Volume 228, number 2, 346-350 FEB 05578 February 1988

Epidermal growth factor-induced phosphoinositide hydrolysis

Modulation by protein kinase C

L.M. Vicentini, R. Cervini, R. Zippel° and P. Mantegazza

Department of Pharmacology and °*Department of General Physiology* and *Biochemistry, University of Milano, Milan, Italy*

Received 7 December 1987

A short-term treatment with phorbol 12,13-dibutyrate (PDBu) was found to inhibit totally the epidermal growth factor (EGF)-stimulated phosphoinositide hydrolysis in A431 cells, whereas long-term pretreatment with PDBu, which is known to down regulate protein kinase C, induced a greater accumulation of the EGF-triggered inositol phosphate accumulation, particularly of Ins(1,3,4,5)P_4. The increased Ins(1,4,5)P_3/Ins(1,3,4,5)P_4 formation in the PDBu long-term pretreated cells was coincident with the increased Ca^{2+} influx stimulated by EGF in the same cells. Since long-term pretreatment with PDBu was found to enhance the EGF signals, an explanation for the synergism between EGF and phorbol esters in the induction of DNA synthesis is provided.

Epidermal growth factor; Inositol phosphate; Phorbol ester; (A431 cell)

1. INTRODUCTION

Binding of growth factors to their specific surface receptors generates a series of early intracellular signals which eventually converge into a final pathway leading to DNA synthesis [1,2]. These intracellular signals should not be considered independent from each other, but as parts of a complicate network of positive and negative controls in continuous operation within the cells. The enzyme protein kinase C (PKC), which is activated by diacylglycerol (DAG) generated by the receptor-triggered hydrolysis of polyphosphoinositides (PPI), or by the DAG analogs, the phorbol esters, appears to play a key role in this network, since it can be responsible for both stimulation and inhibition of mitogenic signals [3]. In particular, PKC is known to profoundly affect the action of EGF, a mitogen for various types of cells. This effect of the enzyme, that is probably mediated by the phosphorylation of the threonine residue 654 in the EGF receptor [4-6], includes the disappearance of the high-affinity binding and a marked inhibition of the tyrosine kinase activity, endogenous to the EGF receptor itself [4-9]. In addition, PKC activation is known to block another signal activated by EGF in the human carcinoid cells A431, i.e. the increase in cytosolic Ca^{2+} [10,11]. The role of the enzyme in these processes, however, had not been precisely defined yet. This was done in the present study, carried out in A431 cells under experimental conditions in which PKC was activated or down regulated by either short (5 min)- or long (24 h)-term treatment with phorbol esters [12].

Correspondence address: L.M. Vicentini, Department of Pharmacology, Via Vanvitelli 32, 20129 Milano, Italy

Abbreviations: InsPs, inositol phosphates; PPI, polyphosphoinositides; PDBu, phorbol 12,13-dibutyrate; $InsP_1$, $InsP_2$, $InsP_3$, $InsP_4$, inositol mono-, bis-, tris-, tetrakis-phosphates, respectively, with assignment of phosphate locants where appropriate (e.g. Ins(1,4,5)P_3)

2. MATERIALS AND METHODS

A431 cells were grown at 37°C in Dulbecco's medium containing 10% fetal calf serum (FCS) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). For labelling with

