Volume 214, number 1, 171–175 FEB 04581 April 1987

Decrease of cellular ATP by dihexanoylglycerol may limit responses to protein kinase C activation

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Received 1 February 1987

1,2-*sn*-Dihexanoylglycerol (HHG) reduced the ATP content of HL-60 cells. This concentration-related (10–100 μM) effect reached a maximum of over 90%, was enantiomerically specific and not accompanied by release of lactate dehydrogenase. Oleoylacetylglycerols (3–100 μM) had no effect on ATP levels while phorbol dibutyrate (PDBu, 0.01–1 μM) decreased ATP content of HL-60 cells by up to 40%. Responses stimulated by HHG became limited as the concentration was increased above 10 μM, this being manifest as either a low maximum response compared to PDBu (superoxide release) or a bell-shaped concentration-effect curve (degranulation). HHG (30–100 μM) inhibited PDBu-stimulated superoxide release, this inhibition being enantiomerically specific. It is probable that the effect of HHG on ATP content impairs cellular reponses mediated through protein kinase C activation.

Protein kinase C; Phorbol ester; Diacylglycerol; Superoxide; (HL-60 cell)

1. INTRODUCTION

Protein kinase C (PKC) is a phospholipid- and calcium-dependent enzyme, activated by diacylglycerols [1] and phorbol esters which interact with the same 'receptor' site on the enzyme [2,3]. Diacylglycerols show enantiomeric specificity for this site, with the 1,2-*sn* configuration being preferred [4]. Naturally occurring diacylglycerols such as 1-stearoyl-2-arachidonoyl-*sn*-glycerol activate isolated PKC, but such molecules do not penetrate cell membranes [5]. Therefore, synthetic diacylglycerols that activate PKC in intact cells have been sought; such compounds include 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) and 1,2-dihexanoyl-*sn*-glycerol (HHG) [5–7]. This paper compares the effects of phorbol dibutyrate (PDBu), OAG and HHG on the ATP content of HL-60 granulocytes and illustrates how the decrease of ATP associated with HHG may limit the magnitude of cellular responses stimulated by this PKC activator.

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2. MATERIALS AND METHODS

The methods used for growth and differentiation of HL-60 granulocytes, superoxide measurement and binding of [^{3}H] phorbol dibutyrate ([^{3}H]PDBu) were as described previously [8]. Cell densities were 0.5×10^6/ml for O_2^- release and ATP measurements, $2–3 \times 10^6$/ml for [^{3}H]PDBu binding, and 15×10^6/ml for measurement of β-glucuronidase release.

2.1. *ATP measurement*

Samples (1 ml) of differentiated HL-60 granulocytes, prepared as for O_2^- release experiments, were incubated with diacylglycerols or PDBu for 30 min at 37°C. After sedimentation in an Eppendorf microcentrifuge, supernatants were removed for measurement of lactate dehydrogen-

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00145793/87/$3.50

ase (see below) and 25 μl perchloric acid (final concentration 2%) was added to cell pellets. Samples were stored at −20°C until ATP was measured by a modification of the method of Stanley and Williams [9]. A sample (5 μl) of each acid extract was added to 1 ml freshly prepared buffer (pH 7.4) composed of equal amounts of $MgSO_4$ (80 mM), KH_2PO_4 (10 mM) and Na_2HAsO_4 (100 mM). The assay was initiated by the automatic addition of luciferin-luciferase (5 mg/ml, Sigma FLE-50) in an LKB 1251 luminometer (integration mode).

2.2. *Lactate dehydrogenase release*

Lactate dehydrogenase (LDH) released into cell supernatants was measured by the method of Bergmeyer and Bernt [10]. Portions of cell suspensions were also incubated with Triton X-100 (0.1%, w/v) to release total LDH content.

2.3. *β-Glucuronidase release*

The release of β-glucuronidase was measured spectrophotometrically, using phenolphthalein glucuronide as substrate [11]. Total enzyme activity was determined in extracts of cells lysed in Triton X-100 (0.2%).

