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Endothelial cell growth factor and ionophore A23187 stimulation of production of inositol phosphates in porcine aorta endothelial cells

(endothelial cells/growth factors/inositol phosphates/mitogenesis)

JORGE MOSCAT, FRANCISCO MORENO, CARMEN HERRERO, CARMEN LOPEZ, AND PEDRO GARCIA-BARRENO*

Medicina Experimental, Hospital General "Gregorio Marañon", c/Dr. Esquerdo 46, 28007 Madrid, Spain

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ABSTRACT **The existence of a bovine brain-derived endothelial cell growth factor has recently been reported, but its mode of action is unknown. We show that the endothelial cell growth factor is a potent stimulant of inositol monophosphate release in porcine aorta endothelial cells. Although the activation of phospholipase C by this factor does not appear to be dependent on Ca^{2+}, the Ca^{2+} ionophore A23187 stimulates release of inositol phosphates. It is suggested that the inositol 1,4,5-trisphosphate 3-kinase/5-phosphomonoesterase pathway could account for the ionophore-induced changes in inositol 1,3,4-trisphosphate.**

Recent reports on the existence of tumor-derived endothelial cell growth factors lend support to the notion that neovascularization is a key event in the development of solid tumors (1). Therefore, a comprehensive study of the mechanisms that control endothelial cell growth is essential for the understanding of the role played by these cells in cancer (2). A growth factor specifically for endothelial cells (ECGF) has been isolated from bovine brain and shown to induce mitosis when bound to the endothelial cell membrane (3). However, the molecular mechanisms of ECGF action are as yet unknown.

Inositolphospholipid metabolism appears to be a ubiquitous transducing system in a number of key cellular functions through the generation of two putative second messengers—namely, inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] through its ability to release Ca^{2+} from intracellular stores and diacylglycerol as activator of protein kinase C (phospholipid- and Ca^{2+}-dependent protein kinase) (for reviews, see refs. 4 and 5). Both compounds are reportedly involved in the regulation of cell growth (5–8). Also, two inositol phosphates (Ins*P*s) have been reported recently to be generated from Ins(1,4,5)P_3—i.e., inositol 1,3,4-trisphosphate [Ins(1,3,4)P_3] and inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4] (9, 10). The former is produced through the action of a 5-phosphomonoesterase on the latter, which in turn is generated by an Ins(1,4,5)P_3 3-kinase (11). Interestingly, Irvine and Moore (12) have provided strong support for the involvement of Ins(1,3,4,5)P_4 in the control of Ca^{2+} influx from the extracellular environment.

We and others have observed a high level of inositolphospholipid metabolism in aorta endothelial cells (13–15). Moreover, previous data from our laboratory suggest that thrombin exerts its mitogenic effect on porcine aorta endothelial cells (PAEC) via phospholipase C activation (16). For these reasons, we decided to investigate the mitogenic properties of ECGF and its possible relation to inositolphospholipid hydrolysis. Evidence is presented here suggesting that ECGF induces mitogenesis in PAEC through activation of phospholipase C.

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MATERIALS AND METHODS

Materials. ECGF and insulin were from Collaborative Research (Waltham, MA), A23187 and cholera toxin were from Calbiochem, and pertussis toxin was from ICN.

Isolation and Culture of Endothelial Cells. Endothelial cells were isolated by collagenase treatment of aortas from large white pigs (17) and were cultured in medium 199 (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin at 50 units per ml and 50 μg/ml, respectively. The cultures were derived from pooled primary cultures (≈7 days) and were passaged with collagenase at a 1:3 split ratio onto plastic culture dishes (35-mm diameter for inositolphospholipid metabolism study or 12-mm diameter for mitogenesis assays). When cell cultures were confluent, the medium was removed and the cells were incubated for 48 hr in serum-free medium 199 to make them quiescent. Endothelial cell cultures showed typical morphology as well as homogeneous staining for factor VIII antigen according to current criteria (18).

Cell-Proliferation Assays. Quiescent cell monolayers were incubated in serum-free medium containing the various agonists for 21 hr, after which 1 μCi (1 Ci = 37 GBq) of [*methyl*-^{3}H]thymidine per ml was added. Incubation was continued for 3 hr, and the incorporation of radioactivity into CCl_3COOH-insoluble material was determined by liquid scintillation counting.