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

myo-[^{3}H]inositol, A431 cells were incubated for 24 h in inositol free basal Eagle's medium (BME) supplemented with 0.1% of FCS and containing *myo*-[^{3}H]inositol (1–2 μCi/ml, Amersham). For the long-term pretreated cells, PDBu (1 μM) was added to the BME medium. On the day of the experiments, the labelled monolayers were suspended by a brief treatment with 0.1% trypsin and 0.8 mM EDTA in Ca^{2+}-Mg^{2+}-free PBS and allowed to recover for 4 h at 37°C in an oscillating bath. The cells were then washed five times in the incubation medium that contained in mM/l: 125 NaCl; 5 KCl; 1 KH_2PO_4 and 2 $MgSO_4$; 2 $CaCl_2$; 24 Hepes NaOH, pH 7.4; 6 glucose (KRH) and 0.1% bovine serum albumin (BSA). After washing the cells were incubated in KRH with or without EGF (Amersham) for the time indicated in the figure legends. The incubations were terminated by addition of an equal volume of 15% trichloroacetic acid, the extracts were washed free of the acid with diethyl ether and inositol phosphates were analyzed by anion-exchange chromatography as described by Berridge [13]. For the separation of the various inositol phosphates contained in the trisphosphate peak, ether-washed extracts were analyzed by high-performance liquid chromatography (HPLC) on a partisil Sax 10 column, eluted with ammonium formate (pH 3.7) (Fluka). The gradient applied was essentially that described by Batty et al. [14] slightly modified by Biden and Wollheim [15]. An ATP standard (50 μM) was always added to each sample. The nature of the radioactive peaks based on the ATP elution time was presumed on the basis of their previously described chromatographic behaviour. The cell culture media and sera were purchased from Flow laboratories and all the other reagents from Sigma.

3. RESULTS

3.1. *EGF-induced accumulation of [^{3}H]inositol phosphates in control and PDBu short- and long-term pretreated A431 cells*

In suspensions of control A431 cells, the addition of EGF (200 ng/ml) in the presence of 10 mM LiCl induced within 5 min a 2-fold increase in total [^{3}H]InsPs (fig.1A). Such an effect was completely prevented by a 5 min pretreatment (short-term pretreatment) with the phorbol ester PDBu (1 μM) (fig.1A). In cell suspensions prepared from cultures long-term (24 h) pretreated with PDBu (1 μM), on the other hand, the EGF-induced accumulation of [^{3}H]InsPs was greater than in controls (3-fold the basal level), and no inhibition was seen when the acute pretreatment with PDBu was given before EGF exposure (fig.1B). These data are in agreement with previous results from our laboratory [11]. In cells first long-term pretreated with PDBu and then cultured without phorbol ester the blocking effect of acutely administered PDBu recovered slowly (50% inhibition after 24 h, not shown).

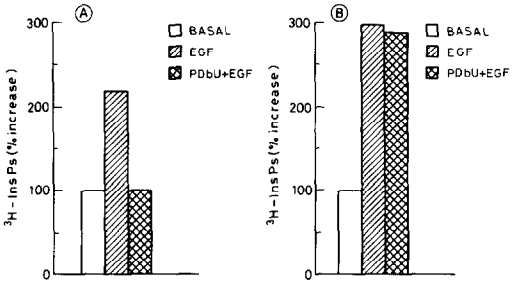

Fig.1. Accumulation of [^{3}H]inositol phosphates stimulated by EGF in control and PDBu short- and long-term pretreated cells. Control (A) and PDBu long-term pretreated (B) A431 cells labelled with *myo*-[^{3}H]inositol (1 μCi/ml) were preincubated for 5 min with either DMSO (0.5%) or PDBu (1 μM) in DMSO and then incubated for 5 additional min ± EGF (200 ng/ml) in KRH containing 10 mM LiCl. Total inositol phosphates were processed as described in section 2. Each result, expressed as % over basal levels, is the mean of duplicate samples from a representative experiment which was repeated twice. Average basal level was 1250 cpm/10^6 cells.

Fig.2 illustrates an ion-exchange chromatography separation of the individual [^{3}H]inositol phosphates accumulated in the presence of LiCl (10 mM) during stimulation with EGF in control and PDBu long-term pretreated cells. After 1 min exposure to the growth factor no apparent difference in the response to EGF was detected, whereas after a longer period (15 min) striking differences appeared, namely the EGF-triggered ac-

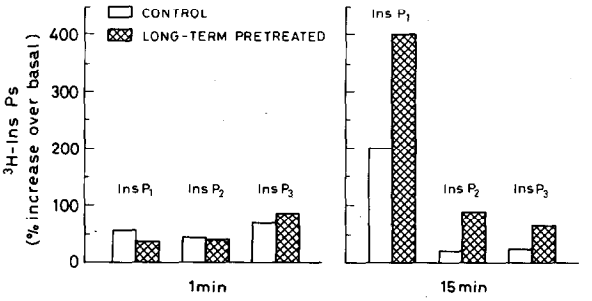

Fig.2. Individual [^{3}H]inositol phosphate accumulation stimulated by EGF in control and PDBu long-term pretreated A431 cells. Control and PDBu long-term pretreated A431 cells labelled with *myo*-[^{3}H]inositol (1 μCi/ml) were incubated ± EGF (200 ng/ml) for 1 min and 15 min in KRH containing 10 mM LiCl. Individual inositol phosphates were separated as described in section 2. Each result, expressed as % over basal levels, is the mean of duplicate samples from a representative experiment which was repeated twice.

