Volume 212, number 2, 242–246 FEB 04443 February 1987

Calcium ionophore A23187 enhances human neutrophil superoxide release, stimulated by phorbol dibutyrate, by converting phorbol ester receptors from a low- to high-affinity state

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Received 27 November 1986

The calcium ionophore A23187 acted synergistically with phorbol dibutyrate (PDBu) to stimulate human neutrophil superoxide production. A23187 shortened the lag period and markedly increased the initial rate of neutrophil superoxide production induced by suboptimal concentrations of PDBu. 1 μM A23187 reduced the EC_{50} value for superoxide release from 56 to 8 nM PDBu. This effect of A23187 was correlated with enhanced binding of [^{3}H]PDBu to its receptor and a reduction in the dissociation constant (K_d) from 27 to 10 nM, without altering the apparent total number of phorbol dibutyrate receptors. These actions of A23187 were abolished in the presence of EGTA or TMB-8, confirming a dependence on Ca^{2+}.

Ca^{2+}; Ionophore A23187; Phorbol ester; Protein kinase C; Neutrophil

1. INTRODUCTION

Tumour-promoting phorbol esters are potent stimulants of neutrophil (PMN) superoxide anion production [1]. The major cellular receptor for phorbol esters is now known to be the phospholipid/Ca^{2+}-dependent protein kinase (protein kinase C) (PKC) [2]; recent evidence suggests that phorbol ester binding to PKC is dependent on phospholipid [2] and that translocation and binding of cytosolic PKC to the plasma membrane may therefore be an important step in activation of PKC by phorbol ester or diacylglycerol [3]. Recent studies have shown that the calcium ionophore A23187 acts synergistically with phorbol esters to elicit a variety of physiological cell responses including guinea pig PMN superoxide production [4]. One suggested mechanism for this synergism is that the rise in cytosol Ca^{2+} produced by A23187 promotes translocation and binding of PKC to the cell membrane, thereby increasing availability of phospholipid cofactor and thus the affinity of binding of PKC for phorbol ester [3,5].

In the experiments described here we have investigated this hypothesis further by comparing the effect of calcium ionophore A23187 on PDBu-induced PMN superoxide anion production with its effect on the binding of PDBu in human PMN.

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Abbreviations: PDBu, phorbol dibutyrate; PMN, neutrophil polymorphonuclear leucocyte; TMB-8, 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester

2. MATERIALS AND METHODS

All reagents were obtained from Sigma except for [^{3}H]PDBu (12.5 Ci/mmol) which was obtained

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

from New England Nuclear. Human PMN were prepared from venous blood by dextran sedimentation followed by fractionation on a Percoll gradient as described [6]. PMN were washed twice and resuspended in RPMI 1640 (Flow Labs) + 10% fetal calf serum (at 10^6 or 5×10^6 PMN/ml for superoxide or PDBu-binding studies, respectively). Unless otherwise stated PMN were preincubated for 10 min at 37°C with A23187 or methanol solvent (0.2%, v/v, final) prior to incubation for 20 min with PDBu or [^{3}H]PDBu in DMSO (0.05%, v/v, final). Final concentrations of methanol or DMSO did not affect the performance of either assay (not shown). Assay of [^{3}H]PDBu binding (in triplicate) was performed as in [7]. Non-specific binding was assayed in the presence of a 50-fold excess of unlabelled phorbol ester.

Superoxide production was measured by monitoring superoxide dismutase-inhibitable ferricytochrome *c* reduction at 550 nm. For kinetic experiments ferricytochrome *c* reduction was

