Ca^{2+} and phorbol ester synergistically induce HL-60 differentiation

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Exposure of HL-60 cells to subthreshold concentrations of TPA caused monocytic differentiation only when cells were cotreated with the Ca^{2+} ionophore A23187. Phorbol ester dose-response curves for growth arrest and enzymatic markers of differentiation were shifted to lower concentrations when the ionophore was present. Expression of a monocyte/granulocyte cell surface antigen also occurred only when cells were treated with both agents. Similar effects were seen with other active but not inactive phorbol esters and with another Ca^{2+} ionophore. The Ca^{2+} component of phosphoinositide-based signalling may thus play a role in HL-60 differentiation.

Synergism *Phorbol ester* *Ca^{2+} ionophore* *Differentiation* *(HL-60 cell)*

1. INTRODUCTION

Signal transduction mediated by inositol phospholipid turnover can be mimicked by phorbol esters and Ca^{2+} ionophores which activate protein kinase C and elevate intracellular Ca^{2+}, respectively [1]. Synergism between these agents in secretory events [1] and in some long-term signalling events such as lymphocyte activation [2] indicates that the affected pathways often act in concert to elicit a full biological response. Although phorbol esters influence terminal differentiation in many cell types [3], synergism with Ca^{2+} ionophores has not been observed. As a result, the role of phosphoinositide turnover in such processes is poorly understood.

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Abbreviations: DMSO, dimethyl sulfoxide; OAG, 1-oleoyl-2-acetylglycerol; PDBu, phorbol 12,13-dibutyrate; PDD, phorbol 12,13-didecanoate; 4α-PDD, 4α-phorbol 12,13-didecanoate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate

The HL-60 human promyelocytic leukemia line [4] terminally differentiates into macrophage-like cells when treated with phorbol esters [5] and other activators of protein kinase C [6,7]. The ability of phorbol esters to induce HL-60 differentiation parallels their biological activity in other systems and activation of protein kinase C in vitro [8]. Inhibitors of the kinase block differentiation [9], implying that its activation is necessary for commitment to a monocytic lineage. However, OAG and bryostatin activate the kinase in situ but do not cause HL-60 cell maturation [10,11], suggesting phorbol esters have other pleiotropic effects which initiate the differentiation program. Since the precise role of protein kinase C in HL-60 differentiation is unclear and since synergism has not been extended to the differentiation of well characterized cell lines, it was of interest to determine the role of Ca^{2+} signalling in HL-60 cell maturation.

2. MATERIALS AND METHODS

HL-60 cells (American Type Culture Collection)

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were grown in RPMI 1640 medium supplemented with 20% fetal bovine serum, 2 mM glutamine, and antibiotic/antimycotic from Gibco. Medium was buffered with 5 mM Hepes and 25 mM sodium bicarbonate in a 5% CO_2 atmosphere so that an equilibrium pH of 7.6 was obtained. Drugs obtained from commercial suppliers were added to cells (2×10^5/ml) from stocks (phorbol esters in ethanol, Ca^{2+} ionophores in DMSO) such that final solvent concentrations were always 0.3%. Solvent alone was added to 0.3% when it was not required as a carrier. Cells used for staining or preparation of enzyme extracts were harvested by gentle scraping and counted with a model ZF Coulter counter. Acid phosphatase activity was determined by the method of Schnyder and Baggiolini [12] while secreted lysozyme activity was measured according to Huberman et al. [7]. Growth inhibition was calculated as 100 − % relative growth (corrected for initial plating density) of treated cells vs control solvent-treated cells. Cell adherence and clumping was assessed qualitatively [range: no adherent cells (−) to essentially all cells adherent and extensive clumping (+++)]. Immunofluorescence was carried out with OKM-1 monoclonal antibody [13] from Ortho Diagnostics Systems and fluorescein-conjugated goat anti-mouse IgG as described by Sauder et al. [14]. Cell viability was determined simultaneously by ethidium bromide fluorescence of nuclei. Positive viable cells were scored as percent of total viable cells (n = 200) by a naive observer for each condition. Immunofluorescence was also quantified with an Ortho Diagnostic Systems Spectrum III flow cytometer. In this case cell fluorescence was corrected for non-specific background staining by subtracting fluorescence from cells treated identically except for the absence of primary antibody. Similar results were obtained with each method of assessment.

