

Continuous presence of phorbol ester is required for its IL-1 β mRNA stabilizing effect

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The protein kinase C (PKC) activating phorbol esters are known to prevent the decay of mRNA of several cytokines and proto-oncogenes. To examine whether the phorbol ester signal is continuously required for this stabilizing effect, THP-1 monocytic cells were stimulated either with phorbol 12,13-dibutyrate (PDBu), which can be removed from the cells by washings, or with the more hydrophobic phorbol 12-myristate 13-acetate (PMA). Both of these stimuli induced high levels of interleukin-1 β (IL-1 β) mRNA. When the cells were washed at the peak of the IL-1 β mRNA expression, this mRNA decayed rapidly in the PDBu stimulated cells while in PMA stimulated cells the mRNA levels were not affected. Moreover, this mRNA degradation induced by the removal of PDBu could be inhibited by readdition of the phorbol ester. This restabilization could be prevented by pharmacologic inhibitors of PKC, but not by inhibiting protein or RNA synthesis. Thus these data suggest that the phorbol ester must be continuously present to exert its mRNA stabilizing effect and that its effect is PKC-mediated but does not require active protein or RNA synthesis.

Phorbol ester; Interleukin-1 β ; Protein kinase C; mRNA degradation

1. INTRODUCTION

The regulation of mRNA turnover plays a major role in the control of gene expression. Many cytokine and proto-oncogene mRNAs are unstable due to *cis*-acting sequences. They contain a single or multiple copies of an adenosine-uridine (AU)-rich sequence (AUUUA) in their 3' untranslated regions [1–3] and this sequence, originally characterized in granulocyte-macrophage colony stimulating factor gene, has been shown to confer instability upon β -globin mRNA if it is fused together with the β -globin gene [1]. Recent reports indicate that protein kinase C (PKC), a calcium and phospholipid-dependent enzyme activated by diacylglycerol (DAG) [4], is involved in controlling of the stability of TNF- α [5] and IL-2 [6] mRNA. The target sequence of the PKC action is, at least in some cases, the AUUUA sequence: it has been shown that phorbol esters activate a cytoplasmic protein which complexes to a variety of labile mRNAs containing this sequence [7].

Now we have used the phorbol ester induced expression of interleukin-1 β mRNA (containing multiple copies of the AUUA sequence [8]) as a model to study the

role of the phorbol ester mediated activation of PKC in mRNA stabilization. Specifically, we wanted to examine whether the phorbol ester must be continuously present to exert its mRNA stabilizing effect and whether this effect requires active protein synthesis.

2. MATERIALS AND METHODS

2.1. Cell cultures

The THP-1 cell line was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI 1640 medium (Flow Labs., Irvine, Scotland, UK) containing 10 mM HEPES, 2 mM L-glutamine, 70 mM 2-mercapto-ethanol, antibiotics and 10% fetal calf serum (Flow Labs.). The cells were tested monthly for mycoplasma and were found to be negative. During the exponential growth phase, cells at the density of 0.5×10^6 /ml were stimulated in 75 cm² tissue culture flasks (Costar, Cambridge, MA) with the indicated concentrations of phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate (PDBu), 1,2-dioctanoyl-*sn*-glycerol C8:0 (DOG), cycloheximide (CHX) and actinomycin D (AD) all from Sigma Chemical Co. (St. Louis, MO) and calcium ionophore A23187 (Calbiochem, La Jolla, CA). Protein kinase inhibitors H-7 (1-(5-isoquinolone-sulfonyl)-2-methylpiperazine dihydrochloride) and HA1004 (*N*-(2-guanidinoethylsulfonamide hydrochloride) were purchased from Seikagaku Kogyo Co. (Tokyo, Japan). After the indicated times, the cultures were harvested and the washings were done with PBS (phosphate buffered saline; pH 7.4) and the cells were used for mRNA analysis.

