Synergism between phorbol ester and A23187 in superoxide production by neutrophils

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Concentrations of phorbol myristate acetate and the calcium ionophore, A23187, which by themselves are minimally effective in stimulating superoxide generation in human neutrophils show marked mutual potentiation when given together. This supports the hypothesis that synergism between cytosolic calcium and protein kinase C is involved in the stimulus/activation coupling of the respiratory burst in the neutrophil.

Neutrophil *Phorbol ester* *Protein kinase C* *Calcium*

1. INTRODUCTION

The production of oxygen radicals such as superoxide during the respiratory burst is essential for effective microbial killing by the neutrophil [1,2] and may also be implicated in tissue injury in complex-mediated disease [3]. However, as has been emphasised in a recent review [4], the transduction mechanisms involved in activation of the burst are not clearly known. An increase in cytosolic calcium is important for $O_2^{\cdot -}$ formation as wel as for other neutrophil responses when the stimulation is by chemotactic peptides, but its significance for $O_2^{\cdot -}$ production by other agents is uncertain (review [5]). Thus, PMA which stimulates the production of $O_2^{\cdot -}$ [6] does not increase cytosolic calcium [7] and a rise in cytosolic calcium to micromolar levels, which is a necessary and sufficient stimulus for exocytosis, does not result in $O_2^{\cdot -}$ production [8].

It has been hypothesized that in many cells in which calcium is a second messenger, an increase of diacylglycerol with subsequent activation of protein kinase C may also be required for signal transduction and that the two 'pathways' may act synergistically [9].

Abbreviations: OAG, 1-oleoyl-2-acetylglycerol; PMA, phorbol myristate acetate

It is possible to activate each pathway independently. A low concentration of a calcium ionophore increases cytosolic calcium without necessarily activating protein kinase C [10] and either PMA or OAG can substitute for endogenous diacylglycerol and stimulate protein kinase C without increasing cytosolic calcium (review [9]). Here, we show that there is potentiation of $O_2^{\cdot -}$ production in neutrophils if the two pathways are simultaneously activated, using concentrations of stimulants which, separately, cause little or no response.

2. MATERIALS AND METHODS

Neutrophils were collected from human volunteers by venipuncture, prepared by Ficoll-Isopaque separation as in [11] and suspended in calcium-free Tyrode solution containing 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 $mg \cdot ml^{-1}$ glucose and 1 $mg \cdot ml^{-1}$ bovine serum albumin. After equilibration for 20 min at 37°C, 2.5×10^6 cells were dispensed into 2.5-ml tubes (Sterilin, NA2S) to which had been added 1 mg ferricytochrome *c* (horse heart type III, Sigma), appropriate dilutions of A23187, PMA and either Tyrode solution or 75 units superoxide dismutase (bovine blood, Sigma). The final calcium concentration in all samples was 3 mM. After 30 min in-

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cubation at 37°C, the reaction was stopped by the addition of 1 mM *N*-ethylmaleimide (Sigma). Following centrifugation at 1400 × *g* for 10 min at 4°C, the absorbance of the supernatant was read at 550 nm in a Perkin Elmer SP-1800 spectrophotometer. The amount of $O_2^{\cdot-}$ produced was calculated by dividing the difference in absorbance of the samples with and without superoxide dismutase by the extinction coefficient for the change between ferricytochrome *c* and ferrocytochrome *c* (E_{550nm} = 15.5 $mM^{-1} \cdot cm^{-1}$) and the resulting value multiplied by 2000 to express the result as nmol $O_2^{\cdot-}$ per 5 × 10^6 neutrophils.

Potentiation of $O_2^{\cdot-}$ generation was calculated by adding together the measurements obtained with ionophore and PMA separately and subtracting this from the measurement obtained when both were given simultaneously. This figure was then expressed as a percentage of the maximum $O_2^{\cdot-}$ obtained, in that particular experiment.

3. RESULTS

The possibility of synergism between A23187 and PMA was tested by measuring the effect of a low concentration of one agent on the lower end of the dose-response curve of the other. It was necessary to confine attention to this part of the dose-response curve because, as has been pointed out [9], at high concentrations each agent causes cell activation by non-specific effects. Variation between subjects occurred, particularly with PMA, but in every case there was marked potentiation of submaximal responses when the two agents were used together.

The mean percentage potentiation of the response to A23187 by PMA is shown in fig.1a, and the converse in fig.1b. As expressing the mean results of 4 separate experiments as percentage potentiation does not necessarily give a clear idea of the actual data obtained, the full results of two representative experiments are also given – the effect of PMA on the A23187 response in fig.1c and A23187 on the PMA response in fig.1d.

PMA (8 × 10^{-9} M) resulted in maximal or near-maximal $O_2^{\cdot-}$ production in the neutrophils from most subjects and it was only possible to obtain a measure of potentiation at this concentration in one subject in whom the response to 8 × 10^{-9} M was clearly submaximal.