3. RESULTS AND DISCUSSION

When HL-60 cells were exposed to 0.5 nM TPA, 300 nM A23187 or carrier solvents alone they were indistinguishable from untreated cells except for degranulation induced by the Ca^{2+} ionophore (fig.1a–d). However, cotreatment with TPA and A23187 caused dramatic changes in morphology as cells aggregated and adhered to substratum (fig.1e). Morphological changes apparent in stained cells included a decrease in nuclear to cytoplasmic ratio and loss of azurophilic granulation (fig.1a–e). These responses are typical of cells exposed to high concentrations of TPA (fig.1f and [5]).

Morphological differentiation in response to TPA and A23187 was accompanied by growth inhibition and induction of the macrophage-specific enzymes acid phosphatase and lysozyme [5,7]. This was manifested as a shift in dose-response curves to lower concentrations of TPA (fig.2a,c,e). At high TPA concentrations differentiation was not altered by cotreatment of cells with A23187 but in such cases a shorter duration of TPA exposure was required for commitment to differentiation (not shown). Ionophore by itself had no effect except for cytotoxic growth inhibition and elevation of lysozyme secretion at high concentrations (fig.2d,f). In this case however, cell morphology was not altered and acid phosphatase activity dropped as cell viability decreased (fig.2b).

Observations with TPA and A23187 were verified with other phorbol esters and another Ca^{2+} ionophore, ionomycin. Differentiation in response to PDBu and PDD, as well as TPA, was strongly enhanced by either 300 nM A23187 or 300 nM ionomycin (fig.3a–c). 300 nM A23187 alone depressed cell growth but synergism with phorbol esters was nonetheless evident in enzyme activities and cell morphology. Ionomycin consistently enhanced cell adherence and growth inhibition but required higher concentrations of phorbol ester to synergistically induce enzyme markers. Ionophores alone or in the presence of high concentrations of the biologically inactive ester, 4α-PDD, did not induce differentiation.

The macrophage and granulocyte membrane antigen identified by OKM-1 monoclonal antibody [13] also appeared upon cotreatment with 0.5 nM TPA and 300 nM A23187 (table 1). Synergistic differentiation was accompanied by OKM-1 reactivity similar to that induced by 10 nM TPA, suggesting that most of the population had differentiated into macrophage-like cells. Cells exposed to 0.5 nM TPA, 300 nM A23187 or carrier solvents alone exhibited reactivity comparable to untreated cells. Thus, neither monocytic nor granulocytic differentiation occurred under these conditions.

