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PROTEIN KINASE C-MEDIATED FEED BACK INHIBITION OF THE CA^{2+} RESPONSE AT THE EGF RECEPTOR

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SUMMARY. Activation of the EGF receptor in A431 cells induces the hydrolysis of phosphoinositides and a transient rise of the cytosolic Ca^{2+} concentration, $[Ca^{2+}]_i$, which are completely inhibited by acute pretreatment with activators of protein kinase C, such as phorbol esters. Down regulation of the enzyme (by long-term pretreatment of the cells with phorbol esters) causes the $[Ca^{2+}]_i$ response to EGF to increase in magnitude and, especially, to become much more persistent (average $t_{1/2}$ of $[Ca^{2+}]_i$ decline 9 min with respect to 2.3 min in controls). These results demonstrate that the activation of protein kinase C induced by EGF in intact A431 cells is sufficient to trigger a feed back, autolimitative regulation of the EGF receptor that might play a prominent physiological role in the definition of the mitogenic activity of the growth factor.

The function of the receptor for the epidermal growth factor (EGF) is modulated by phosphorylations that occur in the cytoplasmic, C terminal domain of the molecule [1]. Both intrinsic tyrosine kinase [1], and protein kinase C (PKC) appear to play important roles in these processes [2]. PKC can be activated by exogenous drugs (such as phorbol esters: phorbol 12-myristate,13-acetate, PMA, and phorbol 12,13-dibutyrate, PDBu) or by treatment with receptor agonists that trigger the generation of diacylglycerol (DAG) through the hydrolysis of membrane polyphosphoinositides (PPI) [3]. In A431 epidermoid carcinoma cells phorbol ester treatment was found to decrease the affinity and tyrosine kinase activity of the receptor [4] and to block another effect induced by EGF, the rise of cytosolic free Ca^{2+}, $[Ca^{2+}]_i$ [5]. In these cells, EGF has been reported to induce PPI hydrolysis within seconds, with detectable accumulation of DAG within

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2 minutes [6-10]. These observations suggested the possibility that EGF receptor activation is autolimited, i.e., that the receptor operates under feedback control mediated by PKC activation [6,7]. Here we report direct experimental evidence for the autolimitation of the EGF receptor, obtained by the use of cells in which PKC activity had been down-regulated by long term pretreatment with phorbol esters.

MATERIALS AND METHODS

The conditions used for the culture of A431 cells, their detachment and dissociation into cell suspensions, loading with quin2 or fura-2, measurement of $[Ca^{2+}]_i$, labelling with ^{3}H-myoinositol, and measurement of released inositol phosphates are described in detail in ref. [6]. Human fibroblasts were cultured in Dulbecco's MEM supplemented with 10% fetal calf serum, and used between 15-25 passages. Down-regulation of PKC was achieved by culturing the monolayers for 24 hr in a medium supplemented with PDBu (final concentration 1 uM, added as a 1600-fold concentrated solution in dimethylsulfoxide). Controls received the solvent only. The incubation medium used for the experiments (KRH) contained (in mmol/litre): NaCl, 125; KCl, 5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2; glucose, 6; Hepes-NaOH (pH 7.4), 25.

Materials EGF receptor grade was purchased from Collaborative Research; media and sera from Flow Labs; quin2/AM and fura-2/AM from Calbiochem; PMA and PDBu from Sigma; ^{3}H-myoinositol and ^{3}H-PDBu from Amersham.

RESULTS AND DISCUSSION

Previous studies in a variety of cell types demonstrated that prolonged (many hours, see ref. 11) treatment with stable activators of PKC, such as phorbol esters, causes a progressive decrease in the activity of this enzyme, that appears to be due to increased breakdown with no compensatory increase of the synthesis [12]. Marked down regulation of PKC occured also in A431 cells treated for 24 hr with 1 uM PDBu (results not shown). Compared to controls, the viability of long term treated A431 cells (indicated by trypan blue exclusion) was unchanged, the resting $[Ca^{2+}]_i$ level was increased only slightly (151 $\pm$10 nM with respect to 112 $\pm$20 nM in controls) and the $[Ca^{2+}]_i$ responses to various agents (ATP, ionomycin) were unchanged (not shown). In contrast, the effects of EGF were drastically modified. A first series of experiments was carried out at a concentration of EGF (16

Table I. Effect of long-term pretreatment with phorbol esters on EGF-induced accumulation of inositol phosphates in A431 cells

Treatment	^{3}H-inositol phosphates (% of basal values)	
	control	long-term pretreated
Basal	100 ±9.5	100 ±2.7
PDBu	109 ±6.8	105 ±6.8
EGF	232 ±8.6	366 ±19.3
PDBu + EGF	96 ±8.4	335 ±33.3