Cell Labeling Procedure. Quiescent cell monolayers grown on 35-mm culture dishes were labeled overnight with 20 μCi of *myo*-[2-^{3}H]inositol (Amersham; specific radioactivity, 50 Ci/mmol) per ml or 5 μCi of [2-^{3}H]glycerol (Amersham; specific radioactivity, 0.5–1 Ci/mmol) per ml; these cells then were washed extensively, equilibrated in serum-free medium 199, and incubated with the different agonists.

Extraction and Fractionation of Ins*P*s. Reactions were stopped with ice-cold CCl_3COOH as described (11). The CCl_3COOH was removed with ether, and the reaction mixture was neutralized with NH_4OH. [^{3}H]Ins*P*s were fractionated by anion-exchange HPLC with an Ultrasil-AX column (4.6 × 250 mm; Beckman). A solvent gradient similar to that described by Irvine *et al.* (11) was used. Standards for [^{32}P]Ins*P*s were obtained from erythrocyte ghosts, and [^{3}H]Ins(1,3,4,5)P_4 was generously provided by Robin F. Irvine (Cambridge, U.K.). When release of unfractionated total Ins*P*s was determined, these metabolites were separated from [2-^{3}H]inositol by anion-exchange chromatography in small Dowex 1 × 8 columns (0.6 ml of bed volume) as described (11).

Abbreviations: ECGF, endothelial cell growth factor; G protein, guanine nucleotide-binding protein; Ins*P*s, inositol phosphates; Ins*P*, inositol monophosphate; InsP_2, inositol bisphosphate; Ins(1,3,4)P_3, inositol 1,3,4-trisphosphate; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; Ins(1,3,4,5)P_4, inositol 1,3,4,5-tetrakisphosphate; PAEC, porcine aorta endothelial cell(s); PtdIns and PtdInsP_2, phosphatidylinositol and its bisphosphates.

*To whom reprint requests should be addressed.

Measurement of Diglyceride Production. For estimation of tritiated glycerol/diglyceride levels, reactions were stopped and lipid was extracted by the method of Bligh and Dyer (19). Lipids were fractionated by thin-layer chromatography in hexane/diethyl ether/acetic acid, 40:60:1 (vol/vol), were visualized by using appropriate standards and exposing the plates to iodine vapor, were scraped from the plates, and were assayed for radioactivity.

RESULTS

Mitogenic Response. PAEC were grown to confluence, made quiescent by serum starvation, and incubated with different amounts of ECGF; the incorporation of [^{3}H]thymidine into the CCl_3COOH-insoluble material was determined. The results confirm that ECGF is a potent mitogen for PAEC. A response similar to that produced by 10% fetal bovine serum was achieved by ECGF (1 ng/ml); this suggests that this mitogen is able to induce both competence and progression in these cells. Hence, the simultaneous addition of ECGF (1 ng/ml) and insulin (1 μg/ml) did not induce a higher mitogenic response than that induced by ECGF (1 ng/ml) alone (data not shown).

Inositolphospholipid Metabolism. To investigate how ECGF promotes PAEC growth, cells were stimulated with ECGF (1 ng/ml) after overnight labeling with [^{3}H]glycerol. ECGF induced diglyceride formation (Fig. 1), indicating that the factor is a stimulant of diglyceride production in PAEC. In another experiment, quiescent [^{3}H]inositol-labeled PAEC were incubated in the presence of ECGF (1 ng/ml) for various times, and the total [^{3}H]Ins*P*s were extracted and fractionated by anion-exchange HPLC (Fig. 2). Tritiated inositol monophosphate ([^{3}H]Ins*P*) was the only one of the Ins*P*s produced after incubation with ECGF. A similar pattern was found at each time point assayed. The time course of [^{3}H]Ins*P* release (Fig. 3) was in keeping with that of diglyceride production.

