). For both R6 C1 cells and R6 PKC3 cells, ET-1 was approx. 1000-fold more potent than ET-3 in competing with specific ^{125}I -ET-1 binding. The greater affinity of ET-1 than ET-3 for the binding sites demonstrates that both R6 C1 and R6 PKC3 fibroblasts express the ET_A subtype of endothelin receptors [23] that is characteristically expressed in cardiovascular tissues, rather than ET_B receptors which bind all endothelin isopeptides with similar affinity [24].

Elevation of $[\text{Ca}^{2+}]_i$ by ET-1

Since PKC- β 1 overexpression increased the magnitude of the InsP_3 response to ET-1, and $\text{Ins}(1,4,5)\text{P}_3$ is known to raise $[\text{Ca}^{2+}]_i$ by mobilizing intracellular Ca^{2+} stores [4], the effect of ET-1 on $[\text{Ca}^{2+}]_i$ was measured to determine whether the increased responsiveness of PKC- β 1-overexpressing cells to ET-1 is functionally significant. As shown in Figure 4, 0.3 nM ET-1 induced only a small increase in $[\text{Ca}^{2+}]_i$ in R6 C1 cells, but induced roughly a 10-fold increase in $[\text{Ca}^{2+}]_i$ in R6 PKC3 cells. The greater responsiveness of $[\text{Ca}^{2+}]_i$ to ET-1 in the PKC- β 1-

Figure 4 Stimulation of $[Ca^{2+}]_i$ by ET-1: enhancement by PKC- β 1 overexpression

$[Ca^{2+}]_i$ was measured with the fluorescent Ca^{2+} indicator fura-2. Ca^{2+} responses to 0.3 nM ET-1 (a) and to 10 nM ET-1 (b) were greater in the R6 PKC3 cells than in the R6 C1 cells. ET-1 was added at 99 s as indicated by the arrows, and was present throughout all recordings. (c) Dose-response curves of the stimulation of $[Ca^{2+}]_i$ by ET-1 in R6 C1 (○) and R6 PKC3 (●) cells. Each point represents the average increase in $[Ca^{2+}]_i$ over basal during the first 60 s of stimulation by ET-1. The results shown in each panel are typical of at least two separate experiments.

overexpressing fibroblasts was also apparent with higher concentrations of ET-1, as illustrated with 10 nM ET-1 in Figure 4(b). PKC- β 1 overexpression appeared to increase both the potency and the efficacy of ET-1 in these cells (Figure 4c).

Effects of various pretreatments on the ET-1-induced $[Ca^{2+}]_i$ response in R6 PKC3 cells were investigated to determine if the $[Ca^{2+}]_i$ response to ET-1 results from intracellular mobilization of Ca^{2+} , and thus whether the greater levels of $InsP_3$ in ET-1-treated R6 PKC3 cells are likely to account for the increased $[Ca^{2+}]_i$ responsiveness of the R6 PKC3 cells to ET-1. Figure 5(a) shows that 1 nM ET-1 induces a biphasic increase in $[Ca^{2+}]_i$, with a rapid peak followed by a decrease to a lower plateau that is still clearly above the basal $[Ca^{2+}]_i$ level. Prior addition of 10 μ M nifedipine, a concentration that is generally maximally effective for dihydropyridine Ca^{2+} -channel antagonists [36], did not affect basal or ET-1-induced $[Ca^{2+}]_i$ levels (Figure 5b). This indicates that ET-1 does not seem to increase $[Ca^{2+}]_i$ by inducing influx

Figure 5 Effects of various pretreatments on ET-1-induced $[Ca^{2+}]_i$ rises

Stimulations of $[Ca^{2+}]_i$ by ET-1 alone (a) or after addition of 10 μ M nifedipine (b), 2 mM EGTA (c) or 1 μ M BQ123 (d) are shown. ET-1 (1 nM) was added during each recording at 201 s as indicated by the arrows. The other test substances were added at 99 s as indicated. All substances were present throughout the recording period.