2.2. RNA isolations and analysis

At the times indicated, cells were harvested and poly(A)⁺ RNA was extracted from 10^8 cells. The poly(A)⁺ RNA was bound to oligo(dT) cellulose (Boehringer-Mannheim GmbH, Mannheim, Germany) directly from cell lysates and eluted as described [9]. The isolated RNA was quantitated spectrophotometrically and samples containing the same amount of RNA were size-fractionated on 1.2% formaldehyde-agarose gels and transferred to a nylon membrane (Pall, Glen Cove,

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Abbreviations: AD, actinomycin D; CHX, cycloheximide; DAG, diacylglycerol; DOG, 1,2-dioctanoyl-*sn*-glycerol; IL-1 β , interleukin-1 β ; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

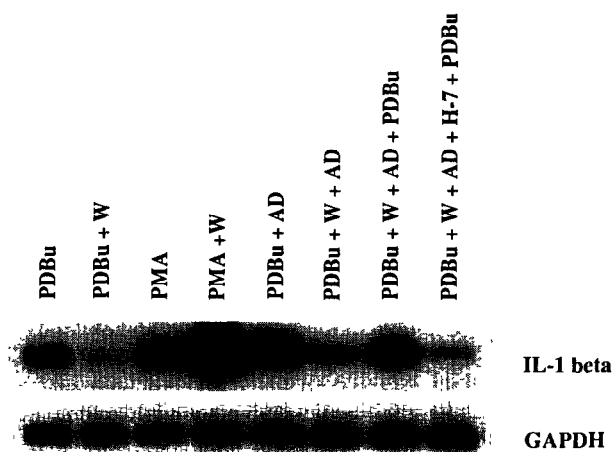


Fig. 1. The effect of washing on PDBu or PMA induced IL-1 β mRNA expression. THP-1 cells were stimulated with either 100 ng/ml PDBu or 10 ng/ml PMA for 19 h. Then the cells in the groups marked with 'W' were extensively washed with PBS and the cells resuspended to a fresh medium. Some cultures received AD (5 μ g/ml). The last two groups were restimulated with 100 ng/ml PDBu with or without the PKC-inhibitor H-7. After 5 h of incubation the cells were harvested and the mRNA was isolated. mRNA samples (5 μ g) were fractionated on agarose gel, transferred to a nylon membrane, and then hybridized to 32 P-labeled IL-1 β and GAPDH cDNA probes.

NY). The IL-1 β cDNA probe (HU-IL-1 β , pcDSR α) used was kindly provided by Dr. Kari Varkila (DNAX Research Institute, Palo Alto, CA). The RNA levels in the nylon membranes were also compared with the signal from a probe to a constantly expressed mRNA, glyceraldehyde phosphate dehydrogenase (pRGAPDH-13). The probes were labeled with [α - 32 P]dCTP (3000 Ci/mol, Amersham International, Amersham, UK) by a random-primed DNA labelling kit (Boehringer-Mannheim GmbH). Prehybridizations and hybridizations were performed in a solution containing 50% formamide, 5 \times Denhardt's solution, 5 \times SSPE and 0.5% SDS. Filters were washed in 1 \times SSC plus 0.1% SDS 2 times for 30 min at room temperature and at 60°C for 30 min. Then the filters were exposed to Kodak AR X-Omat films at -70°C with intensifying screens. Radioactive signals were quantitated from the autoradiograms with a densitometric scanner (Helena Laboratories, Beaumont, TX).

3. RESULTS

To analyze whether the phorbol ester mediated signal is required continuously to prevent the decay of the preformed mRNA we used the relatively water-soluble PDBu, which can be removed from the cells by washing [10–12]. Both PDBu and PMA (the more hydrophobic form) induced a strong IL-1 β mRNA expression in the THP-1 cells (Fig. 1). With both of these agents maximal IL-1 β mRNA levels could be detected ca 24 h after the stimulation (data not shown). When the stimulated cells were extensively washed 5 h prior to the harvesting, IL-1 β mRNA levels strongly declined in the PDBu stimulated cultures but in contrast to this, the PMA induced IL-1 β mRNA levels were not reduced by this treatment (Fig. 1). In fact, the PMA induced IL-1 β mRNA levels were somewhat higher after the washing. Blocking of the RNA synthesis by AD at the same time did not

decrease the PDBu induced IL-1 β mRNA levels, thus suggesting that the observed decline was not due to cessation of the transcription but rather due to accelerated decay of the preformed mRNA. The possible transcriptional effect on the decline of the IL-1 β mRNA levels was excluded by demonstrating that re-added PDBu in the presence of the RNA synthesis inhibitor AD totally abolished the washing effect (Fig. 1).

The half-life of the IL-1 β mRNA after removal of PDBu (20 h after the stimulation) was found to be clearly less than 2 h (Fig. 2). This is in marked contrast to the reported 12 h half-life of the PMA induced IL-1 β mRNA in these same cells [13].