2.4. *Synthesis of diacylglycerols*

A mixture of 3-benzyl-*sn*-glycerol (L-α-*O*-benzoylglycerol, Sigma) (182 mg, 1 mM) and hexanoyl chloride (304 mg, 2.2 mM) in dry toluene (2 ml) and dry pyridine (1 ml) was stirred overnight at room temperature and evaporated under reduced pressure. The residue was partitioned between water and ether and the ether layer was washed successively with ice-cold 4 M sulphuric acid (twice), water, ice-cold saturated $NaHCO_3$ (twice) and water (twice). The ether solution was dried over Na_2SO_4 and evaporated to give an oil which was purified by flash chromatography in ehter/hexane (1:4) on silica gel (Merck, Kieselgel 60 art.9385) to give pure 3-benzyl-1,2-dihexanoyl-*sn*-glycerol. This material (220 mg) was debenzylated by hydrogenation in pure tetrahydrofuran (35 ml) with 10% palladium on carbon catalyst (150 mg). Removal of the catalyst and evaporation gave pure 1,2-dihexanoyl-*sn*-glycerol (160 mg). 2,3-Dihexanoyl-*sn*-glycerol was prepared in the same way, starting from 1-benzyl-*sn*-glycerol. In both cases, thin-layer chromatography and NMR spectroscopy (360 MHz) confirmed the absence of any 1,3 isomer.

2.5. *Preparation of solutions*

Phorbol dibutyrate (Sigma) was stored as a solution (2 mM) in acetone at −20°C. For use, the acetone was evaporated and the compound redissolved in ethanol (Fisons, Spectrograde) at 1000-times the required final concentration. Diacylglycerols were stored at −20°C until use when they were also dissolved in dry ethanol at 1000-times the final concentration. Diacylglycerols and PDBu were diluted 1:1000 on addition to cell suspensions such that the ethanol concentration did not exceed 0.2% (v/v).

3. RESULTS AND DISCUSSION

Concentrations of dihexanoylglycerol (3–100 μM) that stimulated O_2^- release from HL-60 cells also caused a concentration-dependent fall in cellular ATP contents (fig.1). This effect was enan-

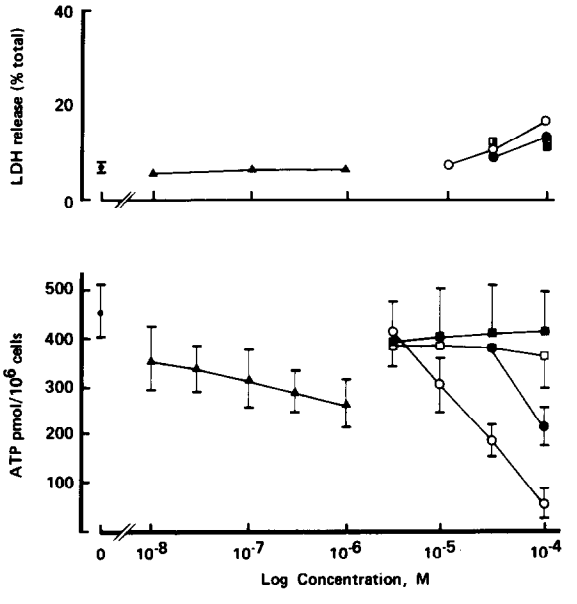

Fig.1. Effect of diacylglycerols and PDBu on the release of LDH from (upper panel) and ATP content of (lower panel) differentiated HL-60 granulocytes. (▲) PDBu, (○) 1,2-*sn*-dihexanoylglycerol, (●) 2,3-*sn*-dihexanoylglycerol, (□) 1-oleoyl-2-acetyl-*sn*-glycerol, (■) 3-oleoyl-2-acetyl-*sn*-glycerol. Each point is the mean ± SD of 3 separate determinations.

tiomerically specific, with the 1,2-*sn* enantiomer (HHG) being approx. 5-times more effective than the 2,3-*sn* enantiomer. At 100 μM, HHG decreased the cellular ATP content by 90%. This fall in ATP was not associated with a major disruption of cells as release of LDH into the supernatant was only increased by HHG (100 μM) to 17% of total, compared with 7% release in controls. The release of LDH by the dihexanoylglycerols was not enantiomerically specific (fig.1). In contrast to the dihexanoylglycerols, the two enantiomers of oleoylacetylglycerol (3–100 μM) did not cause a fall in cellular ATP content. The small increase in LDH release caused by these diacylglycerols (100 μM) was, however, comparable to that induced by HHG. This supports the view that the HHG-induced fall in ATP was not due to cell disruption. Concentrations of PDBu (10 nM–1 μM) that stimulate O_2^- release also decreased the ATP content of HL-60 cells to a minimum of 40% of the control value (fig.1).

