# Signalling mechanisms of endothelin-induced mitogenesis and melanogenesis in human melanocytes

Genji IMOKAWA*, Yukihiro YADA and Mitsutoshi KIMURA

Institute for Fundamental Research, Kao Corporation, 2606 Akabane, Ichikai-machi, Haga, Tochigi 321-34, Japan

To understand the signalling mechanisms involved in the dual stimulatory effects of endothelin-1 (ET-1) on DNA synthesis and melanization in cultured human melanocytes, we analysed the biological profile of ET-1 receptor and determined the effects of ET-1 on the protein kinase C, cyclic AMP system and mitogen-activated protein kinase (MAP kinase) in comparison with their relevant simulants. The photoaffinity labelling of ET-1 receptors with Denny–Jaff reagents revealed an ET-1 receptor with a molecular mass of 51 kDa in human melanocytes. The $ET_A$ receptor subtype-sensitive antagonist BQ123 (50 nM) or pertussis toxin (100 ng/ml) significantly suppressed the ET-1-induced intracellular calcium mobilization, indicating the presence of pertussis toxin-sensitive G-protein-coupled $ET_A$ receptors. An assay of protein kinase C activity revealed that 10 nM ET-1 translocated cytosolic protein kinase C to membrane-bound protein kinase C within 5 min of the start of incubation. In contrast, receptor-mediated melanocyte activation by ET-1 was accompanied by an elevated level of cyclic AMP (4-fold over control) after 10–60 min of incubation, whereas 60 min of incubation of human melanocytes with c-Kit or c-Met ligands such as stem cell factor (10 nM) or basic fibroblast growth factor (10 nM) did not elevate the cyclic AMP level. We have also demonstrated that a specific tyrosine kinase inhibitor, tyrphostin B-42 (10 $\mu$M), inhibited the ET-1-induced growth stimulation, suggesting the involvement of the tyrosine kinase pathway in growth stimulation. Consistently, an assay of MAP kinase revealed that ET-1 caused a 10-fold activation of MAP kinase after 5 min of incubation with human melanocytes in a similar way to tyrosine kinase ligands such as stem cell factor and hepatocyte growth factor. Further, the DNA synthesis stimulated by the c-Kit ligand stem cell factor at a concentration of 1 nM was synergistically enhanced by 5 nM ET-1. These results suggest that ET-induced dual cellular events in human melanocytes are closely associated with cross-talk between the protein kinase C and A and tyrosine kinase pathways.

## INTRODUCTION

We demonstrated that human keratinocytes produce and secrete endothelins (ETs), which can be strong mitogens as well as melanogens for human melanocytes [1,2]. Of considerable interest, the paracrine linkage of ETs between keratinocytes and melanocytes is highly accentuated when keratinocytes are exposed to UVB radiation, implying that ETs are intrinsic mitogens and melanogens for human melanocytes in UVB-induced pigmentation [2]. For melanogenic action, 10 nM ET-1 was found to induce an increase in tyrosinase activity (as measured by release of $^3H_2O$ from L-[3,5-$^3$H]tyrosine and by [$^{14}$C]thiouracil incorporation) in cultured human melanocytes, accompanied by elevated levels of tyrosinase and tyrosinase-related protein-1 mRNA expression as shown by Northern blotting [3]. This dual biological action of ET-1 on human melanocytes is clearly different from those of other growth factors for human melanocytes such as basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF). These growth factors belong to the bFGF family and are likely to express their proliferative action via tyrosine kinase without any effect on melanogenesis [4,5], indicating that the growth-promoting effect on melanocytes is closely associated with the activation of tyrosine kinase. Thus ligands for the tyrosine-kinase receptor (c-Met ligands), namely HGF and bFGF, are powerful mitogens for human melanocytes [4,6] although the involvement of other tyrosine-kinase ligands (c-Kit ligands) such as stem cell factor (SCF) remains unclear. In contrast, the cyclic AMP system was originally described as a stimulatory pathway of melanization in murine pigment cells [7–9]. Recent reports have indicated that $\alpha$-melanocyte-stimulating hormone ($\alpha$-MSH), a typical activator of the cyclic AMP system, stimulates tyrosinase activity in human melanocytes, accompanied by the increased expression of tyrosinase [10]. It is likely that the cyclic AMP system is an essential pathway in accentuating the melanization process. However, recent evidence suggests that the stimulatory effect on melanin synthesis is closely involved with the activation of protein kinase C (PKC); human melanocytes cultured in the presence of 1-oleoyl-2-acetylglycerol, an active analogue of diacylglycerol, showed a higher level of melanogenesis [11]. Furthermore, $\alpha$-MSH-stimulated melanization in murine melanoma cells can also be abolished by depletion of PKC [12]. Because the ET-1 action upon human melanocytes is mediated via the signalling mechanisms involved in the activation of PKC [1], it may be assumed that the dual function of ET-1 is a result of cross-talk between diverse signal transduction pathways. In this study we therefore analysed the biological profile of ET-1 receptor on human melanocytes and determined the effects of ET-1 on PKC, the cyclic AMP system and mitogen-activated protein (MAP) kinase.

## MATERIALS AND METHODS

### Materials

Normal human melanocytes were obtained from Sankou Pure Chemical, LTD (Tokyo, Japan). ET derivatives, HGF, bFGF

Abbreviations used: bFGF, basic fibroblast growth factor; DJR, Denny–Jaff reagents; ET, endothelin; $ET_AR$ and $ET_BR$, two types of endothelin receptor; FITC, fluorescein isothiocyanate; HGF, hepatocyte growth factor; IBMX, isobutylmethylxanthine; MAP, mitogen-activated protein; $\alpha$-MSH, $\alpha$-melanocyte-stimulating hormone; PKC, protein kinase C; SCF, stem cell factor; TR, Texas Red.

* To whom correspondence should be addressed.

and SCF were purchased from the International Reagent Corp. (Tokyo, Japan). All other chemicals were of reagent grade.

### Cell culture

Human melanocytes were maintained in modified MCDB 153 medium supplemented with 1 ng/ml recombinant bFGF, 5 $\mu$g/ml insulin, 0.5 $\mu$g/ml hydrocortisone, 10 ng/ml phorbol 12-myristate 13-acetate, antibiotics (50 $\mu$g/ml gentamycin and 0.25 $\mu$g/ml amphotericin B), 0.5 % fetal calf serum and 0.2 % bovine pituitary extract (MGM) at 37 °C under a 5 % $CO_2$ atmosphere.