To further characterize the stabilizing signal, the cells stimulated for 20 h with PDBu were washed and then

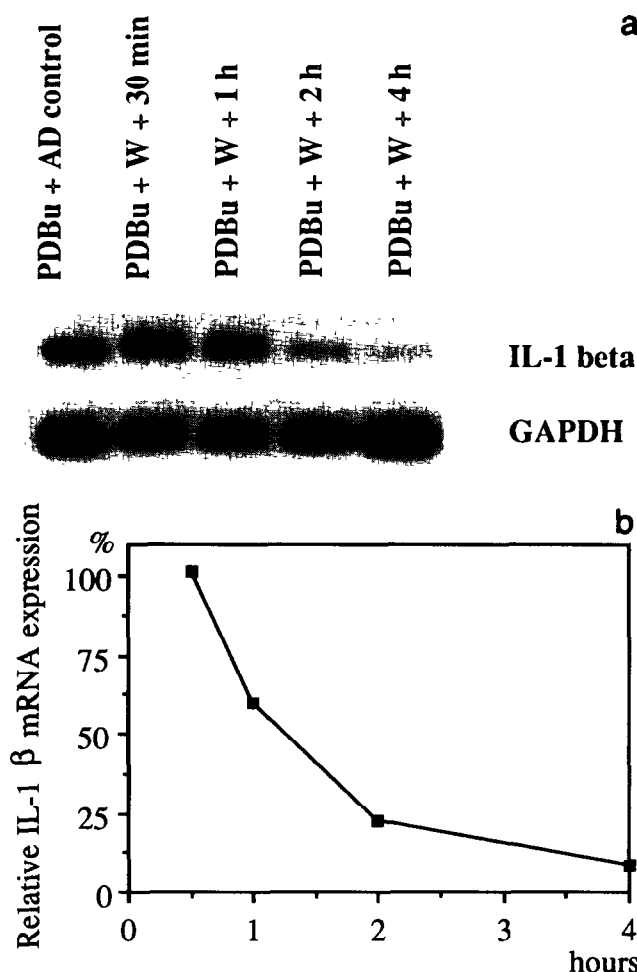


Fig. 2. The degradation rate of the PDBu induced IL-1 β mRNA after washing. THP-1 cells were stimulated with 100 ng/ml PDBu for 20 h, then washed and AD (5 μ g/ml) was added. The control group was harvested immediately and the other groups at the time points indicated. The mRNA samples (5 μ g) were isolated and analyzed as described in the legend of Fig. 1 (panel A). Densitometric scanning of the data are shown in panel B. The levels of IL-1 β mRNA in these groups were standardized according to the expression of the constant gene (GAPDH).