Taken together, the above results suggest that ECGF induces phospholipase C-catalyzed phosphatidylinositol (PtdIns) hydrolysis. Previous results suggested that the phospholipase C attack on PtdIns may be a Ca^{2+}-triggered event (20). Thus, it was possible that ECGF induced PtdIns hydrolysis by promoting Ca^{2+} influx. To test this possibility, the ECGF-induced release of [^{3}H]Ins*P* was determined in the presence or absence of extracellular Ca^{2+}. The results (Table 1) strongly suggest that ECGF-induced Ins*P* release may not be Ca^{2+}-mediated. However, A23187 (a calcium ionophore) activated the reaction in a Ca^{2+}-dependent manner (Table 1). To investigate further the A23187-induced release of Ins*P*s in PAEC, quiescent [^{3}H]inositol-labeled

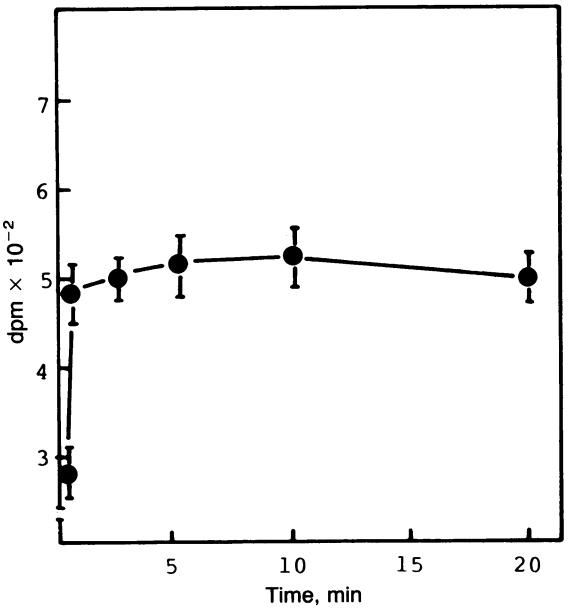

FIG. 1. ECGF-induced formation of diglyceride. Quiescent [^{3}H]glycerol-labeled PAEC were incubated with ECGF (1 ng/ml). At various times, lipids were extracted and fractionated by thin-layer chromatography, and the incorporation of radioactivity into diglyceride was determined. Results are means ± SEM of three independent experiments with incubations in duplicate.

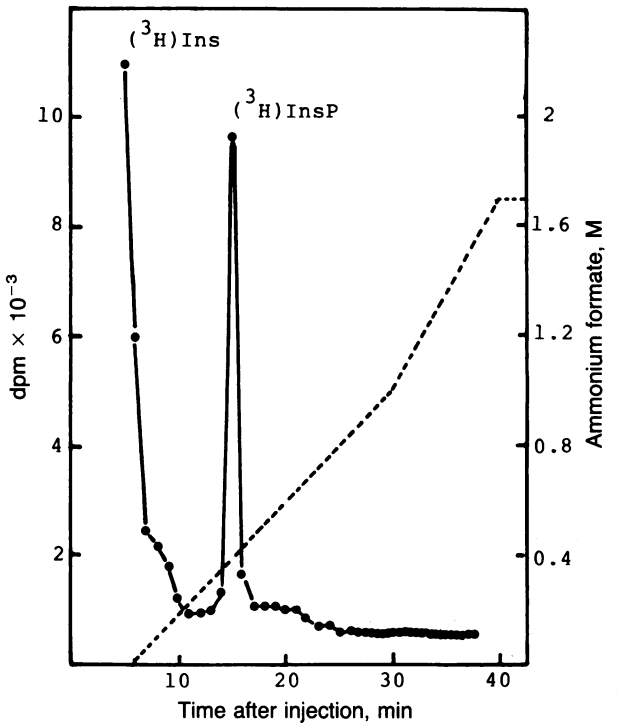

FIG. 2. Representative profile of ECGF-induced release of Ins*P*s. Quiescent [^{3}H]inositol-labeled PAEC were stimulated with ECGF (1 ng/ml) for 30 min and Ins*P*s were fractionated by HPLC. A 0–1.7 M ammonium formate gradient (----) was used. The figure is representative of at least four experiments.