Figure 6 Stimulation of $[Ca^{2+}]_i$ by α -thrombin: inhibition by PKC- β 1 overexpression

α -Thrombin (10 nM) was added to R6 C1 cells or R6 PKC3 cells at 99 s as indicated by the arrow, and was present throughout the recording period. These $[Ca^{2+}]_i$ measurements with the fluorescent Ca^{2+} indicator fura-2 indicate a greater responsiveness of the R6 C1 cells to α -thrombin. Similar results were obtained in two separate experiments.

of extracellular Ca^{2+} through dihydropyridine-sensitive Ca^{2+} channels. Chelation of extracellular Ca^{2+} by addition of 2 mM EGTA (Figure 5c) did not prevent the peak response in $[Ca^{2+}]_i$ to ET-1, although the subsequent plateau phase was virtually abolished, suggesting that the ET-1-induced $[Ca^{2+}]_i$ rise in R6 PKC3 cells results largely from intracellular mobilization, with a measurable contribution from influx of extracellular Ca^{2+} . These partial contributions of intracellular stores and extracellular Ca^{2+} to the ET-1-induced $[Ca^{2+}]_i$ rise are similar to those observed with ET-1-treated Swiss 3T3 fibroblasts and A10 vascular smooth-muscle cells [27,34]. The selective ET_A -receptor blocker BQ123 [37] at 1 μ M concentration abolished the ET-1-stimulated $[Ca^{2+}]_i$ rise (Figure 5d), confirming that this activity of ET-1 is

mediated through interaction with the ET_A subtype of endothelin receptors.

Elevation of $[Ca^{2+}]_i$ by α -thrombin

We have previously found that α -thrombin is a less effective stimulator of inositol phosphate accumulation in R6 PKC3 cells than in R6 C1 cells [15]. Since this is in direct contrast with what was found in the present study with ET-1, we wanted to determine whether an α -thrombin-stimulated rise in $[Ca^{2+}]_i$ would also be suppressed in PKC- β 1-overexpressing rat 6 fibroblasts. Figure 6 shows that 10 nM α -thrombin increased $[Ca^{2+}]_i$ in both control and R6 PKC3 cells, but the effect is dramatically smaller in the R6 PKC3 cells. Therefore, the opposite regulation of ET-1- and α -thrombin-stimulated inositol phosphate accumulation by PKC- β 1 overexpression appears to translate into opposite regulation of $[Ca^{2+}]_i$ stimulations by the two mitogens.

DISCUSSION

In the present study, we demonstrate that ET-1 is a more effective stimulator of inositol polyphosphate accumulation (Figure 1) and $[Ca^{2+}]_i$ rise (Figure 4) in rat fibroblasts that stably overexpress the cDNA for PKC- β 1 than in control cells lacking the PKC cDNA insert. The increased sensitivity of $[Ca^{2+}]_i$ to ET-1 in the PKC- β 1-overexpressing cells probably results directly from an enhanced $InsP_3$ production, since the ET-1-induced $[Ca^{2+}]_i$ rise in these cells is caused primarily by mobilization of intracellular Ca^{2+} stores (presumably $InsP_3$ -dependent) rather than influx of extracellular Ca^{2+} (Figure 5). This increased responsiveness of PKC- β 1-overexpressing cells to ET-1 is in contrast with diminished responsiveness to α -thrombin in the production of $InsP_3$ and DAG [15] and in stimulation of $[Ca^{2+}]_i$ (Figure 6). The selective increase in the responsiveness of cells to ET-1 upon overproduction of PKC- β 1 appears to result largely from an increase in the number of ET_A receptors expressed. ET-1 has also been reported to be a more effective stimulator of phospholipase D hydrolysis of phosphatidylcholine and of mitogenesis in these rat 6 PKC- β 1-overexpressing fibroblasts as compared with control cells [29]. Increased expression of endothelin receptors probably contributes to the greater effectiveness of ET-1 in these actions.