At least two other parameters influenced the ex-

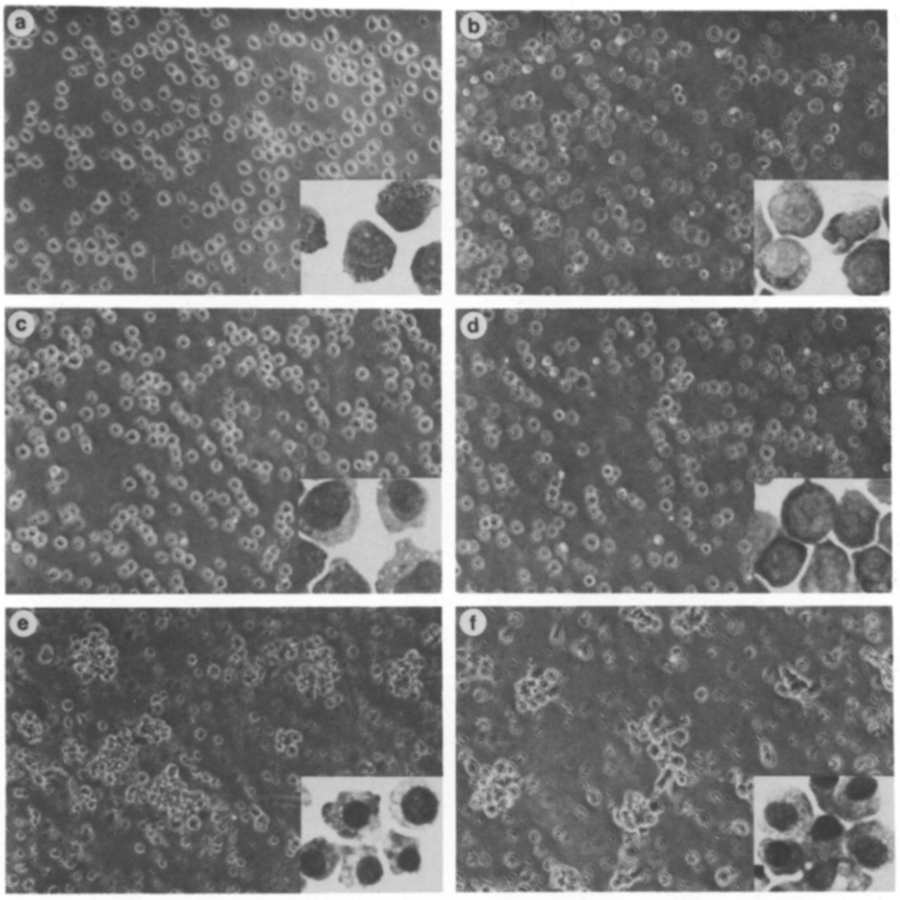

Fig.1. Morphological changes in HL-60 cells after treatment with various combinations of A23187 and TPA. (a) No treatment, (b) carrier solvents alone, (c) 300 nM A23187, (d) 0.5 nM TPA, (e) 0.5 nM TPA and 300 nM A23187, (f), 10 nM TPA. Insets show corresponding May-Grunwald-Giemsa stained cells. All fields were chosen randomly by naive observers.

tent of synergistic differentiation. Media was buffered at pH 7.6 since lower initial pH values increased the cytotoxicity of A23187 and caused a concomitant drop in marker enzyme activity (fig.2b,d,f, triangles). Secondly, DMSO and ethanol, used as carrier solvents for ionophores and phorbol esters respectively, were found to enhance synergistic differentiation. Compared to cells treated with drugs in an aqueous carrier, 20–30% more growth inhibition and increased adherence occurred when solvents were present. Although granulocytic differentiation of HL-60 cells is induced by 1.2% DMSO [15], no evidence of this was seen at 0.3% DMSO (table 1 and [15]). Measurement of intracellular Ca^{2+} by quin 2 fluorescence indicated that neither DMSO nor ethanol affected ionophore activity at the concentrations used (not shown). It remains unclear how these solvents affect HL-60 cells.

Two events that occur early in HL-60 differentiation, transferrin receptor downregulation [16] and activation of the Na^+/H^+ antiporter [17], are synergistically regulated by Ca^{2+} and protein kinase C [16,18]. However, Na^+/H^+ exchange is not a requisite for cell maturation [17] and synergistic downregulation of the transferrin receptor has not been correlated with subsequent differentiation. The results presented here suggest commitment to the complete differentiation program can be initiated by phorbol ester and Ca^{2+}

