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THE EFFECTS OF PHORBOL ESTER AND Ca IONOPHORE ON C-FOS AND C-MYC EXPRESSION AND ON DNA SYNTHESIS IN HUMAN LYMPHOCYTES ARE NOT DIRECTLY RELATED

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SUMMARY. Phorbol ester activates human lymphocyte proliferation as measured by [^{3}H]thymidine incorporation and blast transformation. Phorbol ester and calcium ionophore A 23187 induce c-fos and c-myc expression in these cells as shown by the measure of the specific mRNA levels and of the c-fos nuclear protein amount. The combination of these two agents has a synergistic effect on c-fos but not on c-myc expression. Lymphocyte stimulation by phorbol ester for a short time induces c-fos and c-myc expression, but has no effect on [^{3}H]thymidine incorporation. This result indicates that the induction of c-fos and c-myc expression is not sufficient to commit these cells to DNA synthesis.

A variety of proto-oncogenes has recently been shown to be intimately associated with key functions in the control of cell growth and proliferation (1). Indeed, some of them exhibit homology or identity with growth factors or growth factor receptors (2-4). Stimulation of fibroblasts with mitogenic agents leads to a dramatic induction of the proto-oncogenes fos and myc (5-8). Furthermore we have recently shown that the induction of quiescent hepatocytes to proliferate by a partial hepatectomy is followed by a strong accumulation of c-fos, c-myc and N-myc transcripts a long time before the onset of DNA synthesis (9-10).

The induction of c-myc has also been associated with lymphocyte activation by stimulation of the T-cell-receptor complex (11). However, it is possible to bypass this receptor-ligand stage using phorbol esters such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (12). The effect of TPA on mouse lymphocytes can be potentiated by calcium ionophores (13). It is thought that TPA stimulates protein kinase C and that ionophore A 23187 mimics the action of inositol triphosphate by generating calcium flux (14,15). The suggestion that c-fos and c-myc may be the first genomic

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targets of these membrane signals prompted us to investigate the effects of TPA and A 23187 at the level of gene expression in human lymphocytes and to determine a possible functional link between c-fos and c-myc expression and the capacity of these cells to enter into the cell cycle.

MATERIALS AND METHODS

Cell preparation and incubation. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized (Calciparine Choay Laboratories) venous blood of healthy adult donors by Ficoll-Hypaque density gradient centrifugation (d = 1.077), according to Boyum and to Thorsby and Bratlie (16,17).

Cells were washed in RPMI 1640 medium (Flow Laboratories) and spun at 150 g to remove the platelets. Final cell preparations were adjusted to 5.10^6 cells/ml. The composition of PBMC populations was 98% mononuclear cells, 85% of which were lymphocytes and 15% were monocytes as observed by staining with specific monoclonal antibody (OKT_3-M_5 orthoclone).

Short-term cultures were performed in RPMI 1640 medium without serum in humidified atmosphere of CO_2/air at 37°C. After 1 h of preincubation, TPA at a concentration of 10 ng/ml and/or 10^{-7} M of calcium ionophore A 23187 were added and the cells were incubated for further 30 min to 24 h.

Long term cultures were performed in RPMI 1640 medium including 10% (v/v) heat inactivated human AB serum, 100 IU/ml of specillin G, 100 µg/ml of streptomycin (Specia Laboratories) and 200 mM glutamine. DNA synthesis was assessed by incubation in the presence of 1 Ci/ml of [^{3}H]thymidine/10^6 cells (S. A. 28 Ci/mmole, CEA, France) during the last 18 h of a 24, 48 and 72 h culture with TPA, A23187, PHA (5 µg/ml) or the combination of TPA and A 23187. Cultures were then stopped with a multiple automatic sample harvester (Titertek Flow Laboratories) and ^{3}H incorporation was measured by liquid scintillation spectrometry (Intertechnique S.L.32).