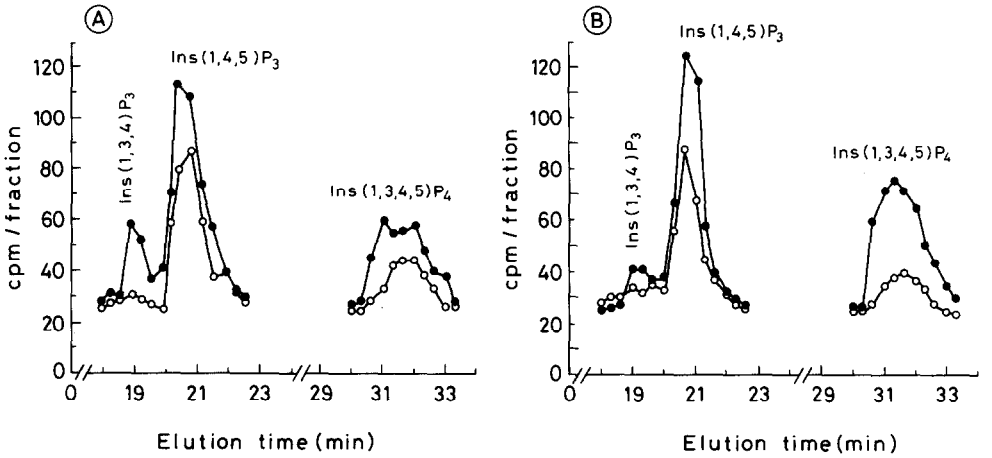

Fig.3. HPLC analysis of tris- and tetrakisphosphate formation stimulated by EGF in control and PDBu long-term pretreated A431 cells. Control (A) and PDBu long-term pretreated (B) A431 cells labelled with *myo*-[^{3}H]inositol (2 μCi/ml) were incubated with (●---●) or without (○---○) EGF (200 ng/ml) for 1 min. The extracts were analyzed by HPLC as described in section 2. The data presented are from a single experiment which was repeated three times giving essentially identical results.

cumulation of $InsP_1$, $InsP_2$ and $InsP_3$ was 2-, 4- and 3-fold greater in long-term pretreated than in control cells, respectively.

3.2. *HPLC analysis of the [^{3}H]inositol phosphates generated by EGF in control and long-term pretreated cells*

[^{3}H]Inositol trisphosphates extracted from control and long-term pretreated cells with or without EGF stimulation were analyzed by means of HPLC anion-exchange chromatography (fig.3), in order to check whether the long-term pretreatment has an effect on the accumulation of the various isomers now known to be recovered together in the anion-exchange column fractions. In particular, by this procedure three important inositol phosphates, Ins(1,4,5)P_3, Ins(1,3,4)P_3 and Ins (1,3,4,5)P_4 were resolved and analyzed separately. As shown in fig.3A, a 1 min stimulation of control cells with EGF caused an appreciable increase in the accumulation of all these InsPs, which was particularly prominent for Ins(1,3,4)P_3. At this point of the experiment the long-term pretreated cells showed two striking differences in the response to EGF with respect to the controls: Ins(1,3,4)P_3 levels were not significantly different from basal, while the levels of $InsP_4$ were higher than in control cells (fig.3B). An even greater difference in the accumulation patterns induced by EGF in long-term pretreated versus control cells was observed after 5 min of stimulation. At this point all InsPs were affected, the largest difference concerning again Ins(1,3,4,5)P_4 (fig.4).