### Preparation of Denny–Jaff reagent-labelled ET-1

A solution of $^{125}$I-labelled Denny–Jaff reagents (DJR) (100 $\mu$Ci per 45 pmol) [13] was evaporated under nitrogen gas, then 100 $\mu$g per 0.2 ml of ET-1 in 20 mM potassium phosphate buffer, pH 7.4, was added. The solutions were vortex-mixed for 1 min and incubated for 2 h at 4 °C. The reaction was terminated with 0.2 ml of glycine (1.0 M), after which the mixture was incubated for 1 h at 4 °C. To isolate the DJR-labelled ET-1, the mixture was applied to an Ultrafree C3GC column (0.5 cm × 2.5 cm) equilibrated with 20 mM potassium phosphate buffer, pH 7.4. The DJR-labelled ET-1 was eluted with the same buffer at a flow rate of 0.2 ml/min, and 0.2 ml fractions were collected. The first fractions were pooled and the radioactivity was measured with a liquid-scintillation counter.

### Photoaffinity labelling of ET receptor by DJR

Melanocytes were seeded in six-well trays at a density of $10^5$ cells/ml and cultured at 37 °C in MGM. After 40 h the medium was exchanged for fresh MGM and the culture continued for 4 h. The cells were incubated for 10 min with 2 $\mu$Ci/ml DJR-labelled ET-1, exposed once to UV (Chromato-UVE C-70G, UV viewing system, UVP Inc.; 365 nm) for 10 min, washed twice with ice-cold PBS, then 4 ml of potassium phosphate buffer, pH 7.4, containing 150 mM dithionate was added. The cells were scraped by using a rubber 'policeman' and the cell suspension was boiled for 5 min. After removal of unbroken cells, the supernatant fraction was centrifuged at 15000 ***g*** for 15 min. The pellet was resuspended in solution for SDS/PAGE [consisting of 50 mM Tris/HCl buffer, pH 7.4, 2.5 % (w/v) SDS, 4 % (v/v) glycerol and 1 % (v/v) 2-mercaptoethanol] and boiled for 5 min. The final solution was applied to SDS/PAGE for analysis of the ET receptor.

### Confocal laser scanning microscopy

Antibodies against $ET_A$ receptor were prepared by immunizing rabbits with a synthetic peptide CIQWKNHEQNNHNTE, with a sequence derived from the $ET_A$ receptor [14]. The $ET_A$ receptor peptide conjugated with keyhole limpet haemocyanin was used to immunize rabbits. Antibodies were affinity-purified by using a synthetic-peptide-conjugated Sepharose 4B column. Melanocytes were seeded in eight-well culture chambers (Lab-Tek, Nunc Inc., IL, U.S.A.) at (5–8) × $10^4$ cells/ml. After 24 h in culture, the cells were fixed with 3.7 % (v/v) formaldehyde and incubated at 4 °C for 24 h with fluorescein isothiocyanate (FITC)- or Texas Red (TR)-conjugated [15] anti-$ET_A$ receptor antibodies, followed by three washes with PBS (20 mM $Na_3PO_4$/150 mM NaCl, pH 7.0). The intensity and localization of fluoresence in cultured melanocytes was observed under confocal laser scannning micrcopy (TCS4D; Leica Lasertechnik, Heidelberg, Germany).

### Measurement of the intracellular calcium content

The cells were loaded with the calcium indicator fura-2/AM (2 $\mu$M) by incubation for 30 min at 37 °C in modified MCDB 153. The cells were washed twice with fresh media and exposed to 5 or 10 nM ET-1 at 37 °C. Fluorescence images were obtained at alternating excitation wavelengths of 340 and 380 nm through a SIT vidicon camera, and processed using an ARGUS-200 image analyser (Hamamatsu Photonics Corp., Hamamatsu, Japan). The fluorescence signal was calibrated in terms of intracellular calcium content by using a digital imaging microscope as described elsewhere [1].

### DNA synthesis

Melanocytes cultured in 24-well trays were incubated with ET-1, isobutylmethylxanthine (IBMX) and SCF at various concentrations. After 20 h, the cells were labelled for 4 h with 1.0 $\mu$Ci/ml [$^3$H]thymidine. After three washes with PBS, the cells were lysed with 2 M NaOH at 37 °C for 15 min and neutralized with 2 M HCl. The acid-insoluble materials were precipitated with 4 vol. of 10 % (w/v) ice-cold trichloroacetic acid for 30 min at 4 °C, collected on a glass fibre filter, washed three times with 10 % ice-cold trichloroacetic acid and once with ice-cold ethanol, and then dried. The filter was mixed with scintillation fluid and the radioactivity was determined with a liquid-scintillation counter.

### Measurement of [$^{14}$C]thiouracil incorporation and $^3H_2O$ release

As exogenous thiouracil has been shown to become part of the melanin polymer by binding to the quinones generated during the synthesis of melanin depending on tyrosinase activity [16,17] and there is a close correlation between the extent of [$^{14}$C]thiouracil incorporation and tyrosine hydroxylase activity, which is measured by the release of $^3H_2O$ as a result of the formation of dehydroxyphenylalanine from L-[3,5-$^3$H]tyrosine [18] in living mammalian pigment cells [1,2] and pigmented skin tissue [19], the incorporation of [$^{14}$C]thiouracil into the trichloroacetic acid-insoluble fraction of melanocytes was measured as an index of functioning tyrosinase activity in living cells. As a result of the preferential incorporation into melanin-synthesizing cells [16,17] and melanin [16], and because of the rare presence of quinones in other eukaryotic cells, it is possible to use this as an index of melanin synthesis in pigment cells.

Melanocytes were seeded in 24-well culture trays at a density of (5–8) × $10^4$ cells/ml, cultured in MGM (without phorbol 12-myristate 13-acetate and bFGF) containing a low level of tyrosine (34.1 $\mu$g/ml), 0.2 % bovine pituitary extract and 0.5 % fetal calf serum, then treated with stimulators at concentrations ranging from 0 to 100 nM. The cells were labelled for 24–48 h with 0.5–1.0 $\mu$Ci/ml [$^{14}$C]thiouracil and 1.0 $\mu$Ci/ml L-[3,5-$^3$H]tyrosine. To measure $^3H_2O$ release [1,18], 500 $\mu$l of the medium was transferred to a tube, then the same volume of 20 % (w/v) charcoal-containing 10 % trichloroacetic acid (charcoal solution) was added. The suspensions were vortex-mixed for 30 s and separated by centrifugation for 10 min at 2000 ***g***. A 750 $\mu$l aliquot of the supernatant was transferred to a tube and 500 $\mu$l of charcoal was added. The suspension was vortex-mixed for 30 s and centrifuged for 10 min at 2000 ***g***. A 1 ml portion of the supernatant was transferred and charcoal-extracted twice. The final supernatant was mixed with scintillation fluid and the radioactivity was determined with a liquid-scintillation counter. For the thiouracil incorporation assay [1,2,19], after three washes with PBS the cells were lysed with 2 M NaOH at 37 °C for 15 min and neutralized with 2 M HCl. The lysate was mixed with

scintillation fluid and the radioactivity was determined with a liquid-scintillation counter.