RNA blot analysis. Total cellular RNAs were isolated from PBMC by the guanidium-isothiocyanate method (18), quantified by absorbance at 260 nm. Samples of 10 µg were denatured at 65°C in electrophoresis buffer : 20 mM morpholinepropane-sulfonic acid, pH 7 (MOPS), 5 mM sodium acetate, 1 mM EDTA, 2.2 mM formaldehyde containing 50% formamide. RNAs were then submitted to electrophoresis in 1% agarose gel containing 2.2 M formaldehyde. Before transferring RNA to Gene Screen Plus filters (New England Nuclear) the gels were stained with 0.5 µg/ml of ethidium bromide in 0.3 M ammonium acetate to determine the position of 28 S and 18 S RNA bands and to assess the integrity of all the RNA samples. Filters, after transfer in 20 X SSC, were dried and baked at 80°C **in vacuo** for 2 h. The prehybridizations were performed for 16 h at 42°C in : 50% formamide, 0.75 M NaCl, 50 µg/ml heparin, 1% SDS, 50 µg/ml herring sperm DNA, 10 µg/ml Poly A and 5% of dextran sulfate. The hybridizations were performed for 48 h at 42°C in the same solution containing 5.10^7 cpm of heat denatured DNA from the human c-fos gene (19) or the human third c-myc exon (20) labelled to high specific activity ($5\text{-}8.10^8$ cpm/µg) with [α-^{32}P]dCTP by nick translation. As a control, after dehybridization of the filters in 0.1% SDS at 68°C for 1 h, they were hybridized with the Ha-ras viral probe.

After washing twice in 0.2 X SSC and 0.2 % SDS at 55°C for 90 min filters were exposed at -70°C for 3 days to Kodak XAR films with intensifying screens. Autoradiograms were analysed by densitometry.

Immunofluorescence assays. PBMC cells were adjusted at 10^6 cells/ml in RPMI 1640 medium without serum, then treated with TPA (10 ng/ml) and/or 10^{-7} M A 23187 for 1 to 24 h. After incubation at 37°C, the cells were

cytospun at 1300 rpm for 10 min, dried by air, fixed in 3% paraformaldehyde and permeabilized by 10% Triton X100. Tumor bearing rat serum (TBRS) [24] or normal rat serum (NRS) were used at 1/50 dilution and rhodamine-conjugated rabbit anti rat IgG (Cappel Laboratories) at 1/100. The cells were then examined through fluorescence LEITZ microscope and photographied using a G x 630 lenz (6.63).

RESULTS

TPA and Ca ionophore A 23187 induce elevation in c-fos and c-myc mRNA levels. Fig.1A shows Northern blots of total cellular RNA extracted from PBMC cultured for 30 min to 5 h with TPA, A 23187 or the combination of the two agents and from control unstimulated cells. Little or no c-fos mRNA was present in quiescent lymphocytes (line 1). Treatment with TPA or A 23187 led to an increase of the 2.2 kb c-fos transcript. The peak of c-fos mRNA was observed 1 h after TPA treatment and only 2 h after A 23187 addition. The transcript level increased a further 4-6 fold when both agents were used together. Elevation of c-fos mRNA was detected at 30 min and the