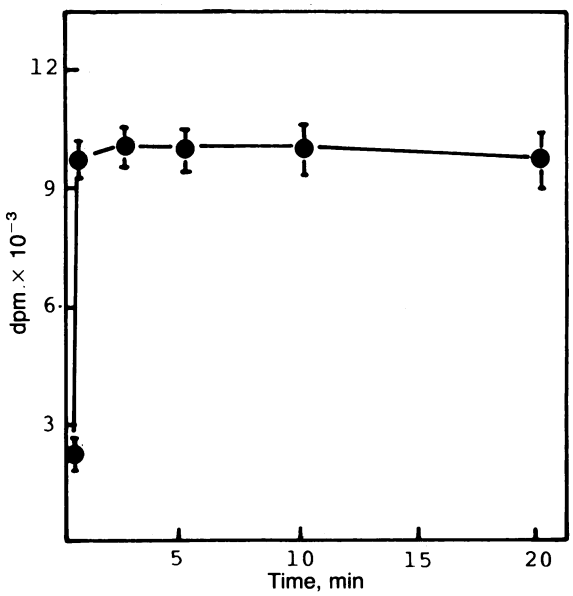

FIG. 3. Time course of the ECGF effect on Ins*P* release. Conditions were as in Fig. 2. Results are means ± SEM of four independent experiments with incubations in duplicate.

Table 1. Effect of Ca^{2+} on ECGF- and A23187-induced release of Ins*P*s in PAEC

Additions	Formation of Ins*P*s, dpm per well	
	Medium with Ca^{2+}	Medium with EGTA (Ca^{2+}-free)
None	2,350 ± 250	1860 ± 260
ECGF (1 ng/ml)	10,480 ± 1300	9750 ± 830
A23187 (10 μM)	14,360 ± 1190	1900 ± 540

Quiescent [^{3}H]inositol-labeled PAEC were incubated in the presence of Ca^{2+} (1 mM) or in a Ca^{2+}-free EGTA-containing medium for 20 min. Stimulants were then added, and release of total Ins*P*s was determined as described after a 30-min incubation period. Results are the means ± SEM of three independent experiments with incubations in duplicate.

PAEC were incubated with A23187 (10 μM) for various times; the [^{3}H]Ins*P*s were then extracted and fractionated by anion-exchange HPLC. Fig. 4 shows a representative HPLC profile after a 5-min treatment of PAEC with A23187. It is clear that ionophore treatment of PAEC induced the release of not only Ins*P*, but also inositol 1,4-bisphosphate [Ins(1,4)P_2], a metabolite tentatively identified as inositol 3,4-bisphosphate [Ins(3,4)P_2], Ins(1,4,5)P_3, Ins(1,3,4)P_3, and Ins(1,3,4,5)P_4. A time course for each of the Ins*P*s released in response to A23187 is shown in Fig. 5. When A23187-induced [^{3}H]diglyceride production was investigated, the biphasic time course shown in Fig. 6 was obtained.

A growing body of evidence indicates that receptor-stimulated phospholipase C activation is a guanine nucleotide-binding protein (G protein)-mediated event (see, for example, refs. 20–23). To test this possibility in PAEC, quiescent [^{3}H]inositol-labeled cells were treated for 30 min with either ECGF (1 ng/ml) or A23187 (10 μM) after a 3-hr treatment with cholera toxin (100 ng/ml) or pertussis toxin (100 ng/ml). The results (Table 2) indicate that, whereas the A23187-induced release of Ins*P*s was insensitive to the treatment with either toxin, cholera toxin abolished the ECGF-induced release of Ins*P*. Pertussis toxin slightly (although significantly) enhanced ECGF-induced release of Ins*P*. Experiments performed with dibutyryl cAMP indicated that the ability of cholera toxin to inhibit ECGF-induced release of Ins*P*s is not secondary to an increase in cAMP levels because noninhibition of ECGF-induced release of Ins*P* was observed when cells were incubated in the presence of dibutyryl cAMP at concentrations (100 μM) at