4. DISCUSSION

It has been suggested that in cells in which calcium rather than cyclic AMP is the second messenger, an additional component necessary for

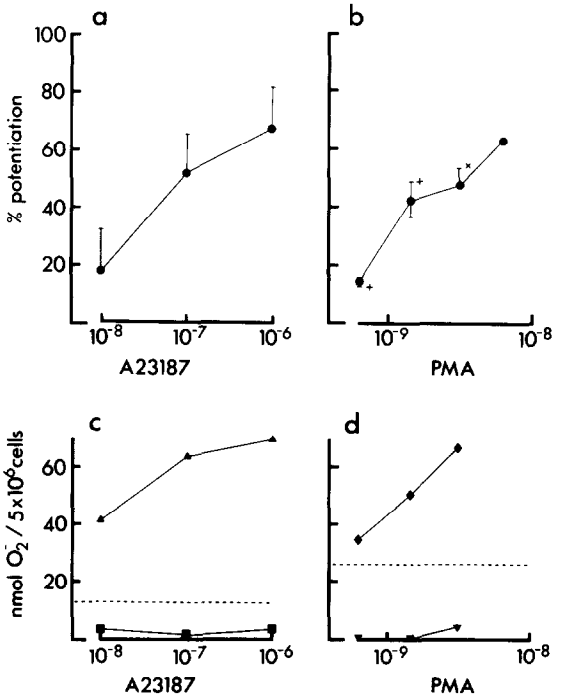

Fig.1. Synergism between A23187 and PMA in superoxide production. (a) Potentiation of the response to A23187 in the presence of PMA (4.9 × 10^{-9} or 8 × 10^{-10} M), expressed as a percentage of the maximum response obtained. The points represent the means and the bars, standard errors. The mean amount of $O_2^{\cdot-}$ produced with the highest concentration of ionophore + PMA was 45 (SE 20.5) nmol per 5 × 10^6 cells (n = 4). (b) Potentiation of the response to PMA in the presence of A23187 (10^{-5} M). The points represent the means and the bars, standard error (x) or range (+). The mean amount of $O_2^{\cdot-}$ produced with the highest concentration of PMA + ionophore was 64 (SE 4.1) nmol per 5 × 10^6 cells (n = 4). (c) Results of an individual experiment showing the effect of PMA on the dose-response curve of $O_2^{\cdot-}$ production with A23187. (■—■) A23187 alone, (▲—▲) A23187 with PMA (4.9 × 10^{-9} M). The dashed line gives $O_2^{\cdot-}$ production with PMA (4.9 × 10^{-9} M) by itself. (d) Results of an individual experiment showing the effect of A23187 on the dose-response curve of $O_2^{\cdot-}$ production with PMA. (▼—▼) PMA alone, (◆—◆) PMA with 10^{-5} M A23187. The dashed line gives $O_2^{\cdot-}$ production with A23187 10^{-5} M by itself.

signal transduction is the turnover of phosphatidylinositol bisphosphate with transient generation of diacylglycerol which results in activation of protein kinase C [9]. There is evidence that both calcium and protein kinase C are involved in receptor-response coupling in several cell types, e.g., in thrombin-induced platelet secretion [10,12], in angiotensin-induced aldosterone secretion [13] and in concanavalin A-induced lymphocyte activation [14]. In the above systems, the relevant response could also be produced by the simultaneous use of a calcium ionophore and a protein kinase C activator at concentrations which, when used separately, caused minimal effects.

In rat neutrophils, OAG-mediated exocytosis was augmented by concomitant administration of A23187 [15]. Thus synergism of the two components occurs for this particular neutrophil response. The question which this study aimed to answer was: are both components involved in the activation of the respiratory burst in human neutrophils? As was emphasized in a recent review 'less is understood about the mechanism of activation of the $O_2^{\cdot -}$-forming oxidase than about any other aspect of the respiratory burst' [4]. The results of some studies have implied that the transduction mechanisms for exocytosis and for $O_2^{\cdot -}$ generation may be different [16,17]. The fact that PMA can cause some stimulation of the NADPH-oxidase in particulate fractions of the neutrophil [17,18] would appear to be evidence for this, as would the fact that some agents (e.g., adenosine) inhibit $O_2^{\cdot -}$ production but not exocytosis [19]. Further support for the idea that the transduction mechanisms of the two responses might be different comes from a recent study in which the authors found a clear dissociation between exocytosis and $O_2^{\cdot -}$ production in that the former was triggered by increasing the cytosolic calcium to micromolar levels whereas $O_2^{\cdot -}$ generation was not elicited even at high levels of cytosolic calcium [8]. These authors concluded that $O_2^{\cdot -}$ generation, unlike exocytosis, required another signalling mechanism, in addition to calcium. The results of the present study imply that the additional signalling mechanism required is protein kinase C activation. Taken in conjunction with the results of the study by authors in [8] they also imply that $O_2^{\cdot -}$ production is more critically dependent on protein kinase C activation than is exocytosis.

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