Fig.2 shows concentration-effect curves for O_2^- release from HL-60 cells stimulated by PDBu, OAG and HHG. The maximum response stimulated by PDBu (1 μM) was twice that stimulated by HHG. Thus, it is clear that there is no direct correlation between the magnitude of O_2^-

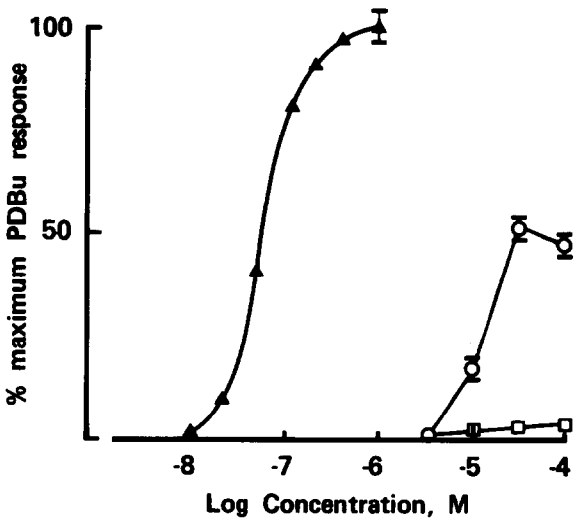

Fig.2. Stimulation of O_2^- production from differentiated HL-60 granulocytes by increasing concentrations of: PBDu (▲) HHG (○), OAG (□). Each point represents the mean (± SE) of 6 separate determinations.

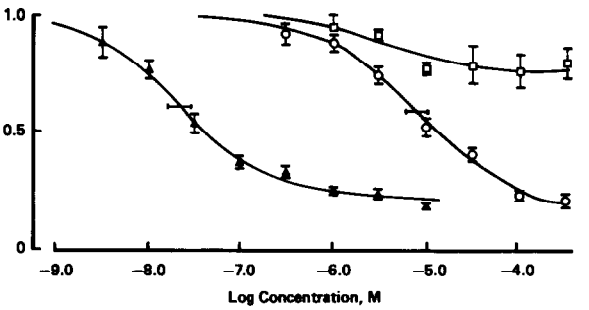

Fig.3. Displacement of [^{3}H]PDBu from undifferentiated HL-60 cells by increasing concentrations of: PDBu (▲), HHG (○), OAG (□). Each point represents the mean (± SE) of 9 separate determinations.

release induced by PKC activators and cellular ATP content. Therefore, it is unlikely that the fall in ATP contributes to the stimulus for O_2^- generation by NADPH oxidase, as was recently suggested by Schinetti and Lazzarino [12]. One possible explanation for the different maximal O_2^- responses to HHG and PDBu is that HHG is unable to penetrate to sites on PKC in HL-60 cells that are accessible to phorbol esters. This interpretation was recently used to explain the weak O_2^- response stimulated by OAG [8]. However, in contrast to OAG, HHG completely displaced specifically bound [^{3}H]PDBu from HL-60 cells (fig.3). Therefore, restricted access to its intracellular 'receptor' on PKC cannot be used to explain the low maximum O_2^- response to HHG.

When added to HL-60 cells concomitantly with PDBu, HHG blocked the O_2^- response stimulated by this phorbol ester (fig.4). This blocking effect occurred with HHG at 30 and 100 μM, concentrations of the diacylglycerol that decreased cellular ATP levels by 55% and 90%, respectively. Furthermore, the blockade of PDBu-stimulated O_2^- release was enantiomerically specific, 2,3-*sn*-dihexanoylglycerol being inactive. The interaction between HHG and PDBu was not competitive since increasing the concentration of PDBu to 10 μM did not surmount the inhibition by HHG 100 μM (not shown). Furthermore, concentration-effect curves for activation of isolated PKC by HHG and PDBu reached identical maxima, consistent with the two activators having equal efficacy (not shown). Thus, there is no evidence that inhibition by HHG of PDBu-stimulated O_2^- production is through antagonism at the PKC 'receptor'.