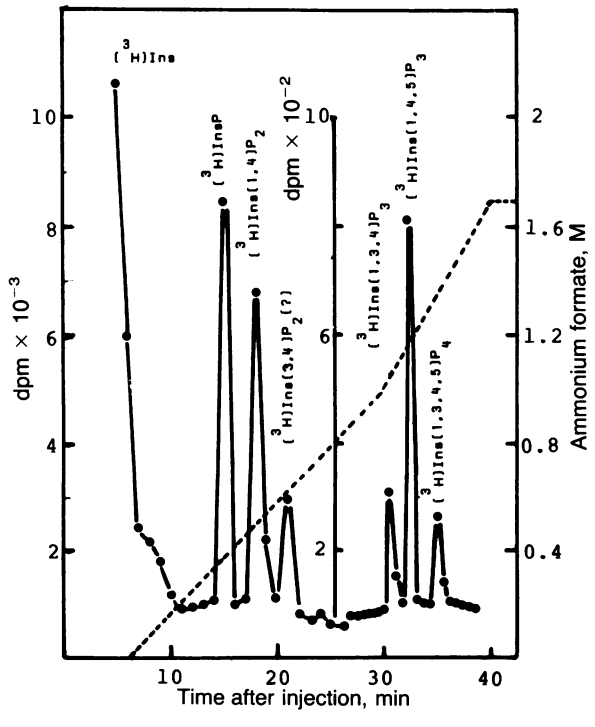

FIG. 4. Representative HPLC profile of A23187-induced release of Ins*P*s. Incubations were carried out for 5 min as in Fig. 2, but with A23187 (10 μM) instead of ECGF.

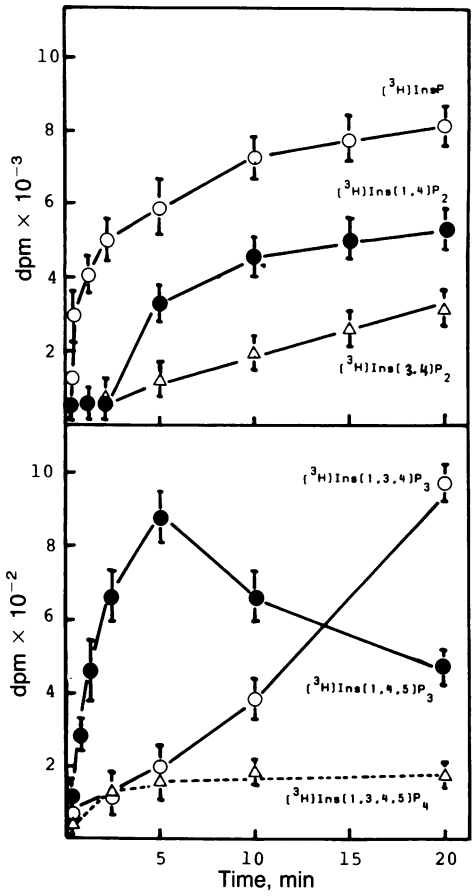

FIG. 5. Time course of the effect of A23187 on the release of Ins*P*s. Conditions were as in Fig. 2, but with A23187 (10 μM) instead of ECGF.

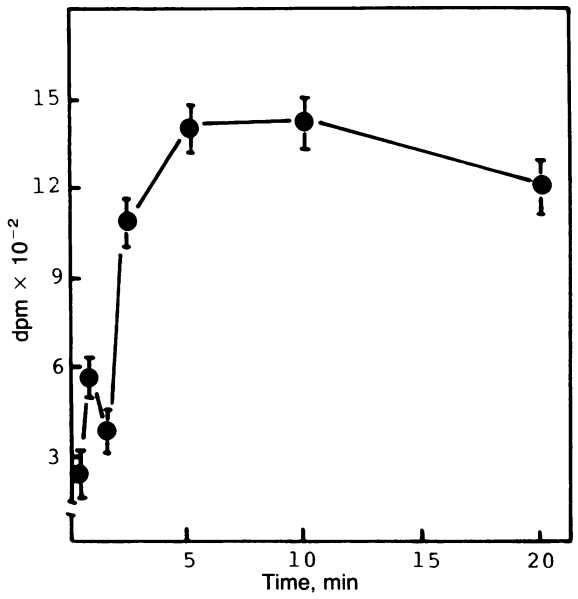

FIG. 6. A23187-induced formation of diglyceride. Conditions were as in Fig. 1, but with A23187 (10 μM) instead of ECGF.