### Measurement of PKC activity and translocation

The cells were incubated with 10 nM ET-1 at 37 °C for the indicated times, washed twice with cold PBS and scraped from the culture dishes. The cell suspensions were centrifuged for 5 min at 200 ***g*** and resuspended in 25 mM Tris/HCl buffer, pH 7.5, containing 2 mM EGTA, 5 mM dithiothreitol and 1 mM PMSF. The cytosolic and membrane fractions were prepared as described elesewhere [20]. PKC activity was assayed by measuring the incorporation of $^{32}P$ from [$\gamma$-$^{32}P$]ATP into histone H-1 (type III-S) as described previously [20].

### Assay of cyclic AMP

The cells were homogenized with 0.1 mM Tris/HCl buffer, pH 7.2, and centrifuged at 2000 ***g*** for 15 min. The concentration of cyclic AMP in the supernatant was measured with a cyclic AMP assay kit (Cayman Chemical Company, Ann Arbor, MI, U.S.A.) and expressed as pg per ml per $10^5$ cells.

### Assay of MAP kinase

MAP kinase activity was measured with a p42/p44 MAP kinase enzyme assay system (Amersham, Bucks., U.K.) as the rate of phosphorylation of a synthetic peptide substrate that is highly selective for p42/p44 MAP kinase. Briefly, human keratinocytes were grown to confluence. After 0–60 min of treatment with ET-1 (10 nM), HGF (10 nM) and SCF (10 nM), cells were rinsed twice with ice-cold PBS and lysed at 4 °C in 10 mM Tris, 150 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 1 mM orthovanadate, 1 mM PMSF, 10 $\mu$g/ml leupeptin and 10 $\mu$g/ml aprotinin, pH 7.4. Cellular debris was precipitated at 25000 ***g*** for 20 min and the supernatant was obtained as cytosolic extracts for MAP kinase assay. The cytosolic extracts (15 $\mu$l) were mixed at 30 °C with the substrate buffer (10 $\mu$l, containing HEPES, sodium orthovanadate, 0.05% sodium azide and the synthetic peptide, pH 7.4) the assay buffer (containing HEPES, ATP and magnesium chloride, pH 7.4) and 1 $\mu$Ci of [$\gamma$-$^{32}P$]ATP. Reactions were terminated after 0, 2, 4, 6 and 10 min by adding 10 ml of stop reagent containing orthophosphoric acid. The terminated reaction mixture was spotted on to the centre of paper discs, which were then washed three times with 250 ml of 75 mM orthophosphoric acid, twice with water, air-dried and counted in a scintillation counter. MAP kinase activity was expressed as pmol of phosphate transferred per minute per mg of protein.

## RESULTS

### Incorporation of [$^{14}$C]thiouracil on melanin synthesis

The incorporation of [$^{14}$C]thiouracil into pigment cells occurs in parallel with the hydroxylation of tyrosine mediated by the activity of tyrosine hydroxylase in living cells [1,2,19], and is a convenient indicator of melanin synthesis owing to its selective incorporation into melanin-synthesizing cells [16,17] as well as its specific inhibitory or stimulatory profile by a tyrosinase inhibitor, phenylthiourea, and melanogenic stimulus, prostaglandin $E_2$ [19]. To confirm the reliability of the incorporation of [$^{14}$C]thiouracil as an index of melanin synthesis in cultured human melanocytes, we compared the extent of its incorporation with the release of $^3H_2O$ (tyrosine hydroxylase activity) under various conditions in cultured human melanocytes. The incorporation of [$^{14}$C]thiouracil was found to occur in parallel with the hydroxylation of tyrosine, with a relation coefficient of 0.878

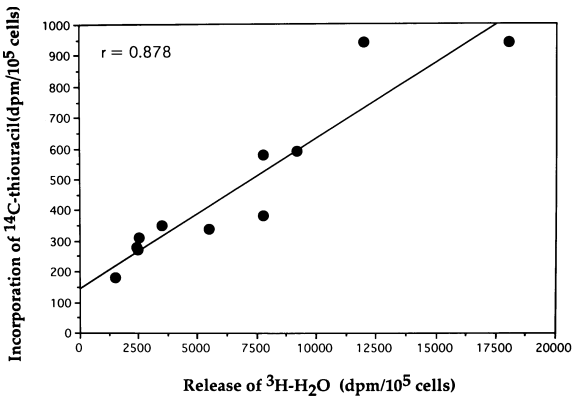


**Figure 1 Correlation between radioactivity released as $^3H_2O$ and incorporation of [$^{14}$C]thiouracil in cultured human melanocytes**

Assays were performed in human melanocytes cultured for 24 h under several conditions.

**Table 1 The effects of ET-1, IBMX and SCF on DNA synthesis and melanogenesis in cultured human melanocytes**

The reagents were added at the indicated concentrations to human melanocytes for 24–48 h. DNA synthesis and melanization were assayed by measuring respectively the amount of [$^3$H]thymidine incorporated for the last 4 h of the 24 h incubation and that of [$^{14}$C]thiouracil for 48 h. The cells were counted when each incubation was terminated. Values are means ± S.D. derived from three wells.

| Reagents | | [$^3$H]Thymidine incorporation (d.p.m. per $5 \times 10^4$ cells) | [$^{14}$C]Thiouracil incorporation (d.p.m. per $5 \times 10^4$ cells) |
|---|---|---|---|
| Experiment 1: | | | |
| Control | | 1218 ± 119 | 1679 ± 98 |
| IBMX | 10 $\mu$M | 1112 ± 191 | 2973 ± 238 |
| ET-1 | 5 nM | 2990 ± 175 | 3289 ± 256 |
| SCF | 10 nM | 2539 ± 241 | 2107 ± 222 |
| Experiment 2: | | | |
| Control | | 1246 ± 137 | 1649 ± 92 |
| ET-1 | 1 nM | 2213 ± 104 | 2250 ± 169 |
| | 10 nM | 2391 ± 214 | 3219 ± 231 |
| | 100 nM | 1954 ± 114 | 4037 ± 319 |
| SCF | 1 nM | 1569 ± 170 | 1742 ± 114 |
| | 10 nM | 2830 ± 140 | 2192 ± 150 |
| | 100 nM | 3020 ± 143 | 2457 ± 130 |