A431 cell monolayers were cultured for 24 hr in a medium supplemented with either solvent (DMSO, 0.25%, controls) or PDBu (1 uM, long-term pretreated), washed with KRH and then treated with solvent or PDBu as above for 10 min, after which EGF (16 nM) or solvent was added together with LiCl (10 mM) for an additional 15 min. Results are the means ±SE of triplicate samples from one single experiment which was repeated three times. Basal radioactivity/mg protein was 1770 and 900 cpm in control and long-term pretreated A431 cells, respectively.

nM) known to induce maximal responses in terms of both PPI hydrolysis and $[Ca^{2+}]_i$ rises [6]. Compared to controls, the cells long-term pretreated with PDBu showed a lower resting level of inositol phosphates and, especially, the disappearance of the total inhibition of the EGF-induced accumulation of inositol phosphates brought about by an acute treatment with phorbol esters (table I). A similar result was obtained when studying $[Ca^{2+}]_i$ (fig. 1). The rise induced by EGF, which in control cells was completely prevented by an acute treatment with either PMA, 100 nM, or PDBu, 1 uM (administered before (trace C) or after (trace B) the growth factor), remained unaffected in the long-term pretreated cells (see fig. 1, traces F and G). Moreover, the down regulation of PKC was found to cause a marked modification of the time course of the $[Ca^{2+}]_i$ rise induced by EGF when administered alone. This modification concerned both the size and the duration of the response. In control cells, $[Ca^{2+}]_i$ levels peaked (323 ±88 nM; n=4) 1-2 min after EGF addition, and then returned to basal within 8-

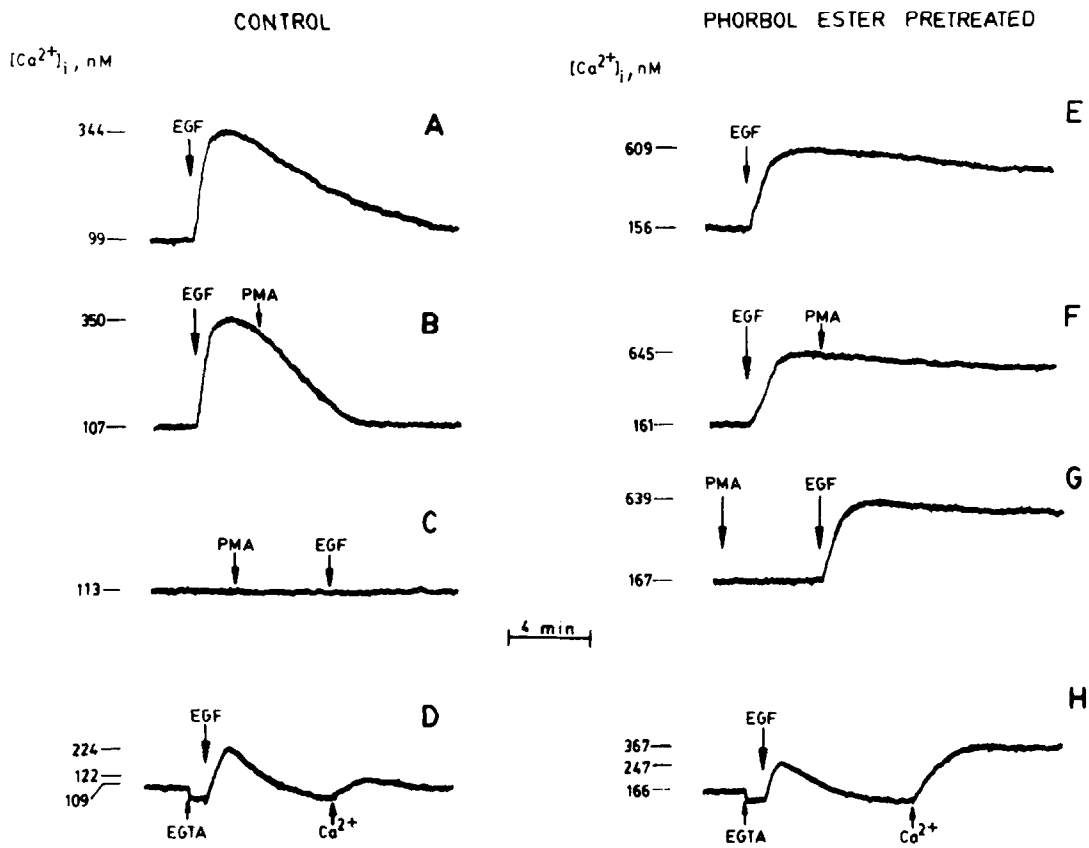

Figure 1. EGF-induced rise of $[Ca^{2+}]_i$ in A431 cells. Comparison between controls and cells long-term pretreated with phorbol ester (PMA, 200 nM, 24 hr) in order to down regulate PKC. Suspensions of A431 cells, loaded with quin2 as described in "Experimental Procedures" were used at a concentration of 500,000 cells/ml. Where indicated EGF was added at the final concentration of 16 nM, PMA of 100 nM. Final free concentrations of EGTA and Ca^{2+} were 1 and 2 mM, respectively.