Table 2. Effect of cholera or pertussis toxin pretreatment of PAEC on A23187- and ECGF-induced release of Ins*P*s

Stimulant	Release of Ins*P*s, dpm per well		
	No pretreatment	Toxin pretreatment	
		Cholera	Pertussis
None	2,350 ± 250	2,560 ± 300	2,160 ± 160
ECGF (1 ng/ml)	10,480 ± 300	2,700 ± 450	12,500 ± 650
A23187 (10 μM)	14,360 ± 1190	14,260 ± 875	11,650 ± 1870

Quiescent [^{3}H]inositol-labeled PAEC were treated for 3 hr with either cholera toxin (100 ng/ml) or pertussis toxin (100 ng/ml), after which they were stimulated with A23187 (10 μM) or ECGF (1 ng/ml) for 30 min, and the release of Ins*P*s was determined as described. Results are the means ± SEM of three independent experiments with incubations in duplicate.

which cAMP has been shown to elicit a biological response (data not shown).

DISCUSSION

Our results show that ECGF is a potent mitogen for PAEC and activates phospholipase C attack on PtdIns, which, in turn, activates the formation of diglyceride and Ins*P*. It has been shown that some growth factors, such as platelet-derived growth factor, need the presence of progression factors for the mitogenic response to be turned on (24). However, ECGF appears to be able to induce in PAEC both competence and progression, since its presence alone is mitogenic for PAEC, irrespective of the presence of insulin.

The view that the metabolism of inositol-containing phospholipids plays a key role in the control of cell growth has received some criticism (25, 26). However, there is little doubt that some growth factors (such as platelet-derived growth factor and bombesin) are potent stimulants of phospholipase C (6, 27), and it is worth noting that more recent data support the role of inositide metabolism in the expression of a number of oncogenes that appear to be involved in the commitment of cells to proliferation (28, 29). Our data strongly suggest that ECGF induces mitogenesis in PAEC through phospholipase C, which would provide the diglyceride necessary for protein kinase C activation. ECGF-induced phospholipase C activation does not appear to be Ca^{2+}-dependent, although the Ca^{2+} ionophore A23187 is a potent stimulant for the release of inositol phosphates in PAEC (see above). It is worth noting that the ionophoretic challenge (absolutely dependent on Ca^{2+}) induces the release of Ins(1,4,5)P_3 concomitantly with the liberation of Ins*P*. This, together with the biphasic time course of A23187-induced diglyceride formation, suggests that ionophore-activated phospholipase C hydrolyzes (presumably in a biphasic way) both PtdIns and PtdIns bisphosphate (PtdInsP_2). Similar conclusions about agonist-activated simultaneous hydrolysis of both phospholipids have been reported by others (20, 30). This suggests that PtdIns is also a physiological substrate for phospholipase C and not only a reservoir for supply of hydrolyzed PtdInsP_2 (30, 31). The fact that ionophore treatment induces PtdInsP_2 hydrolysis is in keeping with recent data showing that, although agonist-induced inositolphospholipid hydrolysis can be triggered at resting cytosolic Ca^{2+} concentrations, Ca^{2+} itself is a potent stimulant of such a pathway (20, 32). It has been shown that an ionophoretic stimulus induces a remarkable rise in Ins(1,3,4)P_3 levels without a previous increase in Ins(1,3,4)P_3 (33). This prompted the speculation that there exist sources other than Ins(1,4,5)P_3 3-kinase/5-phosphomonoesterase for Ins(1,3,4)P_3 synthesis. However, our results clearly support the existence of this pathway, since A23187 induces not only the release of Ins(1,3,4)P_3 but also that of its precursors—i.e., Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4—and at rates compatible with the above pathway.

Regarding the possible existence of G proteins acting as links between ECGF-activated receptors and phospholipase C, our data show that, whereas the ionophore-induced release of Ins*P*s is insensitive to treatment with cholera toxin or pertussis toxin, the ECGF-induced release of Ins*P* is abolished by previous treatment with cholera toxin. As far as we know there are only two reports describing inhibition of the agonist-induced activation of phospholipase C by cholera toxin (34, 35). Two possible explanations may be advanced to account for this effect: (*i*) the existence of a novel G protein that would be an intermediary in the activation of phospholipase C and inhibitable by cholera toxin; and (*ii*) the existence in PAEC of a stimulatory G (G_s)-protein-mediated pathway that would be able to inhibit ECGF-induced phospholipase activation and operate as a feed-back inhibitory mechanism similar to that suggested to account for the effect of high concentrations of glucagon in hepatocytes (35). Elucidation of the mechanism involved is an attractive challenge for future research.

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