**Table 2 Effects of ET-1, bFGF and SCF on the accumulation of cyclic AMP**

Human melanocytes were incubated with the indicated concentrations of each ligand for 60 min. The levels of cyclic AMP were determined as described in the Materials and methods section and are expressed as concentrations per $10^5$ cells. The values are means ± S.D. derived from three wells.

| Ligand | Concentration of cyclic AMP (pg/ml) |
|---|---|
| Control | 0.495 ± 0.094 |
| ET-1 (5 nM) | 2.065 ± 0.138 |
| SCF (10 nM) | 0.560 ± 0.104 |
| bFGF (10 nM) | 0.465 ± 0.136 |

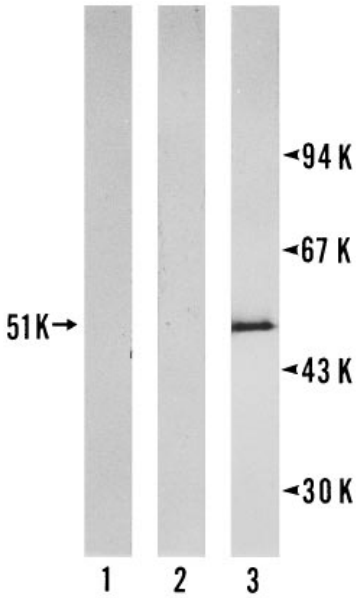


**Figure 2 Photoaffinity labelling of endothelin receptor by DJR**

The cells were incubated for 10 min with 2 $\mu$Ci/ml DJR-labelled ET-1, then exposed to UV (365 nm) for 10 min. The cell pellet suspended in solution for SDS/PAGE was resolved by SDS/PAGE to analyse the ET receptor. Lane 1, incubation with DJR-labelled ET-1 followed by no UV exposure; lane 2, incubation with DJR-labelled ET-1 in the presence of excess of non-labelled ET-1 followed by UV exposure; lane 3, incubation with DJR-labelled ET-1 followed by UV exposure. Abbreviation: K, kDa.

(Figure 1). This indicates that the incorporation of [$^{14}$C]thiouracil is an useful index of melanin synthesis in cultured human melanocytes. In this study, therefore, melanin synthesis was evaluated by the incorporation of [$^{14}$C]thiouracil into human melanocytes.

### Comparison of factors that affect DNA and melanin synthesis in melanocytes

To define the cellular actions of IBMX, ET-1 and SCF, we compared their effects on DNA and melanin synthesis (Table 1). A phosphodiesterase inhibitor, IBMX (10 $\mu$M), significantly increased melanin synthesis as measured by [$^{14}$C]thiouracil incorporation for 48 h without affecting DNA synthesis (as measured by [$^{3}$H]thymidine incorporation for the last 4 h of a 24 h incubation). In contrast, the c-Kit ligand SCF (10 nM) specifically enhanced DNA synthesis with only a slight effect on melanin synthesis. ET-1 (5 nM) had a significant dual effect on both DNA and melanin synthesis, with a higher level of stimulation for each than either IBMX or SCF. Next we compared ET-1 with SCF for their effects on DNA synthesis and melanin synthesis at various concentrations. The c-Kit ligand SCF specifically enhanced DNA synthesis (as measured by [$^{3}$H]thymidine incorporation for the last 4 h of a 24 h incubation) with a slight effect on melanin synthesis (as measured by [$^{14}$C]thiouracil incorporation during a 48 h incubation) at concentrations of 1–100 nM (Table 2). ET-1 had a significant dual effect on both DNA synthesis and melanin synthesis, with a higher level of stimulation of melanin synthesis than SCF at all concentrations tested.

### Receptor-mediated PKC activation by ET-1

To clarify the precise signalling mechanisms of ET-induced mitogenesis and melanogenesis in human melanocytes, we first determined the involvement of PKC activation via a specific receptor in cultured human melanocytes. Photoaffinity labelling of the ET-1 receptor revealed the presence of ET receptors with a molecular mass of 51 kDa in human melanocytes (Figure 2). To determine the type of specific ET-1 receptor in human melanocytes, we studied the effect of $ET_A$-receptor type sensitive antagonist on the mobilization of intracellular calcium (Figure 3b). Incubation for 60 min with the $ET_A$ receptor type-sensitive antagonist BQ123 (50 nM) significantly suppressed the ET-1-induced accumulation of intracellular calcium, indicating the

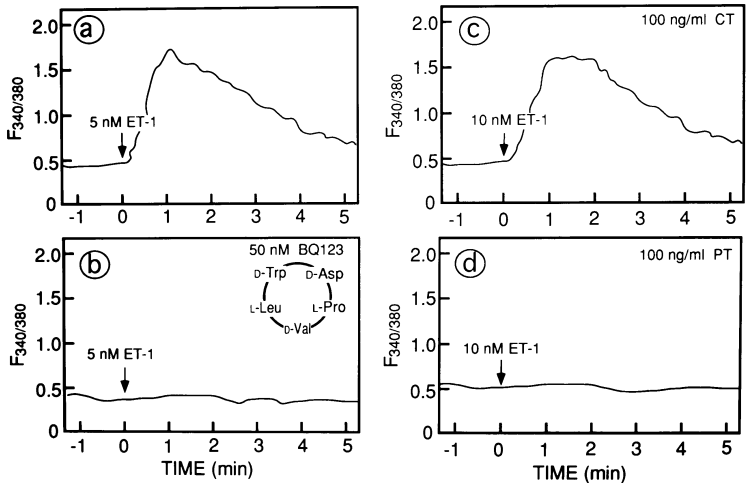


**Figure 3 The effects of the $ET_A$-receptor type sensitive antagonist BQ123 and pertussis and cholera toxins on the ET-1-induced mobilization of intracellular calcium in cultured human melanocytes**

The cells were loaded with the calcium indicator fura-2/AM (2 $\mu$M) for 30 min at 37 °C in modified MCDB 153. BQ123 was added at a concentration of 50 nM for 60 min before incubation with 5 nM ET-1. In this experiment, 5 nM concentration for ET-1 was chosen as one-tenth of 50 nM for BQ123. Pertussis toxin (PT) and cholera toxin (CT) were added at 100 ng/ml 16 h before incubation with 10 nM ET-1. The arrow indicates when the ET-1 was added. The results are the ratios of the fluorescence emitted at 340 nm to that at 380 nm. (**a**) Control; (**b**) BQ123; (**c**) CT; (**d**) PT.