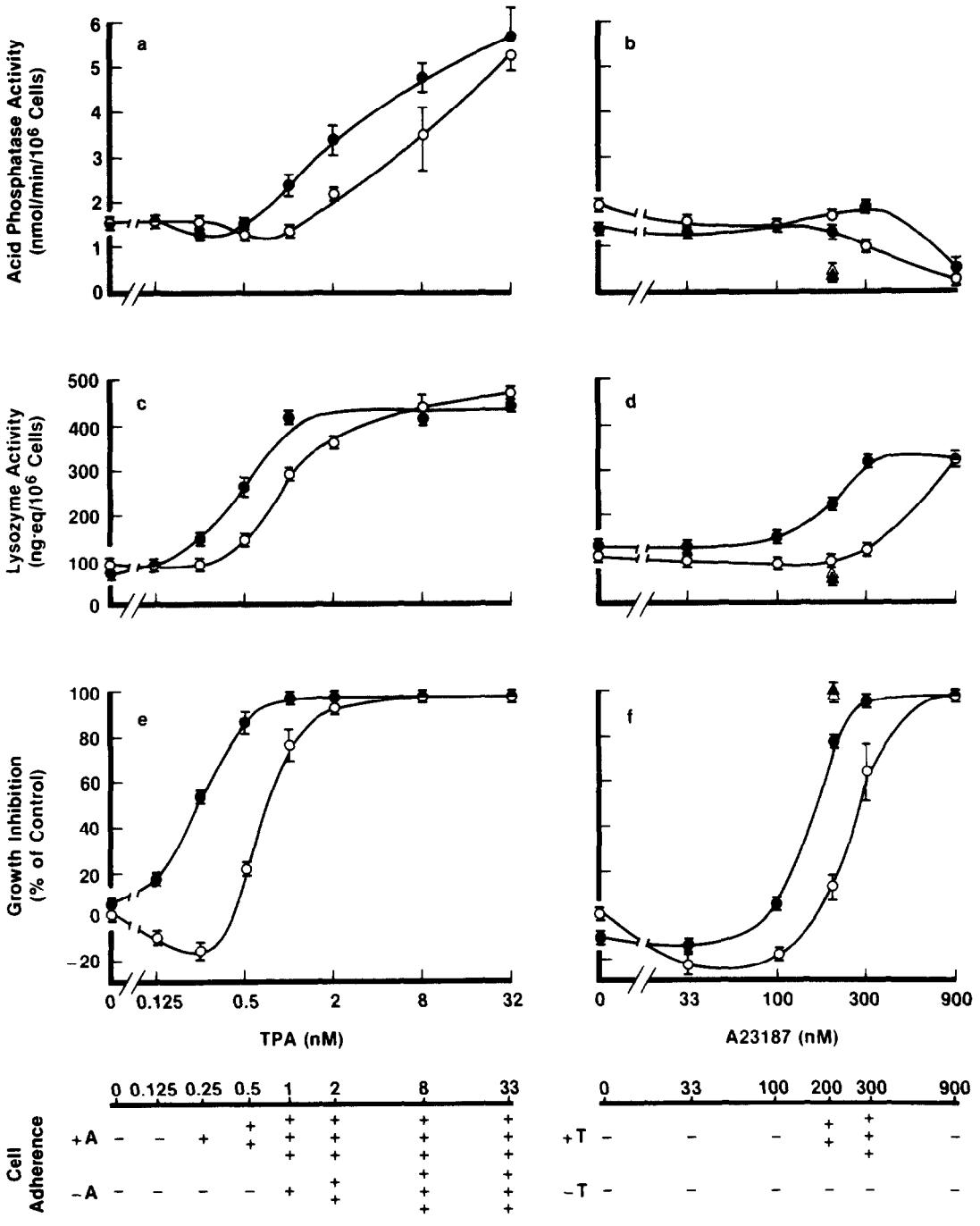

Fig.2. Dose-response curves for synergistic differentiation of HL-60 cells induced by TPA and A23187. Maturation was assayed by cellular acid phosphatase activity (a,b), secreted lysozyme activity (c,d) and growth arrest and morphology (e,f). (a,c,e) TPA in the presence (●, +A) or absence (○, −A) of 200 nM A23187. (b,d,f) A23187 in the presence (●, ▲, +T) or absence (○, △, −T) of 0.5 nM TPA. Triangles represent cells grown in media buffered to pH 7.2 with 10 mM sodium bicarbonate. Experimental points are for duplicate dishes (± SD) and curves are representative of several experiments (n = 3).

