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PDGF - INDUCED RECEPTOR PHOSPHORYLATION AND PHOSPHOINOSITIDE HYDROLYSIS ARE UNAFFECTED BY PROTEIN KINASE C ACTIVATION IN MOUSE SWISS 3T3 AND HUMAN SKIN FIBROBLASTS

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SUMMARY: Short (1-10 min) pretreatment of intact cells with activators of protein kinase C (e.g. phorbol-12 miristate, 13-acetate, PMA) affects the activity of a variety of surface receptors (for growth factors, hormones and neurotransmitters), with inhibition of transmembrane signal generation. In two types of fibroblasts we demonstrate that the PDGF receptor is unaffected by PMA. Exposure to PMA at concentrations up to 100 nM for 10 min failed to inhibit either one of the agonist-induced, receptor-coupled responses of PDGF: the autophosphorylation of receptor molecules at tyrosine residues, and the hydrolysis of membrane polyphosphoinositides. In contrast, the EGF receptor autophosphorylation (in A 431 cells) and the bombesin-induced phosphoinositide hydrolysis were readily inhibited by PMA. Feed-back inhibition of surface receptors by protein kinase C-mediated phosphorylation is therefore not general, and cannot be the only process responsible for the attenuation of receptor-mediated responses in eukaryotic cells.

During the last few years, a number of reports have appeared demonstrating desensitization of surface receptors in various cell types exposed for times as short as 1 min to low concentrations of drugs (such as phorbol esters) known as activators of the Ca^{2+} and phospholipid dependent protein kinase, protein kinase C (PKC). The list of the receptors affected includes both receptors for growth factors (i.e. epidermal growth factor (EGF), insulin, insulin like growth factor I), that are endowed with endogeneous tyrosine-specific protein kinase activity and undergo autophosphorylation in their tyrosine residues when activated (1,2,3,4); and receptors for hormones, neurotransmitters and local factors (i.e. muscarinic M_1, adrenergic α_1,

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receptors for thrombin, angiotensin, the chemotactic factor fMet-Leu-Phe and others) that are coupled across the plasma membrane with a metabolic reaction, the hydrolysis by a specific phosphodiesterase of membrane polyphosphoinositides (5,6,7,8,9,10). Of the two second messengers generated by the latter reaction one, inositol-1,4,5-trisphosphate (IP_3) is known to mediate intracellularly the redistribution of Ca^{2+} from a microsomal store to the cytosol; the other, diacylglycerol, is the physiological activator of PKC (see reviews 11 and 12). At the receptors coupled to phosphoinositide turnover the PKC-induced desensitization can therefore be envisaged as a feedback inhibition.

The receptor for platelet derived growth factor (PDGF) is endowed with both the transmembrane signaling mechanisms described above. Thus, its activation results in autophosphorylation at tyrosine residues as well as phosphoinositide hydrolysis (13,14,15,16,17). For quite sometime this double mode of transduction appeared peculiar to the PDGF receptor, but data from our laboratories (unpublished) have recently revealed that this phenomenon occurs with other receptors as well (for example, EGF and bombesin receptors). This paper deals with another feature of the PDGF receptor, its insensitivity to PKC. In two types of fibroblasts, mouse Swiss 3T3 and human skin fibroblasts, we found that short pretreatment with the potent activator of PKC, phorbol 12-miristate, 13-acetate (PMA), was unable to affect either receptor autophosphorylation or the phosphoinositide hydrolytic responses induced by the application of PDGF. This observation, that in our knowledge is the first concerning a receptor of the tyrosine kinase and/or the phosphoinositide turnover type, opens some interesting insights on trasmembrane signaling and its regulation.

MATERIALS AND METHODS

Cells and growth conditions: mouse Swiss 3T3 fibroblasts, human skin fibroblasts and A431 epidermoid carcinoma cells were cultured in Dulbecco modified Eagle's medium (DME) supplemented with 10% fetal bovine serum (FBS) and grown at 37° in a 95% air and 5% CO_2 atmosphere. Human skin fibroblasts were used between the 15th and 25th passage. Cells were subcultured onto either 35mm dishes or 60mm dishes depending on the type of the experiment.
Inositolphosphates measurement: cells were incubated with ^{3}H-myoinositol (1-2 μCi/ml, Amersham) for 24hr in a inositol-free basal Eagle medium containing 0.1% FBS or 5% platelet-poor plasma. Shortly before the experiments, the cells were washed three times in a modified Krebs Ringer medium (KRH, see ref. 5) containing 0.1% bovine serum albumin (BSA) and incubated in either

KRH or KRH additioned with serum or growth factors. Cells were then extracted with 8% TCA, the extracts were washed free of TCA with diethylether, and inositol phosphate were analyzed by anion exchange chromatography essentially as described by Berridge (15). PMA (Sigma) was dissolved in DMSO and applied at a concentration of 100 nM at the times indicated before the growth factors. Controls received the solvent only (0.5%). Bombesin was purchased from Sigma.