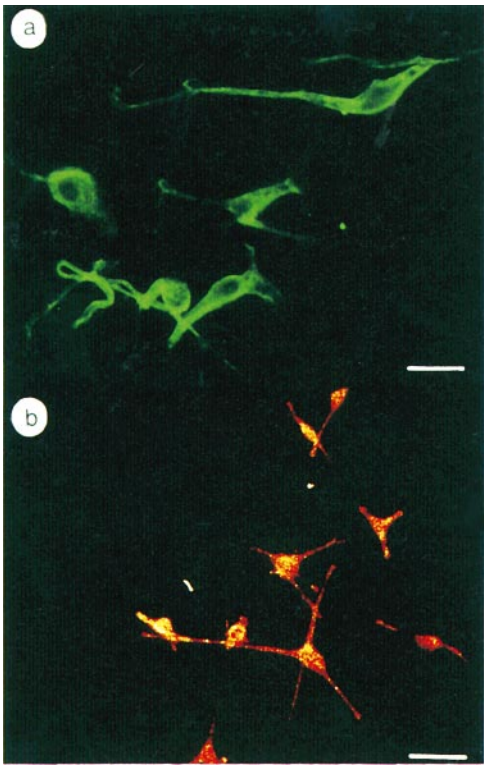


**Figure 4 Confocal laser scanning micrographs of human melanocytes stained with FITC- or TR-conjugated $ET_A$ receptor antibodies**

The cells were fixed with 3.8% formaldhyde at 4 °C for 30 min and washed three times with PBS. FITC- or TR-conjugated $ET_A$ receptor antibodies were incubated at 4 °C for 24 h with fixed human melanocytes and washed three times with PBS. The distribution of fluorescence in human melanocytes was observed under a confocal laser scanning microscope. The original colours of FITC and TR were converted into green (**a**) and red (**b**) respectively. Bar, 15 $\mu$m.

involvement of the $ET_A$ receptor in the ET-1-induced mobilization of intracellular calcium. Consistent with the presence of $ET_A$ receptors, confocal laser scanning microscopic observations with FITC- or TR-conjugated anti-$ET_A$ receptor antibodies revealed that the plasma membrane in the cultured human melanocytes was definitely stained with $ET_A$ receptor antibodies (Figure 4).

To characterize the features of the G-protein linked to the $ET_A$ receptor, we investigated the effects of pertussis and cholera toxins on the ET-1-induced mobilization of intracellular calcium. Incubation for 16 h with pertussis toxin (100 ng/ml) but not cholera toxin (100 ng/ml) significantly suppressed the ET-1-induced accumulation of intracellular calcium (Figures 3c and 3d), indicating that the $ET_A$ receptor is linked to a pertussis-toxin-sensitive G-protein.

Previously we demonstrated that ET-1-induced mitogenesis is accompanied by the formation of inositol 1,4,5-trisphosphate, which is followed by intracellular calcium mobilization [1]. In the present study, an assay of PKC activity revealed that 10 nM ET-1 induced the rapid translocation of cytosolic PKC to the membrane-bound PKC within 5 min, followed by a return to the previous location within 60 min (Figure 5).

### Involvement of the cyclic AMP system in the effect of ET

We determined whether the cyclic AMP system is involved in the ET-induced signal transduction pathway dominating the activ-

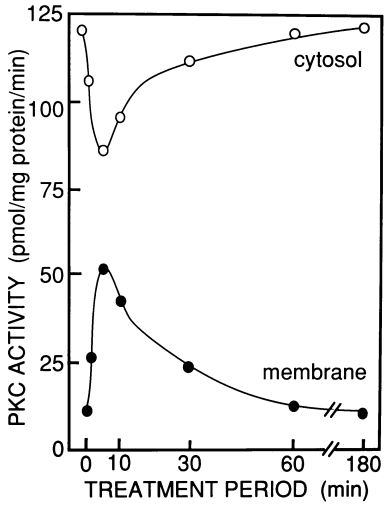


**Figure 5 ET-1 induced translocation of PKC activity from the cytosol to the membrane**

The cells were incubated with 10 nM ET-1 at 37 °C for the indicated periods. The cytosolic and membrane fractions were prepared as described previously [20]. PKC activity was assayed by measuring the incorporation of $^{32}P$ from [$\gamma$-$^{32}P$]ATP into histone H-1 (type III-S) as described elsewhere [20].

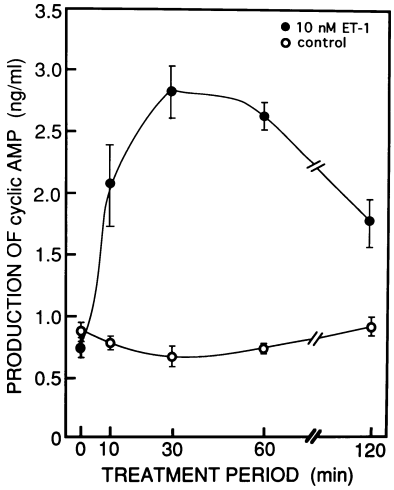


**Figure 6 Time course of the effect of ET-1 on the accumulation of cyclic AMP**

Human melanocytes were incubated with 10 nM ET-1 for the indicated periods of time. The level of cyclic AMP was determined at each incubation time as described in the Materials and methods section and expressed as concentrations per $10^5$ cells. The values are means ± S.D. derived from three wells.

ation of PKC. When human melanocytes were incubated with 10 nM ET-1, the level of cyclic AMP within melanocytes increased 3-fold within 10 min and reached a plateau after 30 min (Figure 6). On the other hand, 60 min incubation of human melanocytes with c-Kit or c-Met ligands such as SCF (10 nM) or bFGF (10 nM) did not elevate the cyclic AMP level, in contrast with the marked increase observed with ET-1 (5 nM) (Table 2). When ET-1 and IBMX were both added to cultured human melanocytes, melanin synthesis as indicated by