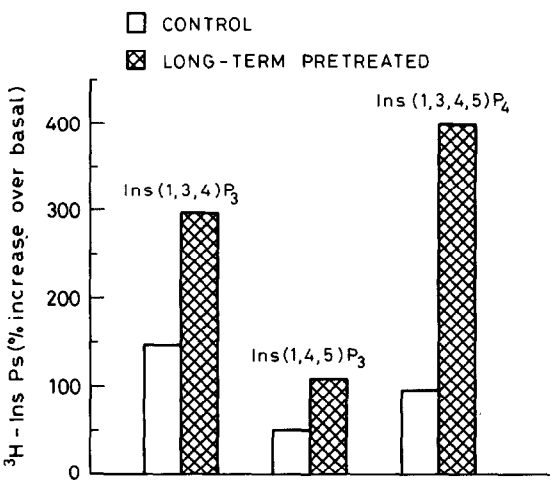

Fig.4. Tris- and tetrakisphosphate accumulation stimulated by EGF in control and PDBu long-term pretreated A431 cells. Control and PDBu long-term pretreated A431 cells labelled with *myo*-[^{3}H]inositol (2 μCi/ml) were incubated ± EGF (200 ng/ml) for 5 min. The extracts were analyzed by HPLC as described in section 2. The data presented, expressed as % over basal levels, are from a single experiment which was repeated twice.

4. DISCUSSION

A definition of the role of PKC in the modulation of a reaction, PPI hydrolysis, triggered by the activation of the EGF receptor has been sought in A431 cells by investigating two well-defined experimental conditions: short (5 min)- and long (24 h)-term pretreatment of the cells with the phorbol ester, PDBu. The short-term treatment is known to cause an activation, the long-term pretreatment a down regulation of the enzyme [12].

We and others previously showed in A431 cells that short-term treatment with PDBu totally inhibits the Ca^{2+} signal induced by EGF [10,11]. Here we report that the same treatment completely prevents the InsP accumulation stimulated by the growth factor. Reduction in intracellular Ca^{2+} rises and a partial inhibition of PPI hydrolysis by phorbol esters is a phenomenon which occurs in many cell types stimulated with various agonists (review [16]). To our knowledge, however, only the EGF signals appear so sensitive to PKC as to be completely inhibited. It is also interesting to note that the transmembrane signalling of another growth factor PDGF is unimpaired by phorbol ester treatment [17,18]. Such a selective action suggests that PKC might exert its negative effect by phosphorylating the receptor itself rather than interfering with common steps between receptor activation and signal generation.

In various cell types, such as A431 and 3T3 cells, both the EGF and PDGF receptors appear coupled to a dual effector system, i.e. tyrosine kinase activity and PPI hydrolysis [17–21]. How this double coupling occurs is still obscure. Since tyrosine kinase activity is only partially inhibited, while the PPI signals are totally inhibited by PKC, it might be suggested that either two types of receptors for EGF exist in the cells, or, alternatively, that the EGF receptor coupling with PPI hydrolysis, differently from the coupling of other receptors, is not a primary, but a secondary event, that includes the involvement of an intermediate step totally sensitive to PKC.

The long-term treatment with PDBu was found to induce a greater EGF-triggered InsP accumulation, an effect that could be due to either increased generation, decreased catabolism or both. Indeed, PKC has been reported to stimulate the 5-monoesterase activity that induces the catabolism of Ins(1,3,4,5)P_4 to Ins(1,3,4)P_3 and of Ins(1,4,5)P_3 to Ins(1,4)P_2 [22]. Irrespective of the cause, our data show that in PDBu long-term treated cells the EGF-induced signalling, particularly the InsP_4 formation, is greatly amplified. Parallel experiments where intracellular Ca^{2+} rises were measured showed that in PDBu long-term pretreated cells EGF stimulated a Ca^{2+} influx which was greater and much more persistent than that in control cells [11]. The correlation between increased Ca^{2+} influx and IP_3/IP_4 formation indirectly supports the recently suggested role of IP_3/IP_4 in the control of Ca^{2+} flux across the plasma membrane [23,24].