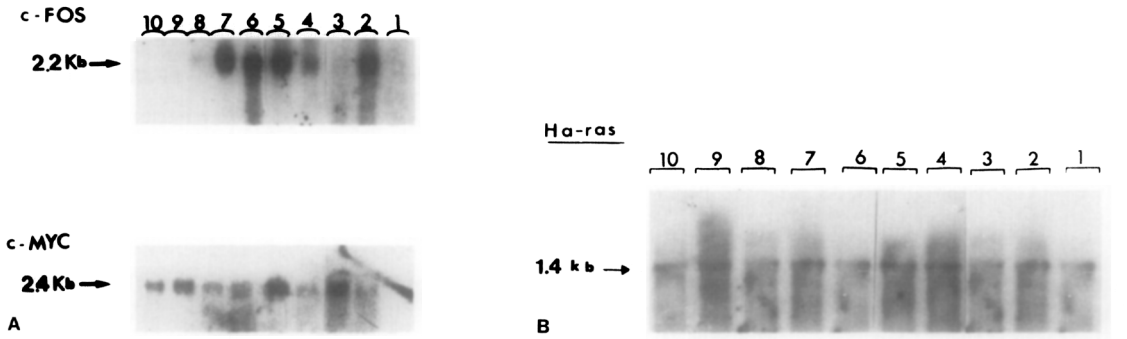

Figure 1A. c-fos and c-myc mRNA levels in mononuclear cells after treatment with PMA and A 23187.
1. Human peripheral blood mononuclear cells (PBMC) were treated either with TPA at 10^{-2} µg/ml for 1 h and 2 h (lanes 2,3) or with A 23187 at 10^{-7} M for the same times (lanes 4,5) or with TPA + ionophore at the same concentrations for 30 min, 1 h, 2 h, 3 h and 5 h (lanes 6-10). Lane 1 represents control cells incubated for 2 h at 37°C in the same media without any of the agents.
Total RNA were extracted using guanidium thiocyanate (21). The samples were equalized for rRNA and 10 µg of each sample were separated on 1% agarose/formaldehyde gel before transfer to Gene Screen Plus membrane. The equalization was confirmed by ethidium bromide staining. The filter was hybridized sequentially with a ^{32}P-labelled 3.5 kb Xho I-Nco I fragment of the human genomic fos plasmid and the Eco RI-ClaI 1.4 Kb fragment of the third human c-myc exon.
After washes in 0.2 x SSC for 1 h at 55°C autoradiograms were exposed for 2-6 days.

Figure 1B. Analysis of Ha-ras mRNA level in activated lymphocytes.
Northern blots which have been hybridized with c-fos and c-myc probes, were dehybridized at 68°C in 0.1% SDS, before their hybridizations with [^{32}P] nick-translated viral Ha-ras, Sac I-Pst I fragment of the BRI clone, at 42°C in a 50% formamide solution. After washing, XAR-5 films were exposed at -80°C with the filters. A two days exposition is shown, the position of the 1.4 Kb Ha-ras mRNA is indicated.

Table I. Effect of TPA and/or A 23187 treatment on the percentage of positive cells after c-fos protein staining by immunofluorescence.

	CONTROL	TPA	A 23187	TPA + A 23187
1 h	nd	5	2	16
2 h	2	15	20	80
5 h	nd	nd	nd	2

nd : not detectable

highest level was observed at 1 h ; thereafter, c-fos mRNA returned to the basal level of unstimulated cells. The time course of c-fos appearance was similar to that previously observed in serum stimulated fibroblasts (8).

TPA or ionophore induced c-myc expression by approximately 10-fold, the same level of the corresponding transcript was obtained when the two agents were used in concert (fig.1A, lines 6-10).

The c-myc mRNA level remained high a longer time than c-fos mRNA since the 3-hour RNA sample (line 9) was found positive. In this experiment, the induction of c-myc mRNA in human lymphocytes stimulated by TPA and Ca^{++} ionophore was comparable in duration to that previously obtained with lectins (11). Similar results were obtained using peripheral blood mononuclear cells from 7 different individuals.

The increase of c-fos and c-myc mRNA levels during lymphocyte activation does not reflect a general increase in proto-oncogene transcription, indeed the level of the 1.4 kb Ha-ras mRNA was unchanged in PBMC exposed to TPA and/or ionophore (fig.1B).

TPA and ionophore A 23187 induce elevation of c-fos protein amount. To confirm and extend these results, we investigated c-fos induction at the protein level. Lymphocytes were examined by immunocytochemistry using antibodies specific for the c-fos product (TBRS) (21). Only a small percentage of quiescent lymphocytes expressed the c-fos protein. The remaining cells exhibited little or no nuclear fluorescence (Table 1). Some non-specific cytoplasmic staining was occasionally observed (fig.2). Lymphocytes treated with either TPA or ionophore for 1 h exhibited a weak nuclear fluorescence. Treatment of lymphocytes with both ionophore and TPA resulted in a larger number of cells which displayed a more intense nuclear fluorescence. Maximum fluorescence was obtained 2 h after the treatment and was significantly decreased after a total of 5 h. Cells stimulated for 24 h