**Table 3 Additive effect of ET-1 and IBMX on melanin synthesis**

Human melanocytes were incubated with 10 $\mu$M IBMX and 5 nM ET-1 for 48 h. Melanin synthesis was assayed by measuring the amount of [$^{14}$C]thiouracil incorporated into the cells during a 48 h incubation as described in the Materials and methods section. Cells were counted when each incubation was terminated; results are means ± S.D. derived from three wells.

| Reagent | [$^{14}$C]Thiouracil incorporation (d.p.m. per $5 \times 10^4$ cells) |
|---|---|
| Control | 1672 ± 104 |
| IBMX | 3258 ± 221 |
| ET-1 | 3862 ± 226 |
| IBMX + ET-1 | 4752 ± 317 |

**Table 4 Effects of tyrosine kinase inhibitors on ET-1-stimulated DNA synthesis**

The inhibitors were added at the indicated concentrations 4 h before treatment with ET-1. DNA synthesis was evaluated by measuring [$^3$H]thymidine incorporation for the last 4 h of 24 h incubation with ET-1. Cells were counted when each incubation was terminated; values are means ± S.D. derived from three wells.

| Reagents | [$^3$H]Thymidine incorporation (d.p.m. per $5 \times 10^4$ cells) |
|---|---|
| Control | 1185 ± 344 |
| Tyrphostin B42 (10 $\mu$M) | 1069 ± 132 |
| ET-1 (10 nM) | 3877 ± 280 |
| ET-1 (10 nM) + Tyrphostin B42 (10 $\mu$M) | 1957 ± 258 |

[$^{14}$C]thiouracil incorporation additionally increased beyond the levels induced by IBMX and ET-1 (Table 3).

### Involvement of tyrosine kinase pathway in the effect of ET

A specific tyrosine kinase inhibitor, tyrphostin B-42, had an inhibitory effect on the ET-1-induced stimulation of DNA synthesis when added at a concentration of 10 $\mu$M 4 h before the onset of incubation with ET-1 (Table 4). Consistent with the inhibitory effect by tyrphostin B-42, an assay of MAP kinase after incubation with human melanocytes revealed that 10 nM ET-1 induced the rapid activation of MAP kinase 5 min later, which declined to the baseline control level after 60 min (Figure 7). Ligands for tyrosine kinase such as SCF (10 nM) and HGF (10 nM) elicited the activation of MAP kinase in a pattern similar to that of ET-1. In addition, DNA synthesis, which can be stimulated by the c-Kit ligand SCF (1 nM), was synergistically enhanced only when 5 nM ET-1 was added to the cells 14 h before SCF and remained in the culture medium during the subsequent 24 h incubation with SCF (ET-1 + SCF, Table 5). This synergism was not induced by 2 h of pre-exposure to ET-1 (results not shown). When ET-1 was removed from the culture medium immediately before SCF (ET-1 → SCF), neither an additive nor a synergistic effect occurred. In contrast, when ET-1 and SCF were added simultaneously with the culture medium (ET-1/SCF), the effect on DNA synthesis was additive.

## DISCUSSION

Endothelins are unique bioactive peptides that can act on melanocytes as melanogens in conjunction with their mitogenic properties [3]. Several agents that elevate intracellular cyclic AMP levels, such as IBMX, dibutyryl cyclic AMP and $\alpha$-MSH, induce melanization in human melanocytes [7,10]. In contrast, ligands such as bFGF and HGF, which are associated with tyrosine kinase, poorly stimulate melanization in human melanocytes despite their definite actions as mitogens [4–6]. Therefore the question has arisen about the signalling mechanisms involved in the dual biological effects of ET-1 in human melanocytes. We demonstrated that ET-induced mitogenesis is accompanied by the formation of inositol 1,4,5-trisphosphate followed by intracellular calcium mobilization [1]. By means of a binding assay using $^{125}$I-labelled ET-1, we also confirmed the presence of a specific receptor for ET-1 on human melanocytes with a dissociation constant of 1.81 nM and $(7.0–8.0) \times 10^4$ binding sites per cell [1]. These findings indicated that the biological action of ET-1 in human melanocytes is mediated via a specific receptor by increased phosphatidylinositide turnover and PKC activation. Despite the involvement of the action of ET-1 with PKC-related

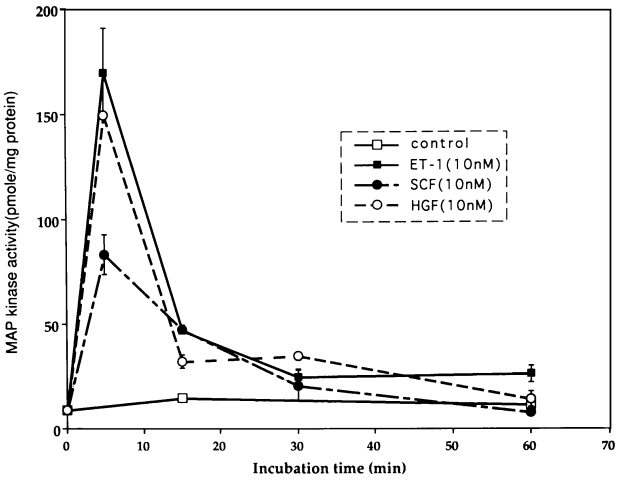


**Figure 7 Comparison of activation of MAP kinase by ET-1, SCF and HGF**

MAP kinase activities in cell lysates were measured by using the p42/p44 MAP kinase assay system as described in the Materials and methods section. The values are means ± S.D. derived from three wells.