This effect of PKC- β 1 overexpression on ET_A receptor number is consistent with recent reports showing that down-regulation of cellular PKC activity by long-term preincubation of vascular smooth-muscle cells with phorbol esters decreases the number of endothelin receptors expressed without a significant effect on receptor affinity for ET-1 [38,39]. Long-term preincubation of rat 1 fibroblasts with phorbol esters has also been shown to decrease the growth response of these cells to ET-1 [28]. However, it is not clear if this results predominantly from a decrease in endothelin receptors in the absence of PKC, or because PKC is necessary to mediate the ET-1-induced mitogenic response. Short-term addition of phorbol esters to vascular smooth-muscle cells [39] or C-6 glioma cells [40] to stimulate PKC has also been reported to decrease endothelin receptor number. This is in apparent contradiction to our present results and the results with long-term phorbol ester treatment. Such contradictory results with phorbol esters might result from lack of selectivity of these agents for a single PKC isoenzyme as well as some PKC-independent effects [41,42]. Thus overexpression of a single PKC isoenzyme, as in the present study, avoids the complications inherent with pharmacological agents.

At present, it is unclear how the level of PKC- β 1 expression regulates the number of endothelin receptors. PKC- β 1 over-

expression might conceivably increase insertion of ET_A receptors into the plasma membrane or prevent internalization and degradation of endothelin receptors, although Cozza et al. [40] have presented evidence with C-6 glioma cells suggesting that acute stimulation of PKC decreases endothelin-receptor insertion and increases the rate of endothelin-receptor endocytosis. It may be more likely that long-term overexpression of PKC- β 1 affects the transcription of the ET_A-receptor gene. Regulation of gene transcription by the level of PKC expression is precedented. Expression of constitutively activated PKC- β in cardiac myocytes stimulates transcription of the β -myosin heavy-chain gene [43], while PKC- β 1 overexpression in C3H/10T $\frac{1}{2}$ fibroblasts decreases transcription of two PKC-regulated genes [44]. Furthermore, overexpression of PKC- α in Swiss 3T3 fibroblasts has been shown to decrease the number of mRNA transcripts for the epidermal-growth-factor receptor, with a corresponding decrease in receptor number [45].

Selective enhancement of ET-1-stimulated $InsP_3$ and $[Ca^{2+}]_i$ signalling with a concomitant decrease in α -thrombin-stimulated $InsP_3$ and $[Ca^{2+}]_i$ signalling have recently been reported to occur in rat fibroblasts in which the protein tyrosine kinase pp60^{v-src} is expressed [46]. Taken together with the present report, this may lead one to speculate that opposite regulation of cellular responsiveness to the mitogens ET-1 and α -thrombin might occur in response to expression of a number of protein kinases that are linked to enhanced growth of fibroblasts. In contrast with our results, the increased responsiveness of v-src-transformed fibroblasts to ET-1 was found not to result from an increase in endothelin receptor number, since membranes prepared from normal and v-src-transformed fibroblasts showed identical ET-1-binding characteristics [46]. Thus there appear to be multiple mechanisms for increasing sensitivity to ET-1 in response to increased protein kinase expression. Although it is not clear what the advantage may be in down-regulating responsiveness to one mitogen (α -thrombin) while up-regulating responsiveness to another (ET-1) in response to protein kinases, these differential effects of kinases on mitogen signalling systems may represent a cellular mechanism to respond differently to various stimuli even when the same signal-transduction pathways are employed.

Since ET-1 is a potent mitogen for vascular smooth-muscle cells, its involvement in the abnormal cellular proliferation characteristic of atherosclerosis has been suggested [25]. In addition, ET-1 is the most potent vasoconstrictor known to date [19], and ET-1 is thought to play an important role in the pathogenesis of numerous other cardiovascular disorders, including hypertension, stroke and myocardial ischaemia [47]. Our present results indicate that an increase in the expression of PKC- β 1 would lead to an enhanced tissue sensitivity to ET-1. A sharp increase in cellular responsiveness to ET-1 could lead to ET-induced pathogenesis in the absence of an increase in ET-1 release. Thus it will be important to determine if the increased expression of PKC isoenzymes observed in some human tumour cells [12] also occurs in association with various cardiovascular disease states, and may play a role in triggering endothelin-induced pathogenesis.

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