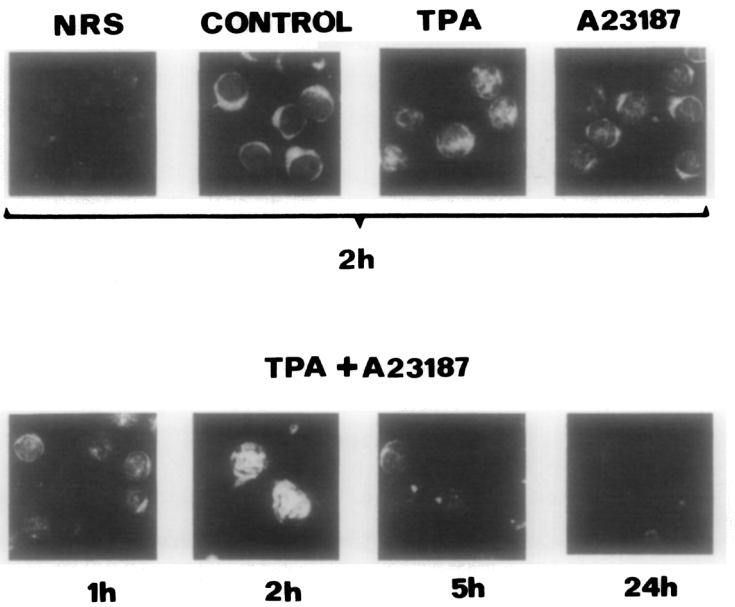

Figure 2. Detection of c-fos protein on human lymphocyte by immuno-fluorescence staining.
PBMC were adjusted at 10^6/ml in RPMI 1640 without serum then treated by TPA (10^{-2} µg/ml) and/or A 23187 (10^{-7}M) for 0 to 24 h. After incubation, the cells were cytospun at 1.300 rpm for 10 min dryed by air, fixed in 3% paraformaldehyde and permeabilized by 10% triton X100. Tumor bearing rat serum (TBRS) (21), or normal rat serum (NRS) were used at 1/50 dilution and rhodamine conjugated rabbit anti-rat IgG (Cappel Laboratories) at 1/100. Cells examined through fluorescence Leitz microscope were photographied using a X63 lenz (G X 630).

were indistinguishable from control cells. As a control, cells treated with both compounds for 2 h were stained with normal rat serum (NRS) (fig.2). Not only fluorescence was enhanced in these cells but, in addition, the percentage of positive cells was increased. TPA or ionophore alone induced the c-fos gene in up to a maximum of 20% of the cell population. However the combination of these two agents induced c-fos protein in 80% of the population (Table I). Interestingly, the pattern of nuclear fluorescence corresponding to c-fos protein in activated lymphocytes was distinct from that previously observed in serum stimulated fibroblasts (8). Fluorescence was not distributed homogeneously throughout the nucleus. Instead, nuclei exhibited a rather patchwork appearance. This pattern has a remarkable similarity to that of dispersed chromatin in lymphocytes 1 h after activation by PHA (22).

Relationship between proto-oncogene expression and progression of lymphocytes into the cell cycle. In order to determine whether the early and transient induction of c-fos and c-myc mRNAs might be directly related