**Table 5 Synergistic effect of ET-1 on SCF-induced stimulation of DNA synthesis**

ET-1 + SCF: ET-1 (5 nM) was added 14 h before 1 nM SCF; both were left in the culture medium for the next 24 h incubation period. ET-1/SCF: ET-1 and SCF were added concomitantly to the culture medium followed by incubation for 24 h. ET-1 → SCF: ET-1 was removed from the culture medium immediately after incubation for 14 h. DNA synthesis was assayed by measuring the amount of [$^3$H]thymidine incorporation for the last 4 h of the 24 h incubation as described in the Materials and methods section. The values are means ± S.D. derived from three wells.

| Reagents | [$^3$H]Thymidine incorporation (d.p.m. per $5 \times 10^4$ cells) |
|---|---|
| Control | 1235 ± 109 |
| ET-1 | 2984 ± 208 |
| SCF | 1512 ± 189 |
| ET-1 → SCF | 2364 ± 74 |
| ET-1/SCF | 3252 ± 291 |
| ET-1 + SCF | 4381 ± 379 |

signal transduction, no direct evidence for the biological properties of ET receptors and for the cellular events, including the nature of the associated G protein and the substantial activation of PKC, has been reported in human melanocytes. In this study we have demonstrated that the ET-induced signal transduction pathway dominates the activation of PKC through a specific ET receptor. Photoaffinity labelling of the receptor showed a specific band with a molecular mass of 53 kDa on SDS/PAGE, which is similar to that reported for the ET-1 receptor in human fibroblasts [21]. Two types of endothelin receptor, $ET_AR$ and $ET_BR$, are known and these are members of the superfamily of heptahelical G-protein-coupled receptors [22–24]. $ET_AR$ and $ET_BR$ differ in their ligand selectivities, with $ET_AR$ preferentially binding ET-1 and ET-2, whereas $ET_BR$ binds ET-1, ET-2 and ET-3 with almost equal affinity [25]. Our previous findings that human melanocytes respond well to ET-1 and ET-2, but not ET-3, to increase their DNA synthesis [1] suggested that mitogenic stimulation was mediated via $ET_AR$. In this study, mediation through $ET_AR$ was also suggested by the marked staining of cultured human melanocytes with FITC- or TR-conjugated anti-$ET_AR$ antibodies as well as by the observation that ET-1-induced calcium mobilization was completely abrogated by the $ET_AR$-type-sensitive antagonist BQ 123.

The characterization of the associated G-protein after receptor–ligand binding by means of ET-1-induced calcium mobilization demonstrated that a G-protein sensitive to pertussis toxin but not to cholera toxin is involved in the effect of ET. Furthermore, the rapid intracellular translocation of PKC activity observed in this study in response to ET-1 application suggested that at least the initial and predominant subcellular events subsequent to agonist–receptor binding is the activation of PKC, which phosphorylates several specific proteins. However, whether or not the downstream cellular events leading to the stimulation of proliferation and melanization are solely involved in the activation of the PKC signal transduction pathway remains unclear.

Evidence that stimulators of the cyclic AMP system such as $\alpha$-MSH and IBMX induce increased melanization in pigment cells without a significant effect upon growth [26–28] suggests that the cyclic AMP system is closely involved in stimulating melanization. However, the growth-promoting effects of tyrosine kinase ligands such as bFGF and HGF on human melanocytes without significant effects on melanization [4–6] also suggest that the tyrosine kinase pathway is closely associated with growth stimulation in human melanocytes. Thus the stimulatory effect of ET-1 on both DNA synthesis and melanogenesis may be attributed to cross-talk between different signal transduction pathways.

For the regulation of cyclic AMP production by PKC, PKC-activating phorbol esters both stimulate and inhibit cyclic AMP levels in a number of cell lines, types and tissues [29]. Various isoenzymes of PKC may have modulatory roles in which for instance the $\gamma$ form may be facilitatory [30]. Phorbol ester selectively stimulates particular isoenzymes of adenylyl cyclase [31]. In our study, as expected, PKC activation induced by ET-1 was accompanied by activation of the PKA pathway as evidenced by a highly elevated level of cyclic AMP after a 60 min incubation, which was not induced by tyrosine kinase ligands such as SCF and bFGF. Similar interactions between PKC and cyclic AMP signal transduction have also been identified in platelets, hepatocytes, adipocytes and lymphocytes [32–35]. Several mechanisms have been proposed for cross-talk of PKC and cyclic AMP, including the phosphorylation of $G_i\alpha$, which decreases its ability to inhibit adenylyl cyclase [32] or the direct phosphorylation of adenylyl cyclase [36].

In contrast, there is evidence supporting antagonism between PKA and MAP kinase cascades: various compounds that increase cyclic AMP levels and PKA activity significantly inhibit the stimulation of MAP kinase kinase and MAP kinase by platelet-derived growth factor in human arterial smooth-muscle cells [37]. In rat adipocytes, the activation of MAP kinase by insulin is attenuated by the $\beta$-adrenergic agonist isoproterenol through activation of protein kinase A, representing a general mechanism for attenuating MAP kinase activation [38]. In the above two cell types, cyclic AMP also inhibits the biological responses induced by each ligand. In contrast, in rat phaeochromocytoma PC12 cells where the elevation of the intracellular cyclic AMP level promotes neuronal differentiation, the stimulation of MAP kinase occurs after elevation of intracellular cyclic AMP on exposure of the cells to IBMX and cyclic AMP analogues [39]. On the basis of the ET-1-inducible dual cellular responses, it is conceivable that neither antagonism nor synergism between PKA and the MAP kinase cascade is involved in the ET-1-induced signalling mechanisms. The observed additive effect of ET-1 and IBMX on melanogenesis in human melanocytes may support the notion of the convergence of PKC and cyclic AMP signal transduction in the melanogenic stimulation process.

Although PKC isoenzymes have no tyrosine kinase activity, there is some evidence that activation of PKC by phorbol esters indirectly induces tyrosine phosphorylation in various cell types [40]. Our studies of the involvement of the tyrosine kinase pathway demonstrated that a specific tyrosine kinase inhibitor, tyrphostin B-42, markedly inhibited the ET-1-induced stimulation of DNA synthesis. In conjunction with the tyrosine kinase pathway leading to activation of MAP kinase, the present study revealed that in human melanocytes ET-1 was capable of activating MAP kinase 10-fold over non-stimulated controls, a higher magnitude than that induced by SCF and HGF. This is direct evidence for convergence of the ET-1-stimulated PKC pathway with the tyrosine kinase pathway including the activation of MAP kinase in human melanocytes.

PKC can directly phosphorylate and activate Raf-1 [41,42], which is regarded as one of the key activators upstream of MAP kinase. In rat mesangial cells and cardiac myocytes, endothelin was reported to activate MAP kinase via a PTX-insensitive pathway that is partly dependent on PKC [43,44]. MAP kinase is also activated through the activation of PKC by phorbol ester [35]. The synergism for growth stimulation between ET-1 and SCF also illustrates that, at least in human melanocytes, the MAP kinase cascade provides meeting points for cross-talk between the different signalling pathways initiated by receptor tyrosine kinases, PKC and cyclic AMP.

In conclusion, our findings suggest that ET-induced cellular events in human melanocytes are closely associated with cross-talk between PKC and other signalling pathways. It is likely that whereas melanogenic properties are closely associated with cross-talk with the protein kinase A pathway, mitogenic properties are relevant to cross-talk with the tyrosine kinase pathway.