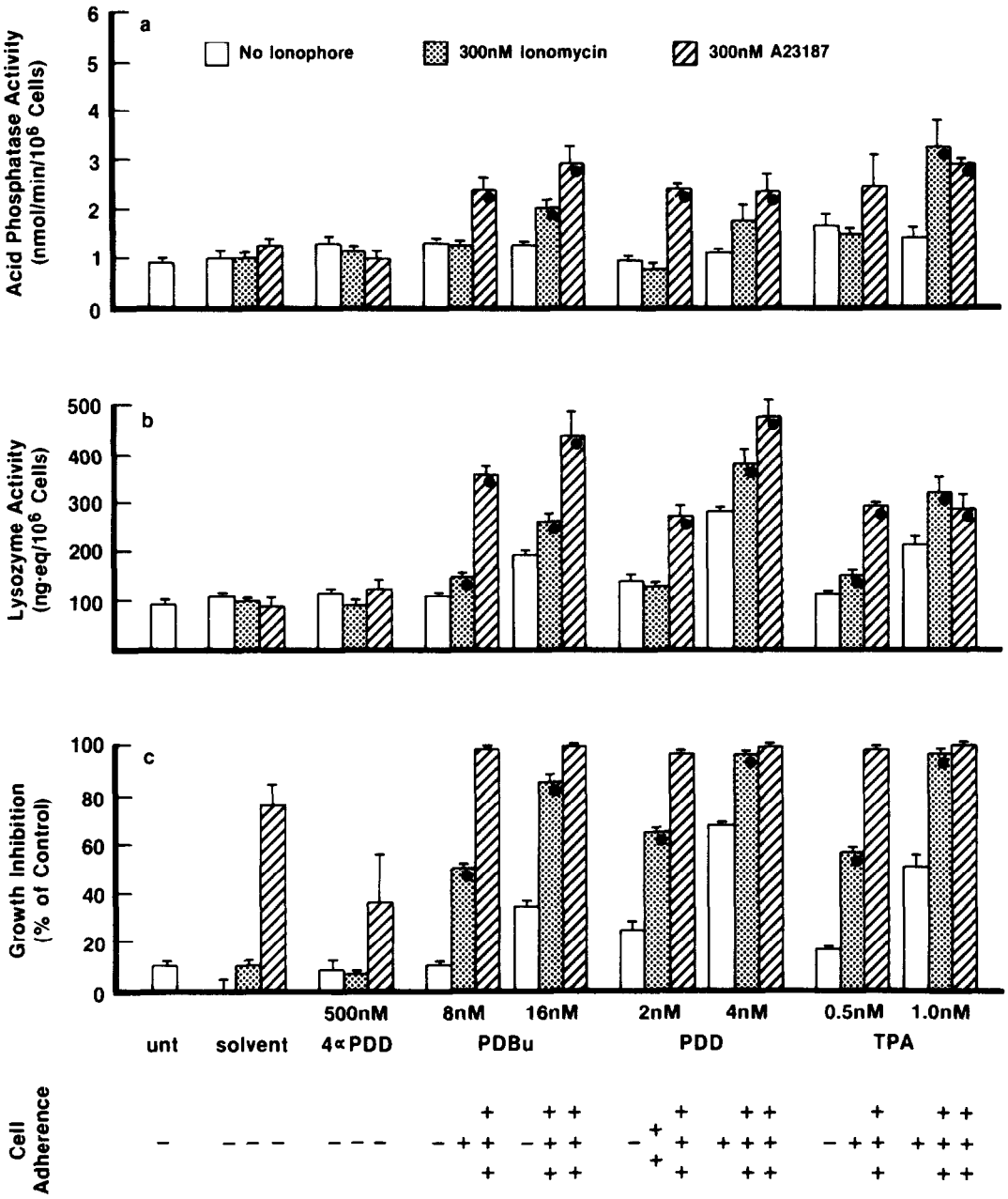

Fig.3. Synergistic differentiation of HL-60 cells induced by various phorbol esters and Ca^{2+} ionophores. Concentrations of active phorbol esters used approximate the inflection point of dose-response curves. Untreated (unt) and solvent (0.3% DMSO, 0.3% ethanol) represent control conditions without phorbol esters. Phorbol esters were added to cells either alone (open bars), or with 300 nM ionomycin (stippled bars), or with 300 nM A23187 (hatched bars). Bars with dots in the upper right corner indicate that phorbol ester and Ca^{2+} ionophore cotreatment had a greater effect than the sum of individual drug effects ($p < 0.05$, t-test). Similar data were obtained in at least 3 separate experiments.

Table 1

OKM-1 reactivity of HL-60 cells after various treatments

Condition	% positive
Untreated	6 ± 2
Solvent	8 ± 3
300 nM A23187	19 ± 7
0.5 nM TPA	12 ± 4
0.5 nM TPA + 300 nM A23187	77 ± 4
10 nM TPA	87 ± 7

Expression of the macrophage-granulocyte cell surface glycoprotein detected by OKM-1 antibody was determined visually and by flow cytometry on HL-60 cells after 3 days continuous treatment. Five independent experiments (3 visual, 2 flow cytometric) were pooled and expressed as mean ± SE. Cotreatment with TPA and A23187 had a greater effect than the sum of individual drug effects ($p < 0.01$, t-test)

ionophore acting together, presumably by mimicking the second messengers of ligand-induced phosphoinositide turnover. It is not clear why agents such as OAG cannot induce differentiation, even in the presence of a Ca^{2+} ionophore (not shown and [10]), but it is evident that subthreshold levels of phorbol ester fulfil whatever requirements are lacking in non-inducing activators of protein kinase C.

Synergism between elevated intracellular Ca^{2+} and protein kinase C was first demonstrated for platelet secretion in which the two pathways act to phosphorylate independent targets [19]. Recent evidence suggests Ca^{2+} enhances the affinity of protein kinase C for membranes and phorbol esters, implying that synergism can also occur at the level of the kinase itself [16,20]. Although transferrin receptor expression is modulated by the latter route in HL-60 cells, some genes known to respond to Ca^{2+} in other cell types, such as *c-myc* and *c-fos* [21,22], are also regulated early in HL-60 differentiation [23,24]. We are investigating the expression of these proto-oncogenes in an attempt to determine how Ca^{2+} and phorbol ester interact during synergistic differentiation of HL-60 cells.

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