Tyrosine phosphorylation of PDGF and EGF receptors: quiescent Swiss 3T3 monolayers were incubated in DME with 0.1% BSA with or without PDGF (partially purified according to Antoniades, ref. 20) for 8 min. A431 cells were incubated in DME with 0.1% BSA with or without EGF (recombinant human EGF, Amersham International) for 10 min. When specified PMA in DMSO was added 10 min before growth factors. Monolayers where then extracted, fractionated by SDS PAGE and immunoblotted with P-tyr antibodies and ^{125}I-protein A as described (19). Molecular weight markers: miosine (200kd), β-galactosidase (116), BSA (66), ovalbumine (45) and carbonic anidrase (31) from Biorad.

Phosphotyrosine antibodies: anti-azobenzyl phosphonate antibodies were produced, affinity purified and characterized as reported (18).

RESULTS

Fig. 1 a-d illustrates the changes in the state of tyrosine phosphorylation of a 170 Kd band, identified as the PDGF receptor, brought about in Swiss 3T3 cells by the addition of PDGF, alone or following short (10 min) pretreatment with PMA, as revealed by a specific antiphosphotyrosine immunoblot assay (19). In agreement with previous results we found that treatment with PDGF greatly increases the labelling in the blot (i.e., causes autophosphorylation of the

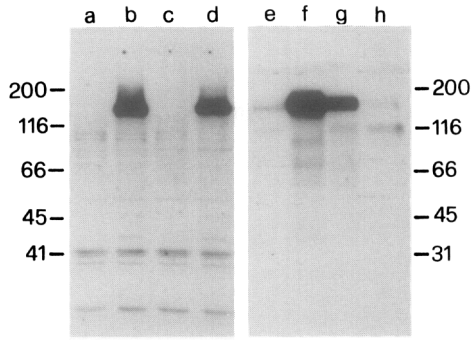

FIG. 1: Differential effect of PMA on tyrosine phosphorylation of the PDGF and EGF receptors.
Quiescent Swiss 3T3 fibroblasts (lanes a-d) received either DMSO (0.5%, lanes a and b) or PMA (100 nM in the same volume of DMSO, lanes c and d) for 10 min, after which PDGF (40 μg/ml) was added to the samples of lanes c and d and incubation was continued for 8 more min.
A 431 cells (lanes e-h) received either DMSO (0.5%, lanes e and f) or PMA (100 nM in the same volume of DMSO, lanes g and h) for 10 min, after which EGF (50 ng/ml) was added to the samples of lanes f and g and incubation was continued for 10 more min.

receptor,compare a and b). On the other hand PMA, that by itself has no effect on tyrosine phosphorylation (lane c) also fails to modify appreciably the PDGF-induced response (compare lanes b and d). Identical results were obtained with another type of cells, human skin fibroblasts (not shown). In order to check wether the experimental conditions of our studies were adequate for our purpose, the well kown effect of EGF and PMA on tyrosine phosphorylation of the EGF receptor in A431 carcinoma cells (1,3) was investigated. As can be seen in Fig. 1, lanes e-h treatment with EGF causes a marked autophosphorylation of the 160 Kd receptor band. At variance with the results obtained with PDGF, however, the effect of EGF is largely inhibited by PMA pretreatment of the cells (compare lanes f and g).

The results obtained by studying phosphoinositide turnover are summarized in Tables I and II. In Table I the effects of PDGF $\pm$ PMA on 3T3 cells and human skin fibroblasts (HF) (incubated in the presence of LiCl to block

TABLE I

Effect of PMA on PDGF- and bombesin- induced accumulation of total inositolphosphates in Swiss 3T3 and human skin fibrablasts

Additions	^{3}H -inositol phosphates (% of controls)	
	3T3	HF
- / -	100	100
- / PDGF	290 $\pm$ 55	170 $\pm$ 18
PMA / PDGF	261 $\pm$ 62	156 $\pm$ 8
- / Bombesin	414 $\pm$ 85	-
PMA / Bombesin	285 $\pm$ 50	-
- / Serum	-	333 $\pm$ 11
PMA / Serum	-	156 $\pm$ 4

Mouse Swiss 3T3 and human skin fibroblasts (HF) were prelabelled with ^{3}H-inositol as described under Materials and Methods. The monolayers were preincubated in a modified Krebs Ringer supplemented either with DMSO (0.5%) or PMA (100 nM) for 10 min and then incubated with or without PDGF (40μg/ml), bombesin (50$_{3}$nM) or serum (10%) for additional 10 min in the presence of 10 mM LiCl. ^{3}H -inositol phosphates were extracted as described under Material and Methods. Results are averages of 4 and 3 experiments for 3T3 and HF respectively and are expressed as % of basal levels $\pm$ S.E.M. Basal radioactivity (cpm/mg proteins) was 4950.