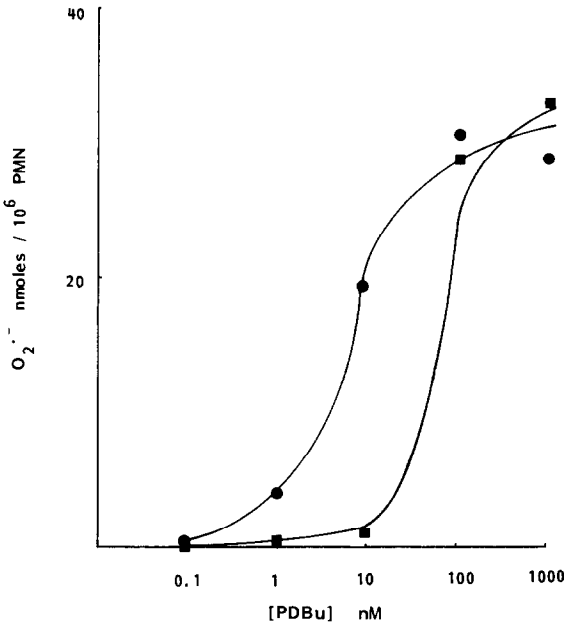

Fig.1. Effect of A23187 on PDBu-stimulated superoxide production. PMN were treated with 1 μM A23187 or an equivalent volume of methanol for 10 min, then PDBu for 20 min. In the presence of A23187 the dose response to PDBu was shifted to the left. A23187 + PDBu (●); PDBu alone (■). Data represent one typical result from three experiments. SE did not exceed 0.8 nmol/10^6 PMN.

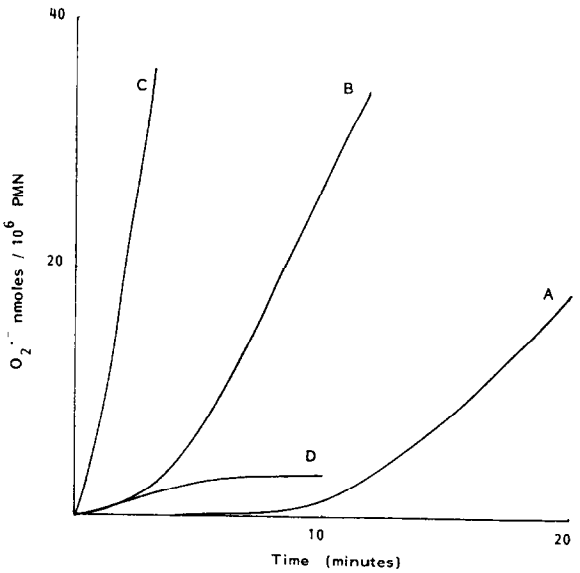

Fig.2. Effect of A23187 on kinetics of PDBu-stimulated superoxide production. The initial rate of PMN superoxide release was increased and the lag time for response to 50 nM PDBu (A) was shortened by 10 min pretreatment with 0.1 μM (B) or 1 μM (C) A23187. 1 μM A23187 alone (D) stimulated only minimal superoxide release.

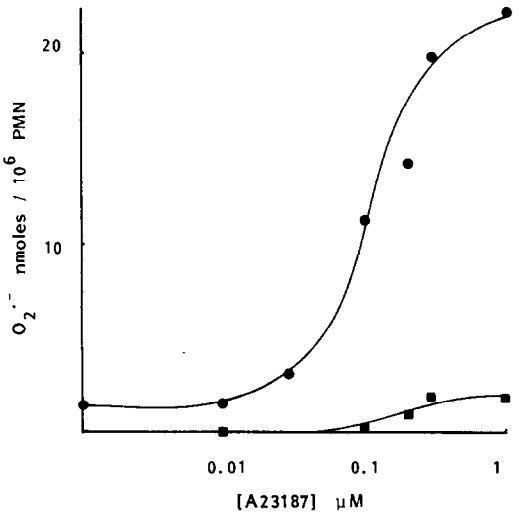

Fig.3. Dose dependence of the synergistic effect of A23187 on PDBu-stimulated superoxide production. 10 nM PDBu + A23187 (●); A23187 alone (■). Data represent one typical result from five experiments.

Table 1

Synergistic action of A23187 on superoxide release and PDBu binding

	(a) Superoxide release (nmol/10^6 PMN per 20 min)		(b) [^{3}H]PDBu binding (% of control)	
	+A23187 (1 μM)	−A23187	+A23187 (1 μM)	−A23187
PDBu	12.8 ± 2.4	1.3 ± 0.6	173 ± 13	100
PDBu + EGTA	0.3 ± 0.1	0.4 ± 0.2	114 ± 14	110 ± 16
PDBu + TMB-8	0.9 ± 0.3	0.2 ± 0.1	111 ± 11	113 ± 24
A23187	1.9 ± 0.7	0	–	–

Effect of 2 mM EGTA or 200 μM TMB-8 on the synergistic action of 1 μM A23187 on mean (± SE) (a) superoxide release elicited by 10 nM PDBu (n = 5); (b) binding of 10 nM [^{3}H]PDBu (n = 3)

monitored continuously at 37°C [8] in a spectrophotometer (LKB) linked to an Apple computer (Ultraspec reaction rate programme). Fixed time point measurements of superoxide release experiments were conducted in duplicate as described in [9].