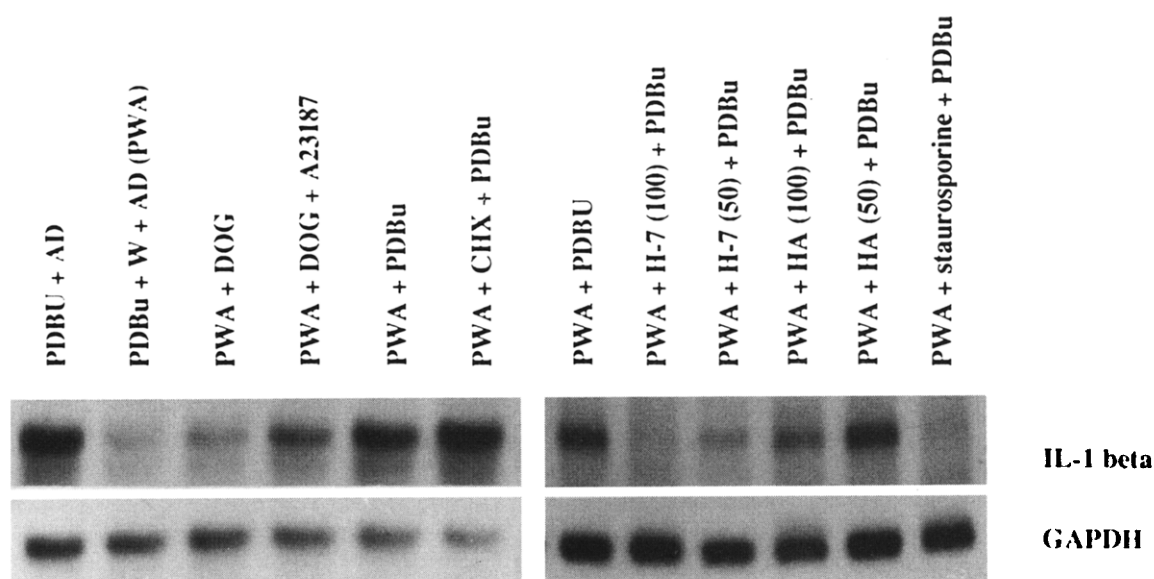


Fig. 3. Characterization of the IL-1 β mRNA restabilizing signal. THP-1 cells were stimulated with 100 ng/ml PDBu for 20 h, then washed and AD (5 μ g/ml) was added (PWA-cells). The cells were then incubated either with 100 nM staurosporine, 50 μ M or 100 μ M H-7 or HA1004 or 10 μ g/ml CHX for half-an-hour before addition of 100 ng/ml PDBu. Some PWA groups were stimulated with 100 μ M DOG in the presence or absence of 500 nM A23187 calcium ionophore. The cells were incubated for 4 h and the mRNA samples were isolated and analyzed as in Fig. 1.

immediately restimulated in the presence of AD. IL-1 β mRNA levels were quantitated 5 h later. As already shown in Fig. 1, re-added PDBu clearly restabilized the IL-1 β mRNA (Fig. 3). Also the structural DAG analog, dioctanoylglycerol (DOG), which in the presence of calcium ionophore causes a short-lived PKC activation [14], was sufficient for restabilization. Moreover, restabilization did not require protein synthesis as CHX could not prevent the restabilizing effect of PDBu. As expected, the PDBu-mediated effect was dependent on PKC activity: PKC inhibitors H7 and staurosporine, but not the preferential protein kinase A inhibitor HA 1004, effectively prevented the restabilizing effect of PDBu.

4. DISCUSSION

The data shown in this report demonstrate that in the absence of the phorbol ester the preformed IL-1 β mRNA is rapidly degraded and that the mRNA stabilizing effect of these agents does not require active RNA or protein synthesis, but is dependent on the PKC activity.

In different cell types stimulation with phorbol esters leads to initial activation and membrane localization of PKC, which lasts a few hours and after this time the enzymatic activity of PKC declines, probably due to proteolytic degradation of this enzyme [4]. In myeloid cell lines, e.g. in the THP-1 cells used in these experiments, this activation induces the cells to differentiate to more mature, macrophage-like cells and to express various cytokine genes, such as IL-1 β (reviewed in [15]).

It is probable that these changes are due to activation of the AP-1 transcription factor complex [16]. Our findings showing that the phorbol ester must be continuously present to exert its mRNA stabilizing effect suggest that this initial PKC activation and the subsequent AP-1 induction are not involved in the stabilization mechanism. This interpretation is further supported by the finding that induction with DOG plus ionophore was sufficient to restabilize the IL-1 β mRNA after PDBu removal; this stimulus causes a very short-lived PKC activation, which activates the AP-1 enhancer activity only very weakly and does not induce differentiation [16]. Thus it can be concluded that the mRNA stabilizing PKC activity differs from the AP-1 inducing PKC activity quantitatively, but it is also possible that it is qualitatively different (e.g. representing the activity of a different PKC isozyme).

The mRNA stabilizing mechanism of phorbol esters described here is fully compatible with the reported mechanism of action of the AU-binding factor (AUBF). It is a cytoplasmic protein which can be activated by phorbol esters and binds to several mRNAs containing this motive [7]. AUBF is posttranslationally activated by phosphorylation and de novo protein synthesis is not required [17] thus being in line with the requirements for the phorbol ester mediated stabilizing effect reported here. In addition to this, when the effect of PDBu removal was tested on mRNAs which can be induced by phorbol esters in the THP-1 cell line but which do not contain the AU-rich sequence (e.g. CD11b), no decline in the mRNA levels was observed (data not shown). Thus the involvement of the AU-rich sequence of the

IL-1 β mRNA in the phorbol ester mediated stabilization seems very likely.

The data reported here have also some practical implications. The mRNA half-life measurements done in PMA stimulated cells after AD blocking are strongly influenced by the stabilizing effect of the PMA remaining within the cells. This effect could mask or modify the influence of other mRNA decay mechanisms. In fact, we have recently reported that the degradative effect of glucocorticoids on IL-1 β mRNA [18] seems to be weaker in PMA stimulated human monocytes than in bacterial lipopolysaccharide stimulated cells [19].

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