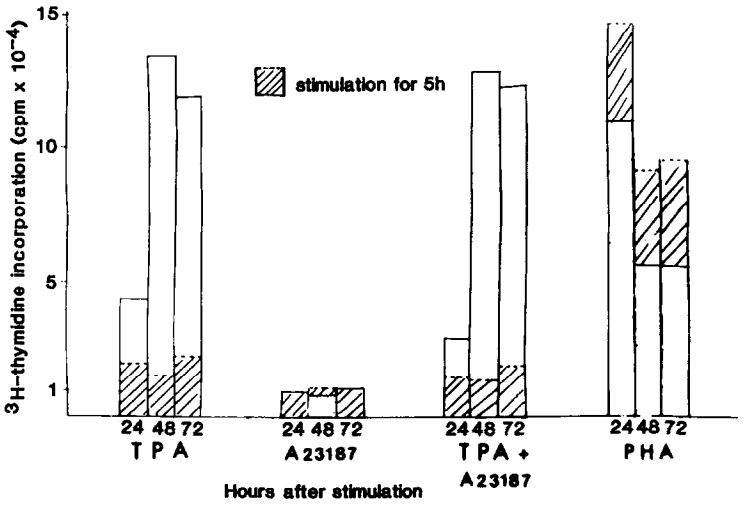

Figure 3. Incorporation of [^{3}H]thymidine by PBMC cells in the presence of mitogenic agents.
10^4 cells were dispersed into 96 well microtitre plates. TPA, A 23187, the combination of the two agents, or PHA were added to give the final concentration, as described in the Materials and Methods section. The cells were incubated for 24, 48 or 72 h at 37°C ; for the last 18 h, all the cells were pulsed with [^{3}H]thymidine, the cells were then collected for counting.
PBMC cells were stimulated for only 5 h with the agents, washed and incubated in a fresh medium for 24, 48 and 72 h, and finally pulsed with labelled thymidine for the last 18 h.

to the capacity of quiescent lymphocytes to proliferate after activation by TPA and Ca^{++} ionophore A 23187, we analysed DNA synthesis, by measuring [^{3}H]thymidine incorporation. Cells were incubated for 24, 48 and 72 h in RPMI medium containing TPA, A 23187, both agents in concert or PHA. In a second type of experiments lymphocytes were incubated with the stimulator agents for 5 h, then washed and incubated further in RPMI medium for the same lengths of time. All the incubations were pulsed with [^{3}H]thymidine for the last 18 h of culture (fig.3).

In contrast to the results obtained with mouse lymphocytes and thymocytes (16,27), TPA alone induced in human lymphocytes cell proliferation as measured by [^{3}H]thymidine incorporation. Indeed a peak of DNA synthesis was detected 48 h after the addition of this agent in lymphocyte cultures. The PHA treatment also allows DNA synthesis, with a high incorporation of labelled thymidine at 24 h, as we have previously described (22). Calcium ionophore A 23187 alone has no effect, but associated to TPA it did not prevent its effect on [^{3}H]thymidine incorporation. Blast transformation was also observed in all cultures when cells replicated their DNA (data not shown). Furthermore figure 3 shows that short time incubation with TPA, or with the combination of both TPA and ionophore are not sufficient to trigger [^{3}H]thymidine incorporation,

whereas PHA is effective at causing lymphocyte DNA synthesis when administrated for only 5 h.

DISCUSSION

In this study we have demonstrated that in human lymphocytes the requirement for antigene- and lectin induced signal for cell activation can be bypassed efficiently by the synergic effects of calcium ionophore and phorbol ester, as previously shown in mouse lymphocytes and thymocytes (13,23). However in contrast with these cells human lymphocytes were efficiently activated by TPA alone which stimulates protein kinase C (24). Lymphocyte activation by TPA and Ca ionophore involves an early transient and strong induction of c-fos and c-myc genes. However this induction is not sufficient to commit the cells to DNA synthesis : (i) treatment of the cells with calcium ionophore only strongly increased the level of c-fos and c-myc mRNAs without any effect on lymphocyte proliferation (ii) treatment of the cells with TPA for 5 h induced c-fos and c-myc genes, but did not allow DNA synthesis. The lymphocytes have to be incubated with these agents for at least 24 h to allow [^{3}H]thymidine incorporation and blast cell transformation. We found a synergistic effect of TPA and calcium ionophore on c-fos expression as measured at the mRNA and at the protein levels, but as opposed to what was observed in mouse lymphocytes, we did not find this synergistic effect on c-myc expression. This suggests that in human lymphotyces the control of c-fos and c-myc expression could occur by different pathways.

Immunofluorescence analysis suggested that in lymphocytes the protein product of c-fos is excluded from condensed chromatin. We hope to confirm this observation by immuno-electron microscopy.

The data presented in this paper suggest that in human lymphocytes the induction of c-fos and c-myc gene is mediated by early membrane modifications involved in cell activation, including protein kinase C activation and calcium flux. However the strong accumulation of c-fos and c-myc products could be necessary but is not sufficient to cause commitment to DNA synthesis. The progression of the cells into the S phase must also depend on and be modulated by other cell responses.

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