3. RESULTS AND DISCUSSION

3.1. *Effect of A23187 on superoxide production*

After pretreatment of PMN for 10 min with A23187 (1 μM), the dose response for PDBu-stimulated PMN superoxide production was shifted to the left (fig.1). The mean (± SE) concentration of PDBu which produced 50% of the maximal superoxide response (EC_{50}) was reduced from 56 ± 8 to 8 ± 2 nM (n = 3) in the presence of 1 μM A23187. At high concentrations of PDBu (>100 nM) A23187 had no synergistic effect (fig.1). As reported in [4] the enhancing effect of A23187 was due both to a shortening of the lag time for response to PDBu and to an increase in the initial rate of superoxide release (fig.2). A23187 (1 μM) alone caused only minimal stimulation of superoxide production (fig.2).

The synergistic action of A23187 on the superoxide response elicited by a 'threshold' concentration of PDBu (10 nM) was dose-dependent (fig.3) with a mean (± SE) EC_{50} of 0.18 ± 0.07 μM A23187 (n = 5). In the presence of EGTA (2 mM) or TMB-8 (200 μM) the synergistic effect of A23187 was inhibited, thus confirming the dependence on Ca^{2+} (table 1). The response to PDBu in the absence of A23187 was also partially inhibited by EGTA or

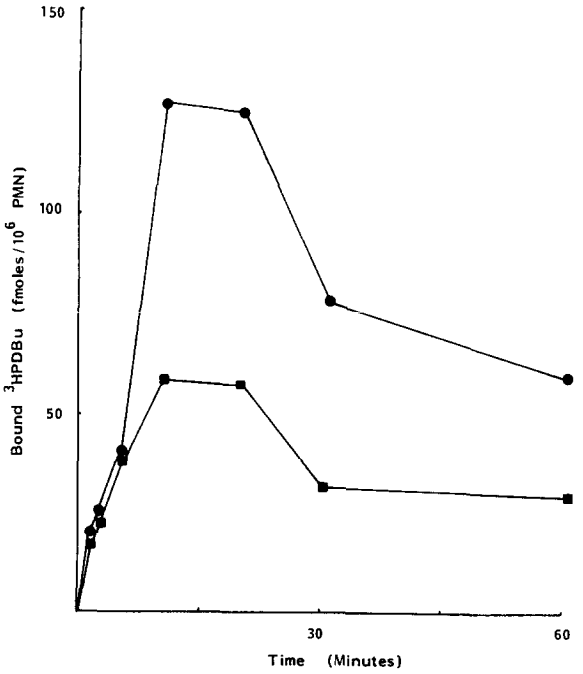

Fig.4. Effect of A23187 on the kinetics of binding of [^{3}H]PDBu to PMN. 10 min pretreatment with 1 μM A23187 increased the extent and rate of PDBu binding but did not alter the overall shape of the kinetic curve. (●) 10 nM PDBu alone; (■) 10 nM PDBu + 1 μM A23187 (SE did not exceed 2 fmol).

the intracellular calcium antagonist TMB-8 [10], emphasizing the importance of Ca^{2+} in the cellular response to PDBu alone.

3.2. *Effect of A23187 on PDBu binding*

To determine whether the synergistic effect of A23187 correlated with increased phorbol ester binding, the action of A23187 on [^{3}H]PDBu binding to PMN was examined. The time course of binding of 10 nM [^{3}H]PDBu to PMN was followed over 60 min. After an initial phase of rapid binding, which lasted 10–20 min, there was a slow decline in PDBu binding. Pretreatment of PMN for 10 min with A23187 (1 μM) increased the initial rate of PDBu binding, but did not alter the overall shape of the kinetic curve (fig.4). Similar binding kinetics have been reported for the HL60 cell line [11], and may reflect proteolytic cleavage of PKC and loss of binding sites following activation of PKC [12,13].