12 min (average $t_{1/2}$=2.3 min, fig. 1A); while in the cells long-term pretreated with phorbol esters the $[Ca^{2+}]_i$ peak (614 ±16 nM; n=4) was followed by a very slow decline, so that $[Ca^{2+}]_i$ remained elevated for at least 30 min (average $t_{1/2}$=9 min, fig. 1, traces E-G).

The $[Ca^{2+}]_i$ rise induced by EGF in A431 cells is due to redistribution from intracellular stores accompanied by the stimulation of a Ca^{2+} influx across the plasmalemma through a voltage-independent Ca^{2+} channel [5,6,10]. Since channel activation lasts longer than redistribution, these two processes can be dissociated by applying EGF in a Ca^{2+}-free medium and by reintroducing Ca^{2+} into that medium a few min

after EGF addition (i.e. after return of $[Ca^{2+}]_i$ to basal). As shown in fig. 1, traces D and H, the initial $[Ca^{2+}]_i$ rise was not significantly different (both in magnitude and time course), whereas the rise caused by Ca^{2+} reintroduction into the medium was greater and much more persistent in the long-term pretreated than in control cells. These results might suggest that of the two mechanisms mediating $[Ca^{2+}]_i$ increase by EGF, only the stimulation of influx is sensitive to feed-back inhibition. It should be emphasized, however, that the acute treatment with PKC activators caused the disappearance of both Ca^{2+} redistribution and Ca^{2+} influx triggered by EGF, and that, in a variety of cell systems, PKC-dependent inhibition of receptors and channels is known to develop after delays of tens of sec to min. We believe, therefore, that the greater sensitivity to PKC of Ca^{2+} influx with respect to redistribution is only apparent, due to the fact that feed-back desensitization of the receptor builds up slowly, to become appreciable only sometime after the application of the growth factor.

The effect of EGF in A431 cells is complex, and includes growth stimulation at subnanomolar concentrations, and inhibition at higher concentrations such as those employed for the experiments so far described [13-15]. Dose-response experiments were therefore carried out. Appreciable increases of $[Ca^{2+}]_i$ were observed beginning at EGF concentrations as low as 100 pM (certainly a non-inhibitory concentration), and these increases were always greater (2-3 fold, fig. 2) and more persistent (not shown) in the long-term pretreated cells. A431 cells are the favourite model for studying EGF effects because of their very large complement of specific receptors. Increases of $[Ca^{2+}]_i$ induced by EGF have however been observed also in other cell types, such as human skin fibroblasts [16]. Also in these cells, long-term pretreatment with phorbol esters caused a marked increase of the EGF-induced $[Ca^{2+}]_i$ responses (not shown).

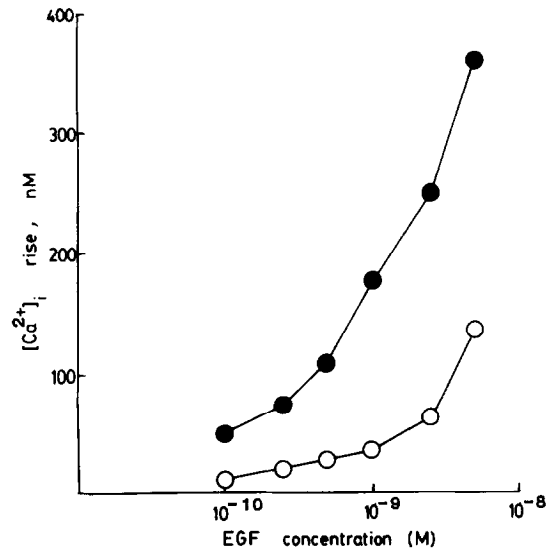

Figure 2. Dose-response of EGF-induced rise of $[Ca^{2+}]_i$ in A431 cells. A431 cells were resuspended as described under "Materials and Methods" and loaded with fura-2. EGF was added either to control (○) or long-term phorbol ester treated (●) cell suspensions at concentrations ranging from 100 pM to 5 nM.

The fact that the increase in size and duration of $[Ca^{2+}]_i$ transients by long-term pretreatment with phorbol esters was demonstrated over a large range of EGF concentrations, and appeared not only in tumoral A431 cells but also in non-tumoral human fibroblasts indicates that the feed back control by PKC represents a basic regulatory mechanism of the EGF-induced transmembrane signalling. In many cell types, full expresion of the mitogenic potential of EGF is known to require the coadministration of additional mitogens [17]. In contrast, other growth factors, such as bombesin and platelet-derived growth factor (PDGF), induce cell proliferation even when administered alone. In this respect it might be important that transmembrane signalling at PDGF and bombesin receptors is affected in part or not at all, respectively, by PKC activation [18]. The latter receptors might therefore operate under a regulation less stringent than that here described for EGF.

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