## REFERENCES


1 Yada, Y., Higuchi, K. and Imokawa, G. (1991) J. Biol. Chem. **266**, 18352–18357
2 Imokawa, G., Yada, Y. and Miyagishi, M. (1992) J. Biol. Chem. **267**, 24675–24680
3 Imokawa G., Miyagishi, M. and Yada, Y. (1995) J. Invest. Dermatol. **105**, 32–37
4 Halaban, R., Langdon, R., Birchall, N., Cuono, C., Baurd, A., Scott, G., Moellmann, G. and McGuire, J. (1988) J. Cell Biol. **107**, 1611–1619
5 Yaar, M. and Gilchrest, B. A. (1991) J. Invest. Dermatol. **97**, 611–617
6 Matsumoto, K., Tajima, H. and Nakamura, T. (1991) Biochem. Biophys. Res. Commun. **176**, 45–51
7 Hearing, V. J. and Tsukamoto, K. (1991) FASEB J. **5**, 2902–2909
8 Fuller, B. B., Lunsford, J. B. and Iman, D. S. (1987) J. Biol. Chem. **262**, 4024–4033

9 Jimenez, M., Kameyama, K., Maloy, W. L., Tomita, Y. and Hearing, V. J. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 3830–3834
10 Abdek-Malek, Z., Swope, V. B., Suzuki, I., Akcali, C., Harriger, M. D., Boyce, S. T., Urabe, K. and Hearing, V. J. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 1789–1793
11 Gordon, P. R. and Gilchrest, B. A. (1989) J. Invest. Dermatol. **93**, 700–702
12 Park, H. Y., Russakovsky, V. and Gilchrest, B. A. (1992) J. Invest. Dermatol. **98**, 645 (abst.)
13 Phillips, M. L., Yip, C. C., Shevach, E. M. and Delovitch, T. L. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 5634–5638
14 Arai, H., Hori, S., Aramori, I., Ohkubo, H. and Nakanishi, S. (1990) Nature (London) **348**, 730–732
15 Titus, J. A., Haugland, R., Sharrow, S. O. and Segal, D. M. (1982) J. Immunol. Methods **50**, 193–197
16 Whittaker, J. R. (1971) J. Biol. Chem. **246**, 6217–6226
17 Farishian, R. A. and Whittaker, J. R. (1980) J. Invest. Dermatol. **74**, 85–89
18 Oikawa, A., Nakayasu, M., Nohara, M. and Tchen, T. (1972) Arch. Biochem. Biophys. **148**, 548–557
19 Imokawa, G. and Motegi, I. (1993) J. Invest. Dermatol. **100**, 47–54
20 Yada, Y., Ozeki, T., Meguro, S., Mori, S. and Nozawa, Y. (1989) Biochem. Biophys. Res. Commun. **163**, 1517–1522
21 Kozuka, M., Ito, S., Hirose, S., Lodhi, K. M. and Hagiwara, H. (1991) J. Biol. Chem. **266**, 16892–16896
22 Sakurai, T., Yanagisawa, M., Takuwa, Y., Miyazaki, H., Kimura, S., Goto, K. and Masaki, T. (1990) Nature (London) **348**, 732–735
23 Lin, H. Y., Kaji, E. H., Winkel, G. K., Ives, H. E. and Lodish, H. F. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 3185–3189
24 Sakamoto, A., Yanagisawa, M., Sakurai, T., Takuwa, Y., Yanagisawa, H. and Masaki, T. (1991) Biochem. Biophys. Res. Commun. **178**, 656–663
25 Sakurai, T., Yanagisawa, M. and Masaki, T. (1992) Trends Pharmacol. Sci. **13**, 103–108
26 Halaban, R., Pomerantz, S. H., Marshall, S., Lambert, D. T. and Lerner, A. B. (1983) J. Cell Biol. **97**, 480–488
27 Friedmann, P. S. and Gilchrest, B. A. (1987) J. Cell. Physiol. **133**, 88–94
28 Ranson, M., Posen, S. and Mason, R. S. (1988) J. Invest. Dermatol. **91**, 593–598
29 Houslay, M. D. (1991) Eur. J. Biochem. **195**, 9–27
30 Gusovsky, F. and Gutkind, J. S. (1991) Mol. Pharmacol. **39**, 124–129
31 Yoshimura, M. and Cooper, D. M. F. (1993) J. Biol. Chem. **268**, 4604–4607
32 Katada, T., Gilman, A. G., Watanabe, Y., Bauer, S. and Jakobs, K. H. (1985) Eur. J. Biochem. **151**, 431–437
33 Naghshineh, S., Noguchi, M., Huang, K. P. and Londos, C. (1986) J. Biol. Chem. **261**, 14534–14538
34 Wiener, E. and Scarpa, A. (1989) J. Biol. Chem. **264**, 4324–4328
35 Bushfield, M., Murphy, G. J., Lavan, B. E., Parker, P. J., Hruby, V. J., Milligan, G. and Houslay, M. D. (1990) Biochem. J. **268**, 449–457
36 Yoshimasa, T., Sibley, D. R., Bouvier, M., Lefkowitz, R. J. and Caron, M. G. (1987) Nature (London) **327**, 67–70
37 Graves, L. M., Bornfeldt, K. E., Raines, E. W., Potts, B. C., Macdonald, S. G., Ross, R. and Krebs, E. G. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 10300–10304
38 Sevetoson, B. R., Kong, X. and Lawrence, J. C., Jr. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 10305–10309
39 Frodin, M., Peraldi, P. and Obbergjem, E. V. (1994) J. Biol. Chem. **269**, 6207–6214
40 Rossomando, A. J., Payne, D. M., Weber, M. J. and Sturgill, T. W. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 6940–6943
41 Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenceller, G., Marne, D. and Rapp, U. (1993) Nature (London) **364**, 249–251
42 Carroll, M. P. and May, W. S. (1994) J. Biol. Chem. **269**, 1249–1256
43 Wang, Y., Simonson, M. S., Pouyssegur, J. and Dunn, M. J. (1992) Biochem. J. **287**, 589–594
44 Bogoyevitch, M. A., Glennon, P. E., Andersson, M. B., Clerk, A., Lazou, A., Marshall, C. J., Parker, P. J. and Sugden, P. H. (1994) J. Biol. Chem. **269**, 1110–1119

Received 17 July 1995/22 September 1995; accepted 10 October 1995