Pretreatment of PMN with A23187 (1 μM) also increased the affinity of binding of phorbol ester receptors for PDBu from a single class of low affinity (K_d 27 nM) to a single class of high affinity (K_d 10 nM), with no apparent alteration of total receptor numbers (fig.5). Thus, as previously reported for other cell types (3,14], A23187 appears to convert low-affinity PDBu receptors to high affinity. These data are consistent with the hypothesis that a rise in cytosol Ca^{2+} converts low-affinity PDBu receptors, believed to be cytoplasmic PKC, to high-affinity receptors representing PKC bound to plasma membrane phospholipid [3,5]. Nonetheless other indirect ef-

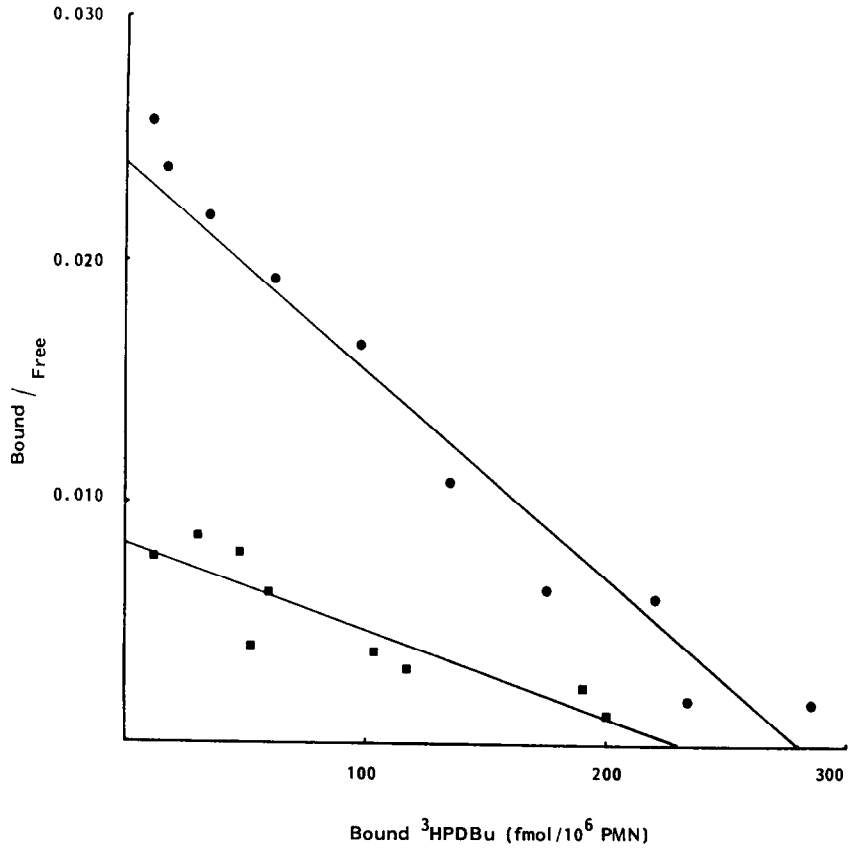

Fig.5. Scatchard plot of [^{3}H]PDBu binding to PMN with or without pretreatment with A23187. 10 min pretreatment with 1 μM A23187 increased the affinity of binding of phorbol ester receptors from a single class of low affinity (K_d 27 nM) to a single class of high affinity (K_d 10 nM). (■) PDBu alone; (●) PDBu + 1 μM A23187. Data represent one typical result from 3 experiments. Each point represents the mean of triplicates, SE did not exceed 2 fmol.

fects of Ca^{2+} on PDBu receptors cannot be excluded.

The action of A23187 on binding of PDBu (10 nM) was dose-dependent with a mean (± SE) EC_{50} of 0.24 ± 0.08 μM A23187 (n = 3), which correlated closely with the EC_{50} (0.18 ± 0.07 μM) for the effect of A23187 on PDBu-induced superoxide release. The effect of A23187 on PDBu binding was inhibited by EGTA or TMB-8 (table 1).

In conclusion these data provide further evidence that the basis for the synergistic effect of A23187 on the initial response of PMN to phorbol ester is due to a Ca^{2+}-dependent increase in binding affinity of receptors for PDBu. In view of the lack of effect of A23187 on the response to maximal doses of PDBu, these data suggest that A23187 affects early events during PMN activation and are consistent with the recently proposed hypothesis that Ca^{2+} promotes translocation and binding of cytosolic PKC to the plasma membrane [3,5].

ACKNOWLEDGEMENTS

J.K.F. is supported by an NH & MRC Medical Postgraduate Research Scholarship. Financial support was also provided by the National Health & Medical Research Council and Australian Rheumatism Association (SA).

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