The attenuation of the EGF receptor function by the PDBu short-term treatment was difficult to reconcile with the reported synergism between EGF and the phorbol esters in the induction of DNA synthesis [25]. Our results provide an explanation for the phenomenon, since long-term treatment with phorbol esters actually enhances the EGF signals. Our results also suggest that the effects of phorbol esters on cell proliferation, which are measured many hours after their application, might be ascribed to the down regulation of PKC, a sort of escape of the EGF-induced transmembrane signalling from the autolimitative feedback control mediated by PKC.

Acknowledgements: This work was supported by CNR special project Oncology to L.M.V. The authors wish to thank Dr J. Meldolesi for critically reading the manuscript and E. Majorana for typing it.

REFERENCES

[1] Rosengurt, E. (1986) Science 234, 161–166.
[2] Vicentini, L.M. and Villereal, M.L. (1986) Life Sci. 38, 2269–2276.
[3] Vicentini, L.M. and Villereal, M.L. (1985) Proc. Natl. Acad. Sci. USA 260, 8053–8056.
[4] Hunter, T., Ling, N. and Cooper, N.A. (1984) Nature 314, 480–483.
[5] Davis, R.J. and Czech, M.P. (1985) Proc. Natl. Acad. Sci. USA 82, 1974–1978.
[6] King, C.S. and Cooper, A. (1986) J. Biol. Chem. 261, 10073–10078.
[7] Brown, D., Dicker, P. and Rozengurt, E. (1979) Biochem. Biophys. Res. Commun. 86, 1037–1043.
[8] Shoyab, M., DeLarco, J.E. and Todaro, G.J. (1979) Nature 279, 387–391.

[9] Cochet, C., Gill, G.N., Meisenheider, J.A., Cooper, J.A. and Hunter, T. (1984) J. Biol. Chem. 259, 2553–2558.
[10] Moolenar, W.H., Aerts, R.J., Tertoolen, L.G.J. and De Laat, S.W. (1986) J. Biol. Chem. 201, 279–284.
[11] Pandiella, A., Vicentini, L.M. and Meldolesi, J. (1988) Biochem. Biophys. Res. Commun., in press.
[12] Collins, M. and Rozengurt, E. (1982) Biochem. Biophys. Res. Commun. 104, 1159–1166.
[13] Berridge, M.J. (1983) Biochem. J. 212, 849–898.
[14] Batty, I.R., Nahorski, S.R. and Irvine, R.F. (1985) Biochem. J. 232, 211–215.
[15] Biden, T. and Wollheim, C.B. (1986) J. Biol. Chem. 261, 11931–11934.
[16] Berridge, M.J. (1987) Annu. Rev. Biochem. 56, 160–193.
[17] Sturani, E., Vicentini, L.M., Zippel, R., Toschi, L., Pandiella, A. and Meldolesi, J. (1986) Biochem. Biophys. Res. Commun. 137, 343–350.
[18] Lopez-Rivas, A., Mendoza, S.A., Nanberg, E., Sinneth-Smith, J. and Rozengurt, E. (1987) Proc. Natl. Acad. Sci. USA 84, 5768–5772.
[19] Pandiella, A., Malgaroli, A., Meldolesi, J. and Vicentini, L.M. (1987) Exp. Cell. Res. 170, 175–185.
[20] Hepler, J.R., Nakahata, N., Lovenberg, T.W., Diguiseppi, J., Herman, B., Earp, H.S. and Harden, T.K. (1987) J. Biol. Chem. 262, 2951–2956.
[21] Wahl, M.L., Sweatt, J.D. and Carpenter, G. (1987) Biochem. Biophys. Res. Commun. 142, 688–695.
[22] Conolly, T.M., Bansel, V.S., Bross, T.E., Irvine, R. and Majerus, P.W. (1987) J. Biol. Chem. 262, 2146–2149.
[23] Irvine, R.F. and Moor, R.M. (1986) Biochem. J. 240, 917–920.
[24] Morris, H.P., Gallacher, D.V., Irvine, R.F. and Petersen, O.H. (1988) Nature, in press.
[25] Dicker, P. and Rozengurt, E. (1980) Nature 287, 607–612.