TABLE II
Effect of PMA on PDGF- and bombesin- induced accumulation of individual inositolphosphates in Swiss 3T3 fibroblasts

Additions	cpm/dish		
	IP_1	IP_2	IP_3
- / -	861	193	110
- / PDGF	3917	770	262
PMA / PDGF	3806	635	244
- / -	505	159	66
- / Bombesin	3140	612	231
PMA / Bombesin	1656	370	130

Mouse Swiss 3T3 fibroblasts were prelabelled with ^3H -inositol as described under Material and Methods. The monolayers were preincubated in modified Krebs Ringer supplemented with DMSO (0.5%) or PMA (100 nM) for 10 min and then incubated with or without PDGF (60 μg/ml) or bombesin (50 nM) for additional 3 min and 2 min respectively. ^3H -inositol phosphates were extracted and fractionated as described by Berridge (15). Results are means of duplicate samples from one single experiment which was repeated twice.

inositol-1-phosphate phosphatase and thus cause accumulation of the released phosphoinositols) are compared with those brought about by the peptide bombesin as well as by serum. As can be seen, all these treatments induce phosphoinositide hydrolysis (revealed by the accumulation of total phosphoinositols) which is greater for bombesin and serum, but still quite marked for PDGF. Short pretreatment with PMA causes the bombesin rsponse in 3T3 cells and the serum response in HF to decrease by 42% and 53% respectively. In contrast the response to PDGF is completely unaffected in both cell types (Table 1). In HF the effect of PMA on the bombesin response could not be investigated since these cells do not respond to the peptide, presumably because they lack the specific receptor.

Three different phosphoinositides, phosphatidylinositol (PI), phosphatidylinositol-4 phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP_2) are known to exist in the plasmalemma and their hydrolysis generates inositol 1-monophosphate (IP_1), inositol 1,4 bisphosphate (IP_2) and inositol 1,4,5 trisphosphate (IP_3) respectively. In order to exclude the possibility that an effect of PMA on the hydrolysis of one such phospholipids had been obscured by the others,the experiment illustrated in Table II were performed. Cell incuba-

tion was carried out for 3 min (the earliest time at which a significant effect of PDGF on total phosphoinositol accumulation could be detected) in the absence of LiCl, and the different phosphoinositols were analyzed separately. The results demonstrate that PDGF causes the increased accumulation of all three phosphoinositols in 3T3 cells and that these accumulations are unaffected by the short pretreatment with PMA. In contrast when bombesin is used as the trigger a 10 min pretreatment of 3T3 cells with PMA induces a marked (over 50%)inhibition of the generation of all three phosphoinositols (IP_1,IP_2,IP_3).

DISCUSSION

Inhibition of receptor activity following activation of PKC is widespread in many cell types and has therefore been suggested as one of the main functions of the enzyme. As far as the molecular targets of this enzyme action, studies on growth factor receptors (insulin, EGF and insulin-like growth factor I receptors) demonstrated that the receptor molecules are themselves substrates of PKC, and suggested that their activity is modulated by their state of phosphorylation. This suggestion has been recently born out for the EGF receptor, whose affinity for the ligand is decreased by PKC-dependent phosphorylation of the threonine residue 654 (21). Direct phosphorylation could also account for the inhibition of receptors coupled to phosphoinositide hydrolysis. So far, however, only the α_1 adrenergic receptor has been reported to be a substrate of PKC (6). Alternatively, receptor inhibition by PMA could be due to phosphorylation not of the receptor molecule, but of the GTP-binding (G) protein(s) that is known to be involved in the coupling of receptors of this class to the specific phosphodiesterase. G proteins are a family of proteins that play strategical roles in transmembrane signaling. At least one such protein is a substrate of PKC, but whether this phosphorylation has physiological importance is not clear yet (22).

Activation of a Na^+/H^+ exchange is one of the early events stimulated by growth factors in fibroblasts. The consequent cytoplasmic alkalinization seems to be crucial for the DNA replication to occur. We recently showed that PMA inhibits the Na^+ flux stimulated by the mitogens bradykinin,vasopressin and serum in human foreskin fibroblasts (23). Moreover,in another cell line, the PDGF-stimulated Na^+/H^+ exchange was reported to be inhibited by a short PMA pretreatment(24). This result was interpreted as an indication of a PKC-

induced desensitization of the PDGF receptor. On the other hand, prolonged (24-72 hr) treatment of 3T3 cells with high concentrations of PMA (a procedure known to down regulate PKC, 25) was found to leave unaffected the PDGF-induced receptor autophosphorylation and phosphoinositide turnover (26). In line with these latter results we have now found that short treatments with PMA (which are known to activate PKC) leave unaffected the functioning of the PDGF receptor. These results differentiate the PDGF receptors from other growth factor and phosphoinositide-coupled receptors, and demonstrate therefore that the feedback regulation, mediated by PKC, is not general. This is an indication that the substrates of the relevant PKC phosphorylations are the receptor molecules and not the G proteins, unless the G protein associated with the PDGF receptor is different from those associated with the other receptors.

The autophosphorylation response of the PDGF receptor is known to be transient, i.e., to decline after 10 min, even in the continuous presence of PDGF (19). The lack of effect of a short pretreatment with PMA in the transductive effects coupled to the PDGF receptor activation suggests that the transiency of the response is not the result of a PKC-mediated feedback regulation, but of another mechanism, that at the moment remains undefined. Thus, although the series of events known to be triggered by PDGF in competent cells is already quite complex, it might be still incomplete.

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