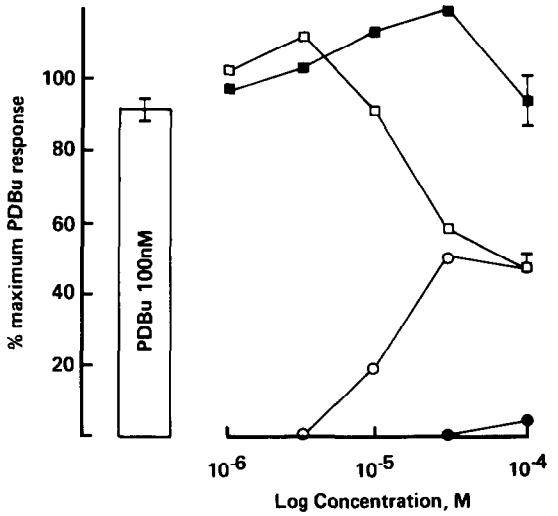

Fig.4. Stimulation of O_2^- production from differentiated HL-60 granulocytes by increasing concentrations of 1,2-*sn*-dihexanoylglycerol, HHG (open symbols) or 2,3-*sn*-dihexanoylglycerol (closed symbols) either alone (circles) or combined with 100 nM PDBu (squares). The response to PDBu alone is shown by the bar. Each point is the mean (± SE) of 6 separate determinations.

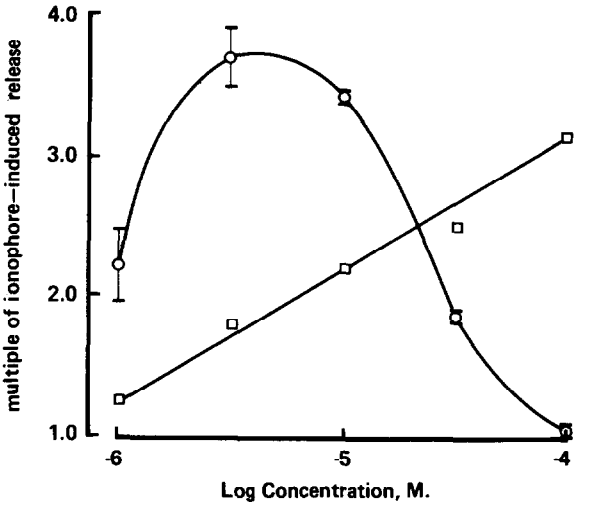

Fig.5. Release of β-glucuronidase from differentiated HL-60 granulocytes stimulated by increasing concentrations of either OAG (□) or HHG (○) added with A23187 (0.1 μM). Each point is the mean (± SE) of 3 experiments, each in duplicate, expressed as a multiple of the control release stimulated by A23187 alone (5.7 ± 0.7% of total β-glucuronidase).

Phorbol esters stimulate degranulation (β-glucuronidase release) of HL-60 cells, a response that also illustrates the synergy between PKC activators and the calcium ionophore A23187 [13]. In contrast, the diacylglycerols OAG and HHG did not directly stimulate degranulation but did synergise with A23187 (fig.5). Degranulation was proportional to the concentration of OAG (1–100 μM). However, the concentration-effect relationship for HHG was bell-shaped, having an optimum at 3 μM with degranulation becoming significantly less as the concentration was increased to 30 and 100 μM. In contrast, the concentration-effect curves for degranulation induced by phorbol esters are sigmoid with asymptotic maxima [13].

In conclusion, this investigation has shown that responses of HL-60 cells stimulated by HHG and phorbol esters differ qualitatively in two ways: (i) the maximum release of O_2^- induced by HHG was less than that induced by PDBu; (ii) the degranulation concentration-effect curve for HHG was bell-shaped rather than a rectangular hyperbola [13]. Both observations can be explained by HHG having an effect on HL-60 cells that opposes responses induced by PKC activation, and this is consistent with the observed inhibition by HHG of PDBu-induced O_2^- release. It is reasonable to speculate that this inhibitory effect is due to the concomitant fall of ATP caused by HHG. It is generally believed that degranulation of neutrophils requires ATP derived from glycolysis [14] as has been shown for secretion in other cells, such as the mast cell [15]. Also, release of O_2^-, which requires NADPH derived from the pentose phosphate pathway, will be impaired by a substantial fall in ATP [16]. It is unlikely that a fall in ATP level of even 90% will limit protein phosphorylation by the kinase itself as the K_m of the isolated enzyme for ATP is reported to be 5 μM [17].

The mechanism by which HHG decreased cellular ATP is not known. It was not through gross cell damage but could be due to increased ion permeability of the plasma membrane, so evoking increased activity of ion pumps and consequent increased consumption of ATP. The metabolism of HHG by diacylglycerol kinase might also contribute to a fall in ATP, and would explain the enantiomeric specificity of this effect. It would, therefore, be interesting to discover the effects of

diacylglycerol kinase inhibitors on responses of HL-60 cells stimulated by HHG.

ACKNOWLEDGEMENTS

We wish to thank Mr G.D. Spacey and Mr R.M. Beams for their